Sperm Function and the Diagnosis of Male infertility.

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Abstract.

This D.Sc. presents a selected series of publications, focusing on the understanding and diagnosis of male infertility. Twenty five articles are presented from work spanning 1990-2018. Experiments assessing the predictive/diagnostic potential of a basic semen analysis and kinematic parameters of sperm motility in the context on *in vivo* conception are described as are studies aimed at improving the understanding of sperm function. This knowledge has been used to potentially develop new diagnostic tests for use at home and in the laboratory. A consistent theme has been involvement with national and international authorities to use evidence based information for making recommendations for both the diagnosis and treatment of the sub fertile male.

Dedication:

To my family, Evelyn, Owain, Fraser and Cerys.

Acknowledgements.

I have been very fortunate to work with some highly talented Post-Doctoral Scientists/Junior Faculty (Allan Pacey, Linda Lefievre, David Hughes, Mark Kelly, Senga Oxenham, Alan Whitmarsh, Sean Brown, Jackson Kirkman-Brown, Paul Andrews, Lars Bjorndahl, Sarah Conner, Sarah Martins Da Silva) and PhD students (JJ Zhu, Neil Chapman, Lisa Thompson, Matt Tomlinson, Steven Mansell, Melanie Williams, Frosso Kessopoulou, Wardah Alasmari, Mojgan Naeeni, and Hannah Williams) who have contributed to the work in this thesis. Moreover I have had some outstanding collaborators such as Ian Cooke, Zaid Kilani, Masoud Afnan, Denny Sakkas and David Hornby. I am truly grateful for all their assistance. I am particularly grateful to my longtime collaborators and friends Christopher De Jonge and Steve Publicover.

My inspiration to follow a scientific career was a direct result of working with my PhD supervisor Dr Jack Cohen (University of Birmingham). His guidance was instrumental to me following an academic career and I fondly remember our frequent scientific exchanges.

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Contents.

Biography of Christopher Barratt.

Barratt graduated with an Honours degree in Zoology (Department of Zoology, University of Wales, Swansea), a Post Graduate Certificate in Education (University of Wales, Swansea) and then a PhD (Department of Zoology, University of Birmingham). Post-doctoral studies and Assisted Reproductive Technology (ART) experience was gained at the University of Sheffield (1985-1997) and, from 1997-2005 he was the Scientific Director of the ART Centre at the Birmingham Women's Hospital/University of Birmingham. Barratt has been awarded a number of honours for outstanding contributions to the discipline e.g. Young Andrologist of the Year (American Andrology Society), the Professor Sir Robert Edwards keynote lecture at European Society of Human Reproduction and Embryology (ESHRE).

He is a regular invited speaker at international scientific conferences/workshops and was a member of the 4th and 5th edition of the World Health Organisation (WHO) laboratory manual for the examination and processing of human semen (WHO 1999; 2010). He was Chair of the WHO Expert Synthesis Group on the Diagnosis of Male infertility (2012–2016) which devised a new system for the diagnosis and treatment of the infertile male and, was recently appointed to the editorial board of WHO for the development of the new Semen Analysis manual (6th edition, due 2020). For six years he was a Member of the Human Fertilisation and Embryology Authority (HFEA), the body which governs ART in the UK. During this time he chaired the Horizon Scanning committee, the Scientific and Clinical Advisory Group. He has been on several research committees e.g. member of MRC committee for quinquennial review (Edinburgh), DFG OFFSpring faculty. He has been on the Editorial Board of Human Reproduction, Biology of Reproduction, Human Reproduction Update and Journal of Andrology. He was Deputy Editor in Chief of Molecular Human Reproduction (2010-2012) and subsequently the Editor-in-Chief (2013-2018). Barratt has published over 230 papers, has

an *h* index of 50 and is currently Head of Division Systems Medicine and Director of Reproductive Medicine at the Medical School, University of Dundee, Scotland.

Background - Male infertility.

Infertility is a significant global problem affecting approximately 80 million couples worldwide and now, male factor infertility is accepted as a significant cause. In a landmark study by Hull and colleagues, sperm dysfunction (lacking ‘normal' function) was identified as the single most common cause of infertility (Hull *et al.*, 1985). Subsequent studies have confirmed these observations and highlighted dysfunctional cells in men with ‘normal' semen parameters and conversely normal sperm function in men with abnormal semen parameters. What is remarkable is that, for the overwhelming majority of sub fertile males, there is no drug a man can take or add to his spermatozoa *in vitro* to improve fertility. The only option is assisted reproductive technology (ART) which usually consists of a graduation of treatment depending on severity, i.e., intrauterine insemination for mild, *in vitro* fertilisation (IVF) for moderate and, since 1992 intracytoplasmic sperm injection (ICSI) for men with severe sperm dysfunction. A primary reason for the lack of progress in this area is our poor understanding of the production, formation and workings of a human spermatozoon. Work presented in this thesis illustrates potential progress in our understanding of sperm function and provides an analysis of the potential diagnostic/prognostic tools available in the discipline.

Synopsis of Selected Publications.

Section 1: Sperm transport in, and dynamic interaction with, the human reproductive tract.

Clinical experience of Intra Cytoplasmic Sperm Injection (ICSI) has shown that injection into the egg of even significantly abnormal sperm can result in successful fertilisation and live births. We can thus conclude that the extreme structural specialisation and unique signalling toolkit of the mature spermatozoon are required to enable the cell to reach the site of fertilisation and successfully interact with the egg vestments. A plethora of data shows that, in humans, this process is frequently dysfunctional, causing infertility, but this can be alleviated by placing the sperm close to egg (In vitro Fertilisation - IVF) or injecting into the egg - ICSI. Understanding how sperm progress through and interact with the female tract is therefore important. In many animals a dynamic and intricate synchronisation of events between the spermatozoon and female reproductive tract environment had been shown.

In Barratt *et al.*, (1990, Paper 1) I critically review the functional significance of white blood cells in the male and female reproductive tract including not only their role in modulating an immune response but in the selection of spermatozoa. The paper uses data from animals but focusses on what is known in humans, potential evidence gaps and outlines areas for further research. This is a key review (cited 160 times) and led to the Tomlinson, Williams and Thompson studies (Tomlinson *et al.*, 1992, 1993 [Paper 7], Thompson *et al.*, 1991, [1992 Paper 4] Williams *et al.*, 1993,b [Paper5]) clarifying the roles these cells play in fertilising ability.

In Barratt and Cooke (1991; Paper 2) I comprehensively and critically review the knowledge of sperm transport within the human female tract and, using the data available from animal species, suggest three key lines of experimentation (1) develop techniques to recover sperm from the female reproductive tract (2) produce an in-vitro model for assessing (or mimicking)

the modulation of sperm function by reproductive tract fluids (3) produce a realistic *in vitro* tissue/cell culture system to investigate and manipulate the interaction between the epithelium and sperm.

In animals, sperm are stored in the caudal isthmus – the so call sperm reservoir. Although there was no data in humans, in fact Jim Overstreet had suggested the cervix was the human sperm reservoir, the overwhelming assumption was that this was the case. Experiments examining sperm transport in the human female reproductive tract are very challenging ethically, logistically and technically such that the type and quantity of data that can be collected are restricted. Having previously established robust techniques for recovery of sperm from the human (Williams *et al.*, 1992), Williams *et al.*, 1993a (Paper 3) accurately determined the number and distribution (intramural, isthmic and ampulla) of sperm within the Fallopian tubes of normal proven fertile women around the time of ovulation. Within ethical limits, experiments were tightly controlled e.g. insemination was carried out after the onset of the LH surge ~ 18hr (17-22 hrs) prior to total abdominal hysterectomy. Spermatozoa were consistently recovered from the Fallopian tubes The primary impact of this paper was to show that cell numbers were relatively low (median of 1:25,000 motile sperm inseminated were recovered) and, in contrast to what was expected, there was no obvious oviductal sperm reservoir. Although this study is over 25 years old it remains the benchmark for information on sperm numbers in the human female reproductive tract (reviewed Sakkas *et al.*, 2015). We (my PhD students Lisa Thompson, Melanie Williams) performed a number of similar experiments recovering sperm from the cervix and uterus. For example Thompson *et al.*, (1992, Paper 4, cited 85 times) confirmed the presence of the physiological leucocytic cells reaction (LCR) to spermatozoa and determined that neutrophils were the primary leucocyte subset involved in this reaction. Moreover, Williams *et al.*, 1993b showed that the LCR extended to the uterus where few sperm could be recovered following insemination (Williams *et al.*, 1993b Paper 5).

The primary impact of these experiments (exemplified by Williams *et al.*, 1993a Paper 3) was to show that, when using appropriate controlled experiments (timing, techniques etc.), relatively few cells consistently enter the uterus and thus have access to the fallopian tubes. There is no reservoir in the uterus or oviduct. The presence of large numbers of leucocytes in the uterus may indicate, as in animals, a potential clearance mechanism but, perhaps surprisingly, we rarely observed active phagocytosis. Ideally, to provide insights into the process of sperm transport and selection, assessment of the functional ability of the recovered spermatozoa would be done. However, this was virtually impossible as numbers were low and technical developments at the time (early 1990's) were very limited e.g. no single cells imaging, patch clamping.

Section 2: Assessment and development of prognostic/diagnostic tools and cell biology of human sperm function.

Semen analysis and the diagnosis of male infertility.

In 1992/1993 we published three substantial studies providing high quality data on the clinical value of semen parameters which were subsequently used in formulating both the 4th (WHO 1999) and 5th (WHO 2010) edition of the WHO laboratory manual for the examination and processing of human semen.

(1) Barratt *et al.*, (1993, Paper 6). Despite widespread use, the predictive value of Computer Assisted Sperm Analysis (CASA) for determining *in vivo* conception of sub fertile couples had not been assessed. We performed a large (222 couples) comprehensive study with detailed quality control for CASA, minimised the influence of female factors and used appropriate statistical analysis (Cox's proportional hazards model). This study showed that the total number of

progressively motile cells and kinematics assessed by CASA were predictive of the time to conception and thus of clinical value. The sentinel marker of impact was inclusion of the data and arguments into WHO (1999) 4th edition.

(2) Tomlinson *et al.*, (1993, Paper 7). The traditional view was that leucocytes, even at physiological numbers (<10^6/ml semen) negatively influence fertility (Barratt *et al.*, 1990, Paper 1). Using a similar experimental design to Barratt *et al.*, (1993, Paper 6) and detailed monoclonal antibodies to identify each leucocyte sub set, this paper demonstrated that neither total number of leucocytes nor any leucocyte phenotypes were negatively associated with conception. In contrast, there was a negative association with immature germ cells in the semen samples which is a reflection of defective spermatogenesis (also previously seen in IVF outcomes by my laboratory - Tomlinson *et al.*, 1992). Tomlinson *et al.*, (1993, Paper 7) demonstrated that in sub fertile men without genital tract infection, normal (physiological) levels of white blood cells have no significant effect on conception. This study is quoted in the 4th and 5th editions of the WHO laboratory manual (WHO 1999; 2010).

(3) Barratt *et al.*, 1992 (Paper 8). The clinical significance of antisperm antibodies for fertility was controversial. This study showed that low (less than 10%) negative binding and moderate (less than 50%) binding had no significant effect on the probability of conception or the time to conception. The impact of the paper was illustrated in its use as the WHO reference value for antisperm antibodies for both WHO (1999) and WHO (2010).

Performing these studies, I was particularly exercised by the fundamental importance of using high quality clinical data to develop and support international guidelines (Barratt 1995a, Paper 9). In Barratt (1995a, Paper 9) I assessed how accurate tests are and emphasised two important issues:

Firstly, the need for comprehensive quality control procedures, training of technicians and effective quality assurance programs. In Clements *et al.,* (1995, Paper 10) we had detailed a comprehensive quality assurance programme. The importance of this was shown both by our ability to collect high quality and robust data for our clinical studies (detailed above) and also by the use of this proposal as part of the basis for establishing national and international training programs for technicians in Andrology. The first course for technicians, and the blueprint for the ESHRE training programs, was organised in my laboratory in Sheffield 1994 (Bjorndahl *et al.,* 2002). The 4th edition of the WHO manual emphasised the importance of quality control by significantly expanding the discussions on practical methods of implementing quality control and assurance in the andrology laboratory and specifically referenced the Clements *et al.,* (1995, Paper 10) study.

Secondly, I stressed the absolute need for clinical data with physiological relevance (Barratt 1995a, Paper 9). For example, in 1992 the WHO (WHO, 1992) had suggested new criteria for scoring sperm morphology but provided no biological data to support the changes. To examine this decision I designed a study (Barratt *et al.,* 1995b, Paper 11) comparing the clinical value of the new criteria to those previously established by the WHO (1987). Semen smears from 166 men attending our infertility clinic, whose fertility status was known [classified as fertile or infertile based on in vivo conception], were scored using both methods and logistic discriminant analysis for compositional data was used to assess the results. No difference between these two sets of criteria with respect to predicting pregnancy outcome was observed. The WHO (1999) modified its assessment of sperm morphology to include biologically relevant assessments (e.g. work by Roelf Menkveld see Menkveld *et al.,* 1990).

Ultimate diagnosis for the male – An over the counter home sperm test.

The development of an over-the-counter home sperm test had been a primary objective in andrology for a number of years. It would allow the patient to obtain an assessment of fertility potential at their convenience. However, any assay must be simple to use, robust, accurate and reliable. Moreover, it is essential to assess sperm motility as well as concentration. Bjorndahl *et al.,* (2006, Paper 12) described key challenges in developing the assay and summarized 7 years work (funded primarily by industry) where I went from generating the principle, to developing a product that was available on the shelves of pharmacists in both Europe and USA. The product was launched in 2006 named Fertell and men for the first time had the option to test their fertility in their own home. A potential new paradigm for sperm testing using a home kit as a screen was presented by Lefievre *et al.,* (2007 Paper 13). There are now several over the counter tests available (Yu *et al.,* 2018) and it will be interesting to see how home testing fits into the patient journey as either a replacement, triage or add on diagnostic test (Lefievre *et al.,* 2007, Paper 13).

A sperm zona pellucida (ZP) binding test and development of the 4 zona pellucida protein model.

A fundamental challenge in diagnostic andrology was the availability of a simple to use, robust predictive/diagnostic test of sperm function. In the 1980's and early 1990's a number of clinical studies showed that the human zona binding test was the most predictive test of sperm function yet the availability of human zona made it an impractical test. To address this I wanted to develop a bead type assay coated with recombinant zona proteins thus mimicking the zona. This would obviate the need for native zona and potentially be a diagnostic system that could be used in any laboratory. There were substantial challenges to achieving this. Firstly the

prevailing view of sperm-zona interaction was based on data from the mouse showing that sperm bind to ZP3 (via glycan residues), acrosome react (AR), bind ZP2 and subsequently penetrate the zona (ZP1 was considered to be purely structural). Moreover, there was little or no information on human sperm zona interaction to ascertain the roles of individual/combined zona proteins. As such, based on information from the mouse it was logical to produce glycosylated human ZP3 recombinant proteins. This was difficult and initial attempts in ours, and other laboratories, struggled to produce ZP3 in a purified form, without a tag, that also retained biological activity (bound to sperm and induced AR). At this time, my PhD student Neil Chapman (Chapman *et al.*, 1998, Paper 14) expressed ZP3 in *Escherichia coli* (non-glycosylated) and consistently observed binding to sperm and induction of the AR clearly demonstrating biological activity. Although controversial it was clear that the non-glycosylated protein was biologically active therefore, we used a commercially available in-vitro transcription and translation system to produce immobilized recombinant human ZP3 on agarose beads (Whitmarsh *et al.*, 1996, Paper 15). This system was chosen as it mimicked what embryology/andrology laboratory staff would encounter every day, allowed easy visualization and required no special expertise/equipment to use. Moreover it was a rapid system that allowed mutation of the protein(s) which could provide real insight into sperm zona interaction. Significantly higher levels of sperm binding and AR were observed indicating biological activity (Whitmarsh *et al.*, 1996, Paper 15). However, the work (and that of Chapman *et al.*, 1998, Paper 14) was very much against the dogma i.e. glycans were critical. Furthermore, I was concerned that, not only was the mouse a poor model for the human but expression of a singular zona protein (ZP3) as a human sperm function test, whilst instructive, was not tenable in the long term. To test the relevance of the mouse model, we examined the evolutionary structure of the zona and showed that humans have a distinct *ZP1* gene and thus the human has 4 ZP genes (Hughes and Barratt, 1999, Paper 16). This was a significant standalone finding,

however, the key question was - are all 4 genes expressed in the human ZP? Lefievre *et al.*, (2004, Paper 17) showed that mRNA for all 4 ZP genes is present in human oocytes and detailed proteomic analysis identified all 4 zona proteins. Moreover, this study showed that the mouse ZPB gene was probably nonfunctional. This paper was very controversial as it highlighted that a new model of sperm-zona interaction was needed and, there were fundamental difference between humans and mice. Lefievre *et al.*, (2004, Paper 17) is a seminal manuscript in the field (200 citations). Remarkably, it's now well accepted that the protein backbone is key for human sperm zona binding and Jurrien Dean and colleagues have recently published in *Science Translational Medicine* ZP peptide beads as a possible human sperm function test (Avella *et al.*, 2016).

Examination of sperm function and nature of defects using an IVF/ART system

To examine the relationship between sperm functional characteristics and fertilising potential we used a simple experimental system to correlate fertilising capacity of cells with sentinel markers e.g. stimulus-induced calcium responses in the cell. Fertilising capacity was assessed directly (outcome of IVF) rather than by using surrogate measures (such as AR, zona binding) and an aliquot of the same sperm population (same ejaculate used for IVF) was used to investigate sperm function, greatly reducing the effects of inter subject variation. Moreover, using this approach large numbers of patients can be screened and some mitigation of female factors (number of eggs, age, and donor eggs) achieved. In Alasmari *et al.*, (2013, Paper 18) we screened more than 200 patients identifying the prevalence of calcium store defects and, as these were negatively correlated to fertilization success, illustrate their biological significance. This paper also showed, as expected, a correlation between calcium responses to progesterone, assessment of hyperactivation and fertilizing success. Alasmari *et al.*, (2013, Paper 17) is the

primary reference for determining the clinical significance of calcium signaling pathways in human spermatozoa (50 citations). It had the highest number of downloads in *Human Reproduction* in 2013 and was therefore selected to be presented as the opening lecture (termed the Professor Sir Robert Edwards award) at the ESHRE 2014 Annual meeting. Although the study identified several men where there was a minimal calcium response to either calcium store mobilizing agent or progesterone who had fertilizing defects, some of these men had suboptimal semen (motility, concentration, morphology) and thus there may have been a number of abnormalities in the spermatozoon out with calcium signalling defects. To demonstrate that CatSper/Calcium stores were absolutely necessary for fertilization, comprehensive data on men with normal semen and no calcium response was required. Lishko had identified a CatSper2 deficiency that had no progesterone potentiated CatSper current but there was no information on calcium flux and the man had severe semen anomalies (Smith *et al.,* 2013). Thus, abnormalities in calcium influx were likely to be due to defective spermatogenesis and not an isolated ion channel defect. Williams *et al.,* (2015, Paper 19) identified a sentinel patient with normal semen (motility, concertation, morphology), no CatSper potentiated CatSper current, or calcium response to progesterone and, failure of progesterone stimulated penetration into viscous media (Williams *et al.,* 2015, Paper 19). This patient was the archetypal CatSper deficient man and, as his sperm responded to calcium store mobilizing agents, the abnormality was confined to CatSper. Critically his sperm did not fertilize at IVF demonstrating that functional CatSper is not necessary for maintenance of normal motility or baseline hyperactivation but is critical for regulation of penetration into viscous media (e.g. egg vestments, detaching from epithelial cells, penetrating zona) and fertilizing capacity. Moreover, it illustrates that CatSper interruption is a feasible target for contraception in humans. Although the paper was published in December 2015 it has been cited 25 times which is an indicator of its importance. Our subsequent genetic studies identified the

lesion in this patient as a Homozygous in-frame deletion in CATSPERE (Brown *et al.,* 2018) so we now understand the functional role of CatSperE.

Parallel experiments in my laboratory have examined the role of KSper (activity assessed using electrophysiology) and identified that abnormalities in KSper are associated with fertilizing potential (Brown *et al.,* 2016). A primary problem working with sperm specific ion channels specifically CatSper is that, even now, inhibitors have off target effects (see Rennhack *et al.,* 2018). Moreover, as there are fundamental differences between the human and the mouse fertilization process, so that studies using this model have limited relevance, identification and investigation of sentinel clinical cases is absolutely vital to understand sperm physiology in humans.

Collaborating with Zaid Kilani (Jordan) we examined the fertilizing capacity of men with globozoospermia (round-headed spermatozoa) (Kilani *et al.,* 2004, Paper 20, 68 citations). Globozoospermia is a well-known rare condition but not a single pathology, given that different men with the condition can show a wide spectrum of effects. In Kilani *et al.,* (2004, Paper 20) we examined the clinical and pathologic details of a unique family, in which the majority of the male siblings (5/7 brothers) had globozoospermia. In addition to making a significant contribution to describing the effects of this rare condition including showing that pregnancies can be achieved with repeated ICSI treatment cycles, this study was the basis for clarifying the genetic basis of globozoospermia. To achieve this, we collaborated with Stephane Viville, showing that the infertile brothers carried a homozygous deletion of 200 kb on chromosome 12 encompassing DPY19L2 (Koscinski *et al.,* 2011) (88 citations). Subsequent studies have confirmed that DPY19L2 deletion men can achieve fertilisation and pregnancy at ICSI and thus assessment of the genetic deletion provides both diagnostic and prognostic information.

Putative *in vivo* treatment of sub fertile men.

In Kessopoulou *et al.*, (1995, Paper 21) we performed a double blind randomized placebo cross over trial using vitamin E to treat reactive oxygen species associated male fertility. This is the only treatment study highlighted in my D.Sc. thesis but is included as it provides the first evidence for any significant improvement in sperm function (manifested as human sperm zona binding) following *in vivo* antioxidant treatment in a well-defined group of men. It is highly referenced (200 citations) and a key study in the justification of the rapidly growing industry of antioxidant treatment of sub fertile men.

Section 3: Scientific leadership in the discipline reflecting substantial and authoritative contributions.

An example of an international position paper.

I have been involved in and led a number of international meetings/consensus workshops on male reproductive health. Barratt et al. (2010, Paper 22) provides an example where I have produced, with colleagues, a comprehensive, timely and highly influential consensus paper (175 citations). This ESHRE position report details the state of play at the time of sperm DNA – basic science and clinical significance. As well as reviewing progress, clear pathways for future research were delineated.

Development of WHO recommendations for diagnosis of male infertility.

In addition to work on the editorial board of the WHO laboratory semen analysis manuals (4th, 5 th and 6th editions) I was Chair of the Expert Synthesis Group (ESG) for the WHO Diagnosis of Male infertility (2012-2016) charged with developing global guidelines for the diagnosis of

male infertility. Barratt et al. (2017, Paper 23) is a very substantive manuscript which reviews the evidence synthesized by the ESG that helped to generate the WHO recommendations and, importantly, defines a number of key challenges and research opportunities. Although published very recently (December 2017) the impact of this analysis is reflected by the high number of citations to date (18).

A new strategy for male reproductive health? A Male Reproductive Health Ecosystem'

While formulating the data for the new WHO guidelines the paucity of high quality data on which to base recommendations was very clear. Simple questions did not have sufficient data to formulate 'low' let alone 'strong' recommendations (Barratt *et al.* 2017, Paper 23). In an attempt to address this, together with Richard Sharpe and Christopher De Jonge we advocate formulation of a detailed roadmap for male reproductive health to facilitate development of a research agenda and deliver effective funding vehicles. This vision we term 'a Male Reproductive Health Ecosystem' (Barratt *et al.*, 2018 Paper 24). The objective is to develop a global network to transform male reproductive health.

De Jonge and Barratt - The Sperm Cell (Editors, 2017).

I have co-written two books and edited 6. De Jonge and Barratt - The Sperm Cell (Editors, 2017 paper 25) is the second edition of an edited book by Christopher De Jonge and myself. We developed the ideas, strategy, selected authors/subjects. Cambridge University Press judged the first edition (De Jonge and Barratt 2006) to be very successful and commissioned a second updated and fully revised version. The book, composed of 18 chapters, is a comprehensive and definitive account of the human male gamete and is primarily designed for

postgraduate students and those starting a clinical/basic science research career. It is a key textbook in the discipline reflecting an authoritative contribution to knowledge.

Conclusions.

The selected publications provide evidence of significant contribution to address key aspects in the investigation of male infertility. One key theme has been continually challenging the predictive/diagnostic potential of a basic semen analysis in the context of *in vivo* conception. An increase in our understanding has been used to potentially develop new diagnostic tests for use at home and in the laboratory. However, unfortunately, there remain dramatic gaps in our knowledgebase and, on reflection of nearly 30 years work, overall progress has been slow. None more so than our understanding of how a spermatozoon successfully negotiates the female reproductive tract to make its way to the egg. Perhaps armed with new technologies, and a new appreciation of the role of the male in the reproductive process, we can start to unravel these mysteries and develop more accurate and easy to use tools for diagnosis and appropriate rational therapy (Barratt *et al.*, 2018 Paper 24).

Selected Publications placed in order of presentation.

Paper 1: Barratt CL, Bolton AE, Cooke ID. Functional significance of white blood cells in the male and female reproductive tract. *Hum Reprod.* 1990 **5**:639-48.

Paper 2: Barratt CL, Cooke ID. Sperm transport in the human female reproductive tract--a dynamic interaction. *Int J Androl.* 1991 **14**:394-411.

Paper 3: Williams M, Hill CJ, Scudamore I, Dunphy B, Cooke ID, Barratt CL. Sperm numbers and distribution within the human fallopian tube around ovulation. *Hum Reprod.* 1993a **8**:2019-26.

Paper 4: Thompson LA, Barratt CL, Bolton AE, Cooke ID. The leukocytic reaction of the human uterine cervix. Am *J Reprod Immunol*. 1992 **28**:85-9.

Paper 5: Williams M, Thompson LA, Li TC, Mackenna A, Barratt CL, Cooke ID. Uterine flushing: a method to recover spermatozoa and leukocytes. *Hum Reprod*. 1993b **8**:925-8.

Paper 6: Barratt CL, Tomlinson MJ, Cooke ID. Prognostic significance of computerized motility analysis for in vivo fertility. *Fertil Steril.* 1993 **60**:520-5.

Paper 7: Tomlinson MJ, Barratt CL, Cooke ID. Prospective study of leukocytes and leukocyte subpopulations in semen suggests they are not a cause of male infertility. *Fertil Steril.* 1993 **60**:1069-75.

Paper 8: Barratt CL, Dunphy BC, McLeod I, Cooke ID. The poor prognostic value of low to moderate levels of sperm surface-bound antibodies. *Hum Reprod*. 1992 **7**:95-8.

Paper 9: Barratt CL. On the accuracy and clinical value of semen laboratory tests. *Hum Reprod*. 1995 **10**:250-2.

Paper 10: Clements S, Cooke ID, Barratt CL. Implementing comprehensive quality control in the andrology laboratory. *Hum Reprod.* 1995 **10**:2096-106.

Paper 11: Barratt CL, Naeeni M, Clements S, Cooke ID. Clinical value of sperm morphology for in-vivo fertility: comparison between World Health Organization criteria of 1987 and 1992. Hum Reprod. 1995 **10**:587-93.

Paper 12: Björndahl L, Kirkman-Brown J, Hart G, Rattle S, Barratt CL. Development of a novel home sperm test. *Hum Reprod*. 2006 **21**:145-9.

Paper 13: Lefièvre L, Bedu-Addo K, Conner SJ, Machado-Oliveira GS, Chen Y, Kirkman-Brown JC, Afnan MA, Publicover SJ, Ford WC, Barratt CL. Counting sperm does not add up any more: time for a new equation? *Reproduction*. 2007 **133**:675-84.

Paper 14: Chapman N, Kessopoulou E, Andrews P, Hornby D, Barratt C. The polypeptide backbone of recombinant human zona pellucida glycoprotein-3 initiates acrosomal exocytosis in human spermatozoa in vitro. *Biochem J*. 1998 **330**:839-45.

Paper 15: Whitmarsh AJ, Woolnough MJ, Moore HD, Hornby DP, Barratt CL. Biological activity of recombinant human ZP3 produced in vitro: potential for a sperm function test. *Mol Hum Reprod.* 1996 **2**:911-9.

Paper 16: Hughes DC, Barratt CL. Identification of the true human orthologue of the mouse Zp1 gene: evidence for greater complexity in the mammalian zona pellucida? *Biochim Biophys Acta*. 1999 **1447**:303-6.

Paper 17: Lefièvre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, Lenton W, Afnan M, Brewis IA, Monk M, Hughes DC, Barratt CL. Four zona pellucida glycoproteins are expressed in the human. *Hum Reprod*. 2004 **19**:1580-6.

Paper 18: Alasmari W, Barratt CL, Publicover SJ, Whalley KM, Foster E, Kay V, Martins da Silva S, Oxenham SK. The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation. *Hum Reprod*. 2013 **28**:866-76.

Paper 19: Williams HL, Mansell S, Alasmari W, Brown SG, Wilson SM, Sutton KA, Miller MR, Lishko PV, Barratt CL, Publicover SJ, Martins da Silva S. Specific loss of CatSper function is sufficient to compromise fertilizing capacity of human spermatozoa. *Hum Reprod.* 2015 **30**:2737-46.

Paper 20: Kilani Z, Ismail R, Ghunaim S, Mohamed H, Hughes D, Brewis I, Barratt CL. Evaluation and treatment of familial globozoospermia in five brothers. *Fertil Steril*. 2004 **82**:1436-9

Paper 21: Kessopoulou E, Powers HJ, Sharma KK, Pearson MJ, Russell JM, Cooke ID, Barratt CL. A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. *Fertil Steril.* 1995 **64**:825-31.

Paper 22: Barratt CL, Aitken RJ, Björndahl L, Carrell DT, de Boer P, Kvist U, Lewis SE, Perreault SD, Perry MJ, Ramos L, Robaire B, Ward S, Zini A. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications--a position report. *Hum Reprod.* 2010 **25**:824-38.

Paper 23: Barratt CLR, Björndahl L, De Jonge CJ, Lamb DJ, Osorio Martini F, McLachlan R, Oates RD, van der Poel S, St John B, Sigman M, Sokol R, Tournaye H. The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities. *Hum Reprod Update*. 2017 **23**:660-680.

Paper 24: Barratt CLR, De Jonge CJ, Sharpe RM. 'Man Up': the importance and strategy for placing male reproductive health centre stage in the political and research agenda. *Hum Reprod.* 2018 **33**:541-545.

Paper 25: De Jonge C and Barratt CLR (2017) The Sperm Cell 2nd Edition. Cambridge University Press. Pp304. ISBN: 9781107126329

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Citations.

The citations and *h* index refer to a SCOPUS search on February 1st 2019. The selected publications presented in evidence in this thesis are published in journals in the top quartile of

their respective category, more often than not in the #1 journal. Citation benchmarking using SCOPUS shows that several of the primary data papers used in the selected publications are in the 98th centile globally in their field.

Declaration of contributions to the papers presented in evidence.

The selected publications are those where I have provided ≥75% of the intellectual contribution e.g. to study design, analysis of data, writing the manuscript, obtaining grant funding. Experiments were either done by one (or several) of my post-doctoral workers and/or one (or several) of my PhD students (where I was the primary supervisor)

I believe there are five exceptions to this, four, where necessarily there are other intellectual contributions such as with consensus/guidelines papers namely:

1. Barratt *et al.,* (2017) where my contribution was ~50%. Several of the PICO analyses were done by members of the EWG group. In this assessment I played a substantial and critical role. I performed detailed analysis of literature on smoking, temperature, and prevalence of male infertility, was a key driving force of the project (I was Chair), wrote the first draft of the paper and made presentations of the recommendations to WHO. As a reflection of my significant contribution I am senior and corresponding author.
2. Barratt *et al*. 2010 where we developed an ESHRE position paper on sperm DNA. In this paper I chaired the meetings, coordinated and wrote a significant degree of the first draft of the paper. As a reflection of my contribution I am senior and corresponding author.
3. Barratt, De Jonge and Sharpe (2018) where the contributions were equal.
4. In De Jonge and Barratt (2007) where the contributions were equal.

In Hughes and Barratt (1999) the contribution was 60:40 respectively.

Selected papers – in full - placed in order of presentation.

Paper 1:

Barratt CL, Bolton AE, Cooke ID. Functional significance of white blood cells in the male and female reproductive tract. *Hum Reprod.* 1990 **5**:639-48.

Human Reproduction vol.5 no.6 pp.639–648, 1990

MINI REVIEW

Functional significance of white blood cells in the male and female reproductive tract

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The functional significance of white blood cells in the modulation of an anti-sperm antibody response, prevention of infection (including HIV) sperm transport/storage and sperm function is extensively discussed. A critical review of the existing literature is presented with future experimental lines of investigation outlined. A lack of controlled clinical studies in the human to validate data from animal species – for example – the involvement of white blood cells in the transport and storage of sperm in the female tract and the possible adverse effect of pathology (i.e. endometriosis) on these functions are presented. In conclusion, with the advent of modern techniques, e.g. monoclonal antibodies and sophisticated sperm function tests, many of the questions raised should be answered in the near future.

Key words: anti-sperm antibodies/cytokines/leukocytes/spermatozoa/transport

Introduction

There is now considerable evidence that leukocytes and their products have significant effects on the functional capacity of spermatozoa in the male and female reproductive tracts (Anderson and Hill, 1988; Witkin, 1988a; Mahi-Brown *et al.*, 1988; Barratt *et al.*, 1990a); As a result, the traditional view of the spermatozoa being the only important cells in semen is changing and more studies are now concentrating on the interactions between spermatozoa and other cells in semen (nucleated cells other than spermatozoa, NCOS; or non sperm cells, NSC). Leukocytes have been shown to modulate an autoimmune response in the male (and possibly the female), to have a deleterious effect on sperm motility and fertilizing capacity *in vitro* and to compromise sperm transport and storage in the female tract.

Our knowledge of the role of these cells in successful reproduction is being enhanced as major advances in biological techniques are available, particularly monoclonal antibodies enabling the accurate detection of these cells and an assessment of their level of activation, the availability of cytokines enabling precise study of their effects on spermatozoa and the development of increasingly sophisticated laboratory tests of sperm function (e.g. quantitative analysis of sperm motility, in-vitro fertilization). With such techniques now available it is possible to further determine the significance of leukocytes in reproduction.

However, the interpretation of studies using monoclonal antibodies is critically dependent on the specificity of the antibodies used and also the uniqueness of expression of surface markers on leukocytes of particular types. In the case of cytokine studies, it is becoming increasingly apparent that target cells react to combinations of cytokines and observing the effect of exposure to sperm, for example, of a single such mediator *in vitro* is probably an over-simplistic experimental approach.

The aim of this review is to critically analyse the evidence for the functional significance of leukocytes in the reproductive process. A precis of the literature will be presented and our current knowledge used to speculate on future lines of research to enhance understanding. With this aim, the review will concentrate on studies involving human subjects although some data from animal studies will be used where necessary.

Distribution of cells in the male reproductive tract

With the application of monoclonal antibodies it is possible to study the distribution of leukocytes in complex tissues, e.g. the testicular interstitium and the epididymis. Such studies have shown the distribution of leukocytes to be compartmentalized throughout the normal genital tract in humans (Ritchie *et al.*, 1984; El Demiry *et al.*, 1987; El Demiry and James, 1988), mice (Nashan *et al.*, 1989) and rats (Miller *et al.*, 1984; Niemi *et al.*, 1986). In the testes of normal fertile men, large numbers of macrophages were detected between the seminiferous tubules in close association with the outer aspects of the tubule wall and around the blood vessels in the interstitium (El Demiry *et al.*, 1987; El Demiry and James, 1988), a finding similar to those in rats where macrophages make up 22–28% of the cells in the testis (Niemi *et al.*, 1986). In the rete testis of man these cells are located mainly in the connective tissue. The majority of macrophages expressed HLA DR antigens (MHC class II) indicating their capacity to initiate an immune response, although it remains to be established if these cells are phagocytic and/or antigen-presenting cells (Roitt, 1988).

El Demiry and coworkers (El Demiry *et al.*, 1987; El Demiry and James, 1988) were unable to detect any lymphocytes in peripheral testicular tissue; however, lymphocytes were present throughout the rete testis located within the lining of the epithelium of the tubules. Suppressor/cytotoxic T cells (CD 8+) were more abundance in the lining of the epithelium while helper/inducer cells (CD 4+) predominated in connective tissue.

They also examined testicular biopsies from selected infertile men. Interestingly, lymphocytes were detected in all men and suppressor/cytotoxic T cells predominated over helper/inducer cells in men with oligozoospermia and obstructive azoospermia. In post-vasectomy patients and those with unilateral testicular obstruction the helper/inducer T cell subset predominated. In most individuals, a predominance of T helper/inducer cells was associated with serum anti-sperm antibodies. However, there was a minority with anti-sperm antibodies and a predominance of T suppressor/cytotoxic cells (El Demiry *et al.*, 1987; El Demiry and James, 1988). In order to obtain a more comprehensive picture these initial results must be extended to include more patients from normal and selected groups of infertile men.

Selected samples of the epididymis and vas deferens of fertile men have also been examined using monoclonal antibodies (El Demiry and James, 1988). T lymphocytes were always seen in the epididymis and vas; again there appeared strict compartmentalization between T cell subsets with suppressor/cytotoxic types being found in the lamina propria and helper/inducer T cells in the interstitial tissue. There was always a predominance of suppressor/cytotoxic cells to helper/inducer cells and none of these cells expressed the interleukin 2 receptor, therefore they were not activated. Monocytes/macrophages were abundant in the epithelium of the vas and epididymis. These studies need to be extended with the additional use of leukocyte activation markers in, for example, men who have a vasovasostomy and anti-sperm antibodies.

In the epididymis of normal fertile mice there also appears a strict compartmentalization of immune cells (Nashan *et al.*, 1989). There is a similar distribution of monocytes/macrophages and T cells between the caput, corpus and cauda epididymis, with macrophages being the most frequently observed cells. T cells were located in the interstitium with a similar number of helper/inducer and suppressor/cytotoxic cells (Nashan *et al.*, 1989).

The main difficulty in studying the distribution of immune cells in the human male tract is the availability of tissue. As initial results from the mouse model show strict compartmentalization of the leukocyte subsets similar to that in humans, the mouse could be an important experimental model for initial studies of, for example, the immunological response to vasectomy, which in many aspects is similar in mouse and man (Barratt and Cohen, 1986, 1987, 1988).

In the human vas deferens there is a special population of epithelial cells in the ampullary region whose primary function is the phagocytosis of spermatozoa (Riva *et al.*, 1981; Murakami *et al.*, 1988). The significance of this mechanism of sperm resorption is unknown but many basic questions need to be answered; for example, how many spermatozoa are resorbed; are only damaged spermatozoa resorbed; is the resorption dependent on sperm numbers in the vas—as in bulls (Amann and Almquist, 1962) and rats (Taylor *et al.*, 1985)? It is interesting to note that a similar population of cells has been documented in monkeys (Cooper and Hamilton, 1977; Murakami *et al.*, 1982) and cats (Murakami *et al.*, 1982). These clear observations of sperm disposal together with the known discrepancy between the number of spermatozoa produced by the testis and the number ejaculated indicate significant resorption of spermatozoa in the normal human male reproductive tract (see Barratt *et al.*, 1988, 1990a). It is therefore surprising that as spermatozoa are antigenic and sperm resorption in the reproductive tract of males is a consistent feature, most males do not make anti-sperm antibodies (Alexander and Anderson, 1987; Barratt and Cooke, 1988). The mechanisms which limit this antibody response are poorly understood. The sequestration of antigens by the blood–testis barrier is undoubtedly important but sequestration of antigens by the blood epididymal barrier is not complete (see reviews, Mahi-Brown *et al.*, 1988; Witkin, 1988a; Lehmann and Emmons, 1989). There is now overwhelming experimental evidence of immunoregulation both systemically and locally in the male tract, where the testicular and epididymal leukocytes play an important role (see above reviews). The mechanisms of this immunoregulation and the precise role of the leukocytes remain to be fully elucidated. It is proposed that the macrophages of the testis and epididymis may restrict antigen exposure by phagocytosis of spermatozoa with the consequent rapid loss of antigenicity (Ball *et al.*, 1984); however, macrophages are typically antigen-presenting cells. It would be interesting to label these macrophages for expression of HLA and sperm antigens to examine if they presented antigen. Rat testis, for example, contains large numbers of MHC class II positive macrophages which possibly have an antigen-presenting function (Pollanen and Maddocks, 1988).

Of particular interest is the proposed role of the T cells in immunoregulation. Ritchie and colleagues (1984) suggest that the predominance of suppressor/cytotoxic cells in the epithelium of the epididymis acts to suppress an immune response. Results from El Demiry and colleagues (1987) support this suggestion and show an alteration in the distribution of the T cell subsets in some men with anti-sperm antibodies where there are more helper/inducer T cells (see above).

Seminal white blood cells and their effects on spermatozoa

Several previous reports using light microscopic studies have documented a tenuous association between male subfertility and high levels of leukocytes in semen (see Auroux, 1984). The validity of these data is uncertain as the techniques used to distinguish the leukocytes from other seminal cells, e.g. germinal cells, and between the various leukocyte subsets are unreliable (Barratt *et al.*, 1988; Kessopoulou *et al.*, 1990).

Using transmission electron microscopy (TEM), a technique which enables a more detailed appraisal of the cells in semen, Hughes and colleagues (1981) clearly demonstrated a heterogeneous population of 'other cells' in the semen of men attending an infertility clinic. They specifically recruited 124 men with a high number of 'round' cells. They were able to classify the men into three groups according to the cell contents of the semen: group 1 contained mainly germinal cells; group 2 'significant' numbers of polymorphonuclear leukocytes, 60% of which contained spermatozoa or sperm fragments and 30% bacteria; in group 3 mainly macrophages were observed which also contained spermatozoa or sperm fragments. Hughes and

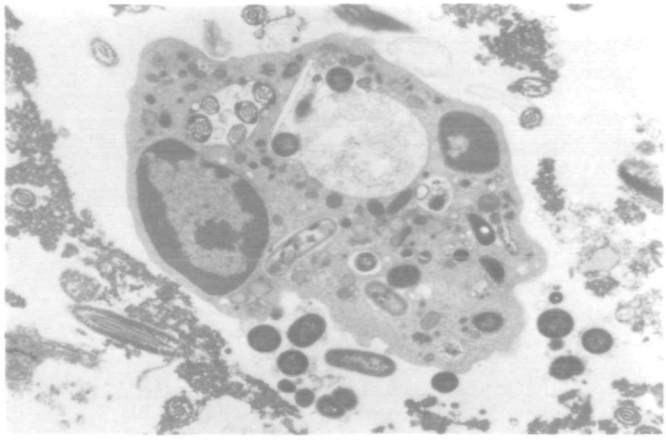

Fig. 1. Phagocytic cell in the semen of a fertile man. Transmission electron micrograph × 9000 magnification.

colleagues (1981) suggested that the primary role of polymorphonuclear leukocytes and macrophages was phagocytosis of spermatozoa or sperm fragments. Similar results have been obtained in a semiquantitative TEM study on the semen of 20 fertile men (Smith *et al.*, 1989). Leukocytes were detected in every semen sample and the variation between individual men was marked. The neutrophils were the predominant leukocytic cell type, accounting for 12% of the non sperm cell population with macrophages at ~1%; lymphocytes were only occasionally observed. As many of the neutrophils and macrophages contained spermatozoa or sperm fragments (Figure 1) the main function of these cells is phagocytosis. Whether these phagocytes engulf normal fertile spermatozoa, degenerate spermatozoa, functionally defective spermatozoa, and/or spermatozoa expressing a genetic imbalance (Cohen, 1984) and how many spermatozoa are disposed of by this mechanism are all simple biological questions which remain unanswered. In fact, the same questions which encompass sperm selection in the male tract equally apply to the female tract where the main fate of spermatozoa is quoted 'to be phagocytozed' (Austin, 1975).

Although such TEM studies provide accurate details of the contents of cells and provide qualitative data, the technique cannot be performed on a routine basis in many laboratories; quantitative data are difficult to obtain and identification of, for example, T cell subsets is impossible.

With the use of monoclonal antibodies to characterize the leukocytes in semen, it is now possible to identify and quantify these cells accurately on a routine basis (Figure 2). However, the two published studies to date using practically the same monoclonals have produced contrasting results. El Demiry *et al.* (1986a,b) examined the seminal leukocyte subsets from 12 fertile men and 65 men attending an infertility clinic. The semen of fertile men contained significantly more leukocytes than that of the infertile men. No T lymphocytes were detected in the fertile men and were only present in 13 out of 63 infertile men, where the predominant subset was the suppressor/cytotoxic phenotype. In contrast, Wolff and Anderson (1988a) reported significantly more leukocytes in the semen of infertile men. Suppressor/cytotoxic and helper/inducer T cells were detected in 71 and 77% of infertile men and 53 and 71% of fertile men. In both studies, granulocytes were reported to be the predominant leukocytes although in each case, identification was made difficult by non-specific staining.

The marked contrast between these two studies is probably due to three reasons. Firstly, no details were presented of the antibody status of these men [El Demiry *et al.* (1986a,b) only reported testing 19 out of 81 men, Wolff and Anderson (1988a) listed none] and the antibody status is known to affect the leukocyte numbers and subsets (Witkin and Goldstein, 1988; Barratt *et al.*, 1990a). Secondly, no information was presented about the presence or absence of genital infection, which also influences the number and sub-types of leukocytes (Wolff and Anderson, 1988b), and thirdly, the classification of men as fertile or infertile undoubtedly requires refining, e.g. between 10 and 50% of couples attending an infertility clinic can achieve a pregnancy, regarding men attending an infertility clinic as infertile will undoubtedly bias the results and make comparisons between studies difficult (Barratt *et al.*, 1990b).

Using monoclonal antibodies, Wolff *et al.* (1990) provided the first evidence of a significant relationship between elevated leukocyte concentrations ($>1 \times 10^6$/ml), their sub-types and a reduction in total number of spermatozoa, percentage motility, sperm velocity and total number of motile spermatozoa. Semen samples from 179 men attending an infertility clinic were analysed. Men with a high concentration of monocytes/macrophages ($>5 \times 10^5$/ml) had a lower volume, men with a high number of T cells ($>10^5$/ml) a lower sperm velocity, and men with a high granulocyte elastase concentration (indirect evidence for a high number of granulocytes, >1000 ng/ml, see Wolff and Anderson, 1988c) had a lower ejaculate volume, total sperm number and total motile sperm number. They concluded that leukocytospermia may occur in male infertility patients and that high levels of white blood cells in semen are associated with poor semen quality.

The effect of the soluble products of leukocytes on sperm motility has been preliminarily investigated *in vitro* using purified cytokines (Hill *et al.*, 1987). Interferon-γ (IFN) and tumour necrosis factor (TNF) had significant anti-motility effects at very high concentrations ($100\times - 1000\times$ those which are cytotoxic to trophoblast cells; Berkowitz *et al.*, 1988) so the relevance to the physiological situation remains uncertain. To critically assess the influence of cytokines on spermatozoa, the concentrations in semen and the male tract need to be determined, although to date the presence of proteases in semen has made accurate determination of these cytokines difficult (Anderson and Hill, 1988). Once these concentrations have been established, their functional effects can be elucidated by using in-vitro assays of sperm function. Interestingly, the concentration of TNF in the peritoneal fluid of women with mild endometriosis has been determined. High concentrations of TNF (800 U TNFα/ml) significantly reduced progressive motility and total motility after 4 and 21 h incubation *in vitro* (Eiserman *et al.*, 1989). However, when performing such studies, two important points need to be taken into account. First, cytokines are local effector substances and it is the concentration of these molecules in the immediate locality of the cells producing them that is of importance.

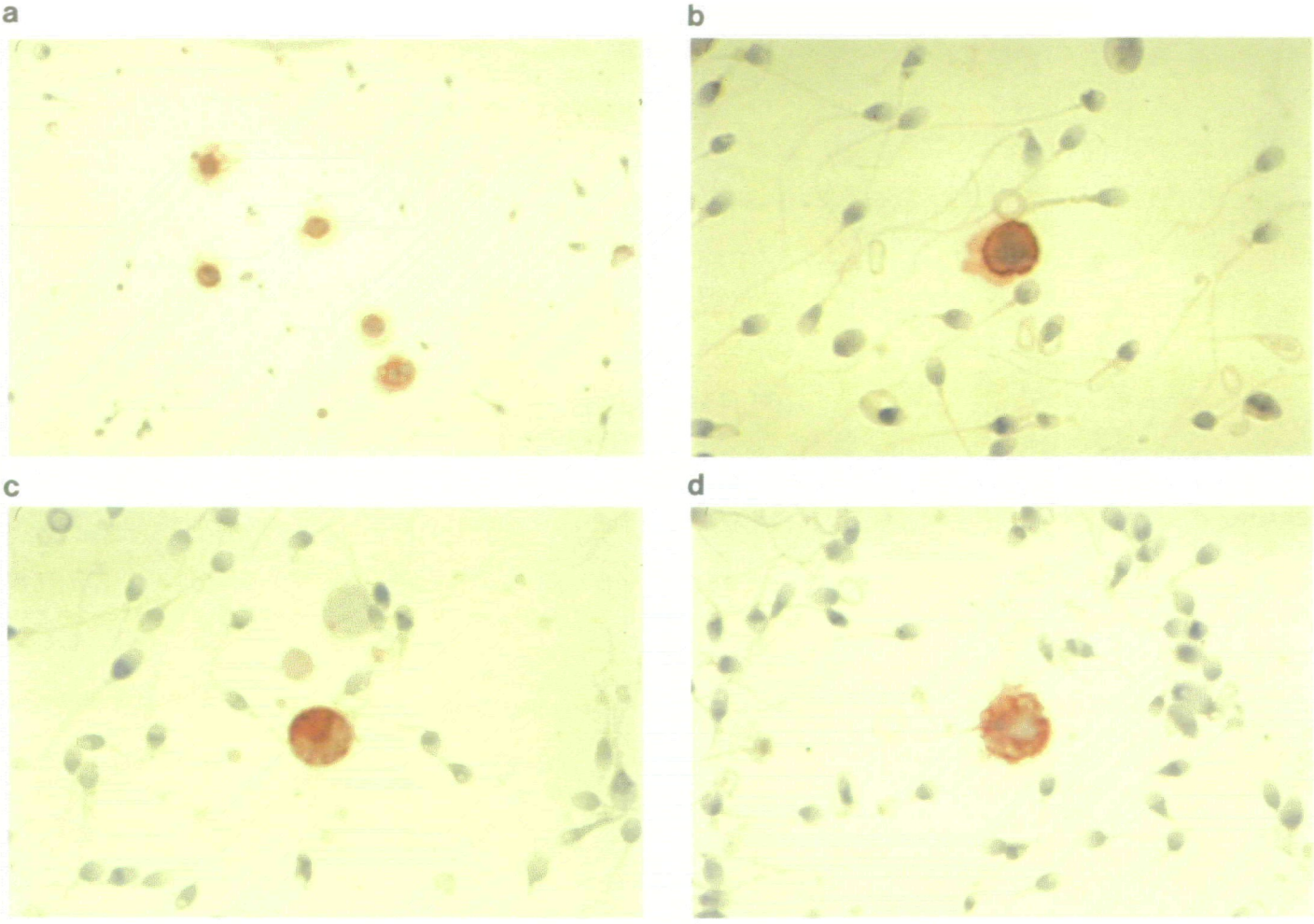

Fig. 2. Alkaline phosphatase—anti-alkaline phosphatase (APAAP) immunocytochemical staining of white blood cells in the semen of a fertile man stained using monoclonal antibodies. (**a**) Pan leukocyte marker × 400 magnification. (**b**) T lymphocyte × 1000 magnification. (**c**) Macrophage × 1000 magnification. (**d**) Neutrophil × 1000 magnification exhibiting sperm phagocytosis.

Second, possible interactions between various cytokines should be investigated as *in vivo* these products rarely act in isolation (Roitt, 1988).

These important in-vitro observations need to be expanded and are likely to provide mechanisms by which white blood cells can affect sperm function, possibly through changes in the sperm plasma membrane with implications for sperm motility and fertilizing capacity. Using the hamster oocyte penetration test as a test of sperm function, IFN and TNF over a wide concentration range significantly reduced the ability of human spermatozoa to penetrate hamster eggs (Hill *et al.*, 1989). This effect on penetration was independent of sperm motility although an effect on oocyte toxicity could not have been ruled out. These observations are concordant with other authors who documented a significant inverse relationship between the presence of white blood cells and hence possibly elevated local cytokine concentrations and fertilization of the human egg *in vitro* (Van der Ven *et al.*, 1987).

It is interesting to note that in the above studies, some men with a high number of leukocytes had normal semen parameters and fertilizing capacity. Undoubtedly the heterogeneous aetiology of the leukocytes is important, e.g. infection, inflammation, autoimmune. Whether these cells in semen originate from the reproductive tract (testis, epididymis, vas) and/or the accessory glands will be important. If the leukocytes originate from the tract they are likely to have been in contact with the spermatozoa for a long period of time (possibly days) and presumably a deleterious effect on spermatozoa would be more likely. To date, the origin of these cells is uncertain although clearly in some non-inflammatory situations, the cells originate mainly from the reproductive tract (Anderson and Hill, 1988). An examination of the leukocyte profile in the semen of men prior to and then subsequently post-vasectomy would determine the contribution from the testis or epididymis and vas to the seminal leukocytes.

The leukocyte profile in men with anti-sperm antibodies is complex and controversial. Witkin and Goldstein (1988) specifically compared the T lymphocyte population between men with anti-sperm antibodies post-vasovasostomy and 'fertile' men.

In vasovasostomized men, the levels of T suppressor/cytotoxic cells were significantly reduced and the helper/inducer subset predominated. The reduction in suppressor/cytotoxic cells was suggested as a major factor in the genesis of anti-sperm

Table I. White blood cells ($\times 10^6$/ml) in the semen of men without anti-sperm antibodies (controls and vasovasostomized men −ve) and with anti-sperm antibodies (vasovasostomized +ve and idiopathic)

Patient group	*n*	All leukocytes	Suppressor/cytotoxic T cells	Helper/inducer T cells	Monocyte/macrophage
Control	17	0.8	0.17	0.16	0.31
		(0.5−16.06)	(ND − 2.56)	(ND−1.63)	(ND−9.33)
Vasovasostomy −ve	4	1 44	0.31	0.21	0.69
		(0.83−3.86)	(0.10−0.61)	(0.05−0.27)	(0.39−0.91)
Vasovasostomy +ve	12	1.80	0.08	0.32	0.32
		(0.34−9.87)	(ND−1.87)	(ND−6.84)	(ND−3.21)
Idiopathic	11	1.48	0 32	0.53	0.48
		(0.48−8.03)	(ND−4.01)	(ND−3.10)	(ND−1.76)

ND, none detected.
+ve, anti-sperm antibodies on spermatozoa or in seminal plasma detected by immunobead test ≥10% binding = positive.
−ve, no anti-sperm antibodies on spermatozoa or seminal plasma.
Idiopathic, men with anti-sperm antibodies but with no known cause—no previous genito-urinary tract infection or vasectomy.
Control, semen donors free from infection, no anti-sperm antibodies and proven fertile.

Table II. White blood cells (x 10^6/ml) in the semen of men with and without urethral tract infection (median and ranges)

	All leukocytes	T suppressor/cytotoxic T cells	Helper/inducer T cells	Monocytes/macrophages	B cells
Fertile semen donors	0.8	0.17	0 16	0.31	0.08
	(0.5−16.06)	(ND−2.56)	(ND−1.63)	(ND−9.53)	(ND−1.02)
Active urethral infection	2 07	0.15	0.06	0.56	ND
	(0.16−29.89)	(ND+3.6)	(ND−5.33)	(0.09−21.52)	(ND−0.66)

ND, none detected.

antibodies. This is concordant with the observations of El Demiry and colleagues (1987) in the human tract (see above). However, an alteration in the predominant subset of T cells does not occur in all men with anti-sperm antibodies, a predominance of suppressor/cytotoxic T cells in the ejaculate can be associated with anti-sperm antibodies either post-vasovasostomy or in men with no known cause of antibody formation (Barratt *et al.*, 1990a; see Table I).

Interestingly, there appear to be notably more leukocytes in the semen of men with anti-sperm antibodies (El Demiry *et al.*, 1986a,b; Barratt *et al.*, 1990a) although the cause for this is unknown. An increase in the phagocytic activity of the leukocytes in men with anti-sperm antibodies may be expected as many of the spermatozoa would be opsonized by antibody. Previous studies have indicated an increase in phagocytosis of antibody-coated spermatozoa by peripheral blood leukocytes (London *et al.*, 1984, 1985).

It is now apparent that anti-sperm antibodies may have a multitude of effects on spermatozoa, not only on sperm transport through the female tract but also on sperm function (Alexander and Anderson, 1987). Witkin and Chaudhry (1989) reported significantly higher serum IFN levels in women with anti-sperm antibodies. As IFN, at high concentrations, adversely affects sperm motility (Hill *et al.*, 1987), and men with anti-sperm antibodies have lower sperm motility (Barratt *et al.*, 1989a) the mechanism of the effect of anti-sperm antibodies on sperm motility may be via the action of IFN. This hypothesis needs to be specifically tested.

Many other products of white blood cells may be deleterious to spermatozoa, for example, high levels of reactive oxygen species which can be associated with significant leukocyte infiltration (Aitken *et al.*, 1989). Aitken and colleagues (1989) demonstrated a significant relationship between the generation of reactive oxygen species by spermatozoa and defective sperm function in men with oligozoospermia. It is very important in such studies to determine the role of the granulocytes as these cells are copious producers of free oxygen radicals (Aitken *et al.*, 1989). Bearing in mind the observations of Wolff and colleagues (1990) that men with high numbers of leukocytes have a significant reduction in sperm numbers, this is a very important distinction which remains to be specifically addressed.

Leukocytes in semen and genital tract infection

Traditionally, an increase in or the presence of leukocytes in semen has been taken as one of the main clinical signs of genital tract infection. However, the relationship between the number of leukocytes and a genital tract infection is controversial (Barratt *et al.*, 1988). Comhaire *et al.* (1980) reported that ejaculates with $>1 \times 10^6$ peroxidase-positive cells/ml (mainly neutrophils and macrophages) contained significantly more pathogenic bacteria isolates than a group with $<1 \times 10^6$/ml peroxidase-positive leukocytes. However, the demarcation between pathogenic and non pathogenic bacteria is uncertain and the relationship between a semen culture and an active genital tract infection also remains to be elucidated (Barratt *et al.*, 1988). We have consistently been

unable to demonstrate a significant relationship between either the number or concentration of peroxidase-positive cells in the semen of sperm donors and a genital tract infection—mainly manifest as non-specific urethritis (Monteiro *et al.*, 1987; Barratt and Cooke, 1989; Barratt *et al.*, 1990c). The controversy over the clinical value of seminal leukocytes in detecting genital tract infection is in part due to the inaccurate techniques used to detect the leukocytes. El Demiry and colleagues (1986a,b) used monoclonal antibodies to detect white blood cells and failed to demonstrate a relationship between leukocyte numbers, concentrations or specific types and a positive semen culture including the detection of *Chlamydia* and *Ureaplasma*. Our results are in agreement with these conclusions, as 35% of our semen donors had leukocytospermia ($>1 \times 10^6$/ml) (Table II). All these donors were fertile, had no anti-sperm antibodies and were free from genital tract infections (Chauhan *et al.*, 1988; Barratt *et al.*, 1989b). In our studies on the seminal leukocyte profile of men with a clinically diagnosed active genital infection (men with urethritis and epididymitis) out of 20 men examined 80% had leukocytospermia (Table II). Interestingly, in many of the men with a urethral infection, the main leukocyte subset appears to be granulocytes. From our initial observations, two important points emerge. First, it is clearly apparent that the traditional criterion of $>1 \times 10^6$/ml leukocytes in semen as an indicator of a genital infection needs to be readdressed and second, a reliable monoclonal antibody to detect seminal granulocytes is urgently required.

The roles of seminal leukocytes in the transmission of human immunodeficiency virus (HIV) infection deserved further attention. It is now known that human immunodeficiency virus (type 1 HIV 1) can be transmitted sexually through semen (see Kreiss *et al.*, 1988; Alexander, 1990). However, there is controversy whether the virus is transmitted by the spermatozoa, by the leukocytes and/or free in seminal plasma (Anderson *et al.*, 1990). HIV has been clearly identified in the mononuclear cell fraction and free in seminal plasma (Anderson *et al.*, 1990).

Anderson and colleagues (1990) examined 49 HIV sero-positive homosexual men, 152 HIV sero-negative homosexual men and 17 normal controls. There was a significant increase in the number of white bood cells in the semen of sero-negative men compared to controls. Semen from HIV-positive men with symptoms contained more white blood cells than either of the other two groups. This increase may be a result of increased levels of genital tract infections due to the severe immunosuppressed status of these patients. It will be interesting, when further information becomes available, to see whether semen from patients in the advanced stages of the disease have more leukocytes and if as a result these men are more infectious. It may then be possible to identify 'super transmitters' of the virus.

Undoubtedly, an important area of future research on leukocytes will centre on establishing the activation status of the cells in the reproductive tracts and semen of selected subgroups of men. As the technology to examine these receptors in peripheral blood is well established then the techniques can easily be adapted for use in semen. For example, it would be very interesting to investigate the activation status of the lymphocytes (e.g. Il 2 receptor) in the semen of anti-sperm antibody positive men and compare these with controls. One might expect the cells in the antibody-positive men to be activated and thus with the production of cytokines a deleterious effect on spermatozoa may result.

When the activation status of these cells has been determined, and the concentration of their products, it may be possible to identify men where the leukocytes are most likely to damage the spermatozoa. When such information is available, appropriate rational therapy of such patients may begin.

Female reproductive tract

Leukocytes have a multitude of parallel functions in the male and female tract, e.g. modulation of an immune response, phagocytosis of spermatozoa. Their role before and after implantation has received widespread attention and has been documented in several excellent articles (see Clark, 1988). This section will specifically consider the function of leukocytes in sperm transport to the site of fertilization.

Sperm transport in the female tract

There is now clearly documented evidence for the infiltration of leukocytes after insemination in humans (Moyer *et al.*, 1970; Mortimer, 1983; Hunter, 1987; Sinosich and Saunders, 1987) and animals (Austin, 1957, 1975; Mortimer, 1983; Overstreet *et al.*, 1989)—the leukocytic cell reaction. The leukocytic cell reaction in the human cervix, which is a specific response to spermatozoa, is very rapid with a large number of leukocytes being detected even 15 min after insemination (Pandya and Cohen, 1985). Order-of-magnitude calculations suggest $>10^9$ leukocytes at the human cervix within some 4 h after insemination so spermatozoa are considerably outnumbered ($<3 \times 10^8$) (Cohen, 1984; Pandya and Cohen, 1985). This massive increase in leukocytes is a physiological response and not a pathological one and has also been documented in rabbits and mice (Tyler, 1977). The main cell types involved in this reaction are thought to be phagocytes (polymorphonuclear leukocytes and macrophages); however, the proportions of these cells and the involvement of other cell types, e.g. lymphocytes, is unknown. Interestingly, in rabbits, fertile spermatozoa have been inseminated into the cervix after the initiation of the leukocytic cell reaction and still found capable of producing live progeny (Taylor, 1982). At least then, at the cervix, the phagocytes are selective in sperm destruction, although there is no evidence about the senility, motility or fecundity of either the phagocytosed or the surviving populations in many animal species with a clear paucity of data in the human [see Cohen (1984) and Cohen and Adeghe (1987) for detailed discussion on sperm selection]. It would be of specific interest to examine why some spermatozoa are not phagocytosed. Sinosich and Saunders (1987) detected PAPP-A on only 1.7% of ejaculated spermatozoa, and since PAPP-A specifically inhibits leukocyte elastase, they suggested that spermatozoa coated with PAPP-A were 'selected' to overcome localized phagocytic proteolytic degradation. This attractive hypothesis remains to be specifically tested.

The function of the leukocytic cell reaction at the cervix and

the uterus (see Austin, 1975; Hunter, 1987) is unknown but there are several, not necessarily mutually exclusive, possibilities.

Firstly, these leukocytes may 'prime' the female tract to prepare for fertilization and implantation. An interesting series of experiments in mice showed that there were significantly fewer 2-cell embryos (44% reduction) when they were artificially inseminated with only 5% of the normal number of spermatozoa (Chaykin and Watson, 1983). This reduction could be substantially reversed by inseminating with the normal number of dead spermatozoa 12 h later than the live aliquot. Also, labelled spermatozoal DNA from live and dead spermatozoa was detected in the adrenals, ovaries, heart and transiently in the draining lymph nodes (Watson *et al.*, 1983). The authors suggested that phagocytosis of spermatozoa in the female tract either at the cervix, uterus and/or the oviduct, can accumulate genetic information from the sperm DNA which changes maternal physiology to achieve better fertilization-to-cleavage percentages. This hypothesis is an extension of that previously presented by Reid (Reid, 1965a,b, 1966; Reid and Blackwell, 1967). Interestingly, in corroboration of this idea, it has now been demonstrated that insemination of spermatozoa improves implantation and birth rates after embryo transfer in rats (Carp *et al.*, 1984) and women (Bellinge *et al.*, 1986).

These series of experiments definitely raise more questions than answers. It would be fascinating to elucidate possible mechanisms by which the spermatozoal signal is transmitted to the maternal tract; perhaps, as Chaykin's group suggest, this may be an explanation for the high spermatozoa to ova ratios characteristic of mammalian reproduction. Such hypotheses need to take into account the low number of spermatozoa inseminated *in vivo* (10% normal ejaculate in humans, 1−0.1% of the ejaculate in bulls; Amann, 1989) which are adequate to achieve successful pregnancies in artificial insemination. When further details are available, an examination of implantation and pregnancy rates in men with functional spermatozoa but severe oligozoospermia, with comparison to controls would be interesting.

A second function of the massive infiltration of leukocytes after insemination may be in the prevention of infection. This may be manifest as 'cleaning up' after intromission. However, the leukocytic response in rabbits does not occur after mating to vasectomized bucks (Tyler, 1977). In women, the leukocytic response is specifically to spermatozoa following artificial insemination but no quantitative data are yet available after intromission.

A third function has been suggested, i.e. the modulation of an immune response to spermatozoa. As for the male tract, the mechanisms by which the leukocytes affect this response are very poorly understood. One component of the immunosuppression is cervical mucus which, like seminal plasma, has immunosuppressive properties *in vitro* although the nature of this suppression is as yet undetermined (Pockley *et al.*, 1989; Dalton *et al.*, 1990). Witkin (1988a,b) suggests that a decrease in the numbers of suppressor/cytotoxic cells in the vagina and cervix or a deficiency in the responsiveness of these lymphocytes to semen immunosuppressive factors can increase the chances of a deleterious immune response to spermatozoa. Anti-sperm antibodies may arise in some women as a consequence of a failure of spermatozoa from their male partners to inhibit lymphocyte activation (Witkin, 1988b). Interestingly, in-vitro studies using peripheral blood lymphocytes from female donors clearly demonstrated that spermatozoa coated with anti-sperm antibody induced interferon production (Witkin, 1988b); interferon induction of Ia antigen expression on macrophages may be required for the recognition of processed antigens by the female's helper cells—this suggested a mechanism for anti-sperm antibody production. Later work by Witkin (Witkin and Chaudhry, 1989) demonstrated a significant association between serum IFN levels and the presence of anti-sperm antibodies in the serum of women. These complex interactions are only now being unfolded and are likely to be an area of very productive future research.

One very interesting area of active research is the role of leukocytes and their products on fertility in patients with endometriosis (Barratt *et al.*, 1990b). It is now clear, at least in cases of mild endometriosis (stage I and stage II), that there is a significantly higher number of macrophages and T helper cells in the peritoneal fluid of these patients (Haney *et al.*, 1983; Halme *et al.*, 1987; Hill *et al.*, 1988). Products of these cells, e.g. TNF and γ-interferon, adversely affect a number of reproductive functions, e.g. sperm motility, fertilization, embryo development and trophoblast proliferation (see earlier). Eisermann and colleagues (1989) detected elevated levels of tumour necrosis factor (TNFα) in peritoneal fluid from infertile women with mild endometriosis. Using peritoneal fluid and recombinant human TNF, high concentrations of TNF (500−1000 U of recombinant TNFα; and 800 U in peritoneal fluid) significantly affected sperm motility when incubated *in vitro* for 4 and 21 h. Interestingly, the addition of anti-TNF antibody in some patients reversed this inhibitory effect indicating that in these patients TNF was the sole factor responsible for the reduction in motility. In the near future, a better understanding of the role of these leukocytes associated with endometriosis is likely to provide an insight into the immunological mechanisms underlying their infertility.

The distribution of leukocytes in the human oviduct remains an unknown quantity. Haney *et al.* (1983) cannulated and flushed the distal oviducts of patients undergoing sterilization, patients with infertility and those with proximal oviductal obstruction. They suggested that oviductal macrophages arise, at least in the large part, from peritoneal macrophages that migrate into the oviducts via the fimbrial ostia. Their preliminary (unpublished) observations suggest that oviductal macrophages phagocytose and destroy spermatozoa from fertile men. These interesting observations need to be confirmed.

There is now clear evidence in animal species of a specific storage region for fertilizing spermatozoa in the caudal isthmus of the oviduct (see Hunter, 1987). In this region of pigs, there is a remarkable absence of leukocytes (Hunter *et al.*, 1987). This would seem an essential strategy at least until the time of ovulation and penetration of the eggs. Hunter provides evidence in pigs that after ovulation polymorphs appear in the terminal folds of the isthmus which probably arise from the uterus with the relaxation of the uterotubal junction after ovulation (Hunter *et al.*, 1987). In many species of bats where there are long periods of sperm storage in the various portions of the female tract

depending on the species (uterus, oviduct, utero tubal junction) there is a noticeable absence of phagocytic cells in such storage sites (Racy, 1975). If leukocytic infiltration occurred it would lead to phagocytosis of capacitated spermatozoa which is incompatible with sperm storage (Bedford, 1965). In the human, the potential of the caudal isthmus of the oviduct to act as storage site for fertilizing spermatozoa requires urgent examination. The factors regulating this storage, if it exists, also need to be examined. These investigations should include an examination of the leukocyte profile of normal fertile women and then to compare this to women with endometriosis where a population of white blood cells may be expected in their oviducts which would interfere with sperm storage.

It is now essential to accurately categorize the leukocyte cell types (neutrophils, macrophages, T lymphocyte subsets, B lymphocytes) in the vagina, cervix, uterus and oviducts throughout the menstrual cycle and their response to spermatozoa. As for the male reproductive tract, the activation status of these cells and the products they secrete will be areas of active research in the near future.

In conclusion, leukocytes appear to play a very important role in successful reproduction. With the advent of modern techniques in reproductive biology many of the questions addressed above should be successfully answered in the very near future.

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Review

Sperm transport in the human female reproductive tract — a dynamic interaction

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Summary

The interaction between sperm and the human female tract has been largely ignored. This review summarizes the data available from animal species with specific reference to sperm in the oviduct. Our knowledge of sperm transport within the human female tract is explored and, using the data available from animal species, three lines of future experimental design are suggested. Firstly, there is the need to improve and develop techniques to recover sperm successfully from the tract. Second, an in-vitro approach which examines the modulation of reproductive tract fluids on sperm function is advocated. Third, an in-vitro tissue/cell culture system is required to investigate in more detail the interaction between the epithelium and sperm. Using such approaches many of the questions posed in this review can be addressed confidently in the near future.

Keywords: capacitation, female tract, humans, oviduct, spermatozoa, sperm transport.

Introduction

In 1951 Chang concluded that 'further study (of the physiological change of sperm in the female tract) may explain aspects of sterility due to incompatability between the environment of the female tract and spermatozoa' (Chang, 1951). Unfortunately, 40 years later we are still ignorant of the conditions or factors which control sperm function within the human female genital tract (Overstreet & Drobnis, 1991). In contrast, the interaction between sperm and the female tract in some animal species is now an area of active research, from which it can be concluded that there is intricate co-operation and synchrony.

The assessment of human infertility has progressed by more detailed assessment of ejaculated sperm and more elaborate evaluation of pituitary/ovarian endocrinology and oocyte maturation (Aitken, 1990; Lenton, 1990). However, there is a marked paucity of data on the transport of sperm to the site of fertilization and the mechanisms controlling this. In the human, we still do not have answers to some very basic scientific questions, for example, does the oviduct act as a sperm

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reservoir? Is capacitation of sperm an ovulation-related event? Recent evidence in animals provides some likely answers to these questions.

In the animal models studied, the oviduct acts as a functional sperm reservoir and conserves sperm function if mating takes place prior to ovulation, and immediately before ovulation, activates a very small proportion of the sperm to progress to the site of fertilization (Hunter, 1987; Smith & Yanagimachi, 1989; 1990; 1991). This suggests strongly that, in animals, capacitation of sperm is an ovulation-related event. This review will firstly discuss some of these studies in animals, concentrating on the specific role of the oviduct in an attempt to provide an insight into the remarkable process of sperm transport. A precis of the literature available in the human will be presented, and then an outline of in-vitro studies which have examined the interaction between sperm and secretions of the female tract. Throughout this manuscript possible lines of experimental investigation in the human will be explored.

Sperm transport in animals

Background

There is now clear evidence in all of the mammalian species studied to date, even in those with very different modes of sperm transport through the lower tract (e.g. vaginal, cervical and intra-uterine inseminators) that the isthmus of the oviduct acts as a functional sperm reservoir, and that the movement of sperm from this region to the ampulla is a highly synchronized event (Hunter, 1987; Smith & Yanagimachi, 1991; Overstreet & Drobnis, 1991). Our knowledge of this remarkable synchronization, at least in mammals, comes mainly from the elegant studies of Hunter, Yanagimachi and Overstreet.

Transport in the oviduct

Hunters' group used mating during spontaneous oestrus cycles and transection of the different parts of the isthmus at varying times after mating to study sperm transport. A minimum period of 6–8 h was required for a functional population of sperm to be established in the fallopian tube of sheep and cows mated at oestrus (Hunter & Wilmut, 1984; Wilmut & Hunter, 1984; Hunter & Nicol, 1986a,b; Hunter *et al.*, 1982). Sperm are sequestered in the caudal isthmus until just before ovulation where they then redistribute for transport to the ampullary region for fertilization, (Hunter & Nicol, 1983). The latter authors examined sperm in the oviduct of sheep by transecting the oviductal isthmus 1.5–2.0 cm proximal to the utero-tubal junction at increasing time intervals after mating. The incidence of fertilization and numbers of accessory sperm (sperm attached or embedded in the zona pellucida) were examined in eggs recovered 1–3 days after surgery. None of the eggs recovered from the oviducts transected up to 21 h after mating were fertilized (ovulation occurs 24–26 h after the onset of oestrus). Two of the 33 eggs from the oviducts transected between 22 and 24 h after mating were fertilized yet, 3/14 eggs and 13/16 were fertilized with transections at 25 and 26 h after mating.

Hunter & Nichol, (1986a) further refined their experimental design by ligating

the oviducts of sheep more proximally i.e. 3–5 cm from the utero-tubal junction after ovulation. Failure of fertilization following ligation immediately after ovulation (within 1 h) indicated that insufficient viable sperm had moved along towards the site of fertilization at that stage, yet remarkably, 1 h later (transection 27 h post mating) all of the eggs were fertilized.

These studies demonstrate that the movement of viable sperm towards the ovary is controlled *tightly* in the first hours after ovulation. A similar controlled process has been documented in pigs (Hunter, 1981; 1984; Hunter & Nicol, 1988; Hunter *et al.*, 1987) and cattle (Wilmut & Hunter, 1984; Hunter & Wilmut, 1984).

Sperm capacitation in the oviduct and epithelial attachment

The oviduct may regulate the speed of sperm capacitation. For example, in hamsters, when sperm were recovered from the isthumus of the oviduct in animals mated shortly after the onset of oestrus (1, 2, 4 and 6 h after mating) they required additional time *in vitro* before being able to penetrate the eggs. However, for females mated immediately after ovulation, sperm recovered from the oviducts could penetrate eggs within 30 min (Smith & Yanagimachi, 1989). Therefore, sperm capacitation proceeds at a faster rate when mating occurs after ovulation. This implies either that some essential factor may be missing from the oviductal milieu until about the time of ovulation or that there is a temporal arrest of capacitation.

Interestingly, many of the sperm recovered from the hamster oviduct are immotile and very fragile. Sequential flushing of oviducts, before ovulation, showed that the majority of sperm in the first flush (containing mainly sperm from the lumen) and the second flush (containing mainly sperm from the mucosal surface) were dead, yet over 50% of the sperm from the third flush (containing mainly sperm in the crypts) were alive (Smith & Yanagimachi, 1990). The origin of the sperm in the flushings was determined by direct observation through the oviduct wall. As only live sperm traverse the utero-tubal junction the majority of sperm in the hamster isthmus die before ovulation (Smith *et al.*, 1988).

Notably a high proportion of sperm which were attached to the mucosal surface were alive, indicating that sperm attachment to the epithelial surface may play an important role in maintaining sperm viability in the oviduct. The importance of attachment and release of sperm from the epithelium at the caudal isthmus in hamsters has been addressed recently (Smith & Yanagimachi, 1991). The oviducts were flushed *in situ* to remove sperm from the lumen, leaving only those firmly attached to the isthmic mucosa of the oviduct. When eggs were recovered from the oviducts at 20 h after flushing, the majority were fertilized indicating that sperm which are attached firmly to the mucosa are capable of detaching and ascending to the ampulla to fertilize eggs. When uncapacitated sperm were introduced into the oviduct, many attached to the oviductal mucosa, unlike capacitated sperm which did not. This suggests that a change in the sperm surface, rather than in the mucosal surface, may cause the release of sperm (Smith & Yanagimachi, 1991). In hamsters, it would therefore appear that sperm remain attached to the isthmic mucosa until they become capacitated, after which they then detach and migrate to the ampulla to fertilize the eggs.

Fecundity of sperm populations in the tract

The results of these animal studies illustrate several important points. First, the primary role of sperm transport is the formation of adequate sperm reservoirs and it is the factors controlling these processes that we should be focusing our attention on; questions such as the phenomenon of rapid transport (see Overstreet & Drobnis, 1991 for details) are of secondary importance. Secondly, of the thousands of sperm stored in the isthmus, only a few are transported to the ampulla at the time of fertilization. Detailed calculations of the number of sperm in the respective portions of the oviduct are only available for hamsters in which approximately 1 in 10,000 sperm reach the site of fertilization (Smith & Yanagimachi, 1987), yet examination of the crude data in other animals suggests similar proportions would be expected. Thus, sperm wastage occurs in the oviduct as well as in the cervix and utero-tubal junction (Overstreet *et al.*, 1989; Overstreet & Drobnis, 1991). The low ratio of sperm to eggs (approximately 1:1) in the ampulla at the time of fertilization (rats: Shalig & Phillips, 1988; mice: Peitz & Olds Clarke, 1986; hamsters: Cummins & Yanagimachi, 1982; rabbits: Overstreet & Cooper, 1978; Overstreet *et al.*, 1978; pigs: Hunter *et al.*, 1987; sheep: Hunter & Nicol, 1986a), suggests strongly that these sperm are likely to be very fertile (see Cohen, 1991; Cohen & Adeghe, 1987 for critical reviews on sperm selection). It is interesting to compare the low sperm:egg ratios *in vivo* to the in-vitro situation in humans, where commonly 100,000 sperm are placed in the vicinity of the egg (Barratt *et al.*, 1989). One way to improve our in-vitro fertilization procedures would be to refine the number of *effective* sperm that are used. It is therefore a matter of importance to examine the character of sperm which reach the site of fertilization *in vivo*. In this context, an important starting point is the experiment of Siddiquey & Cohen (1982) which compared the fertilizing capacity *in vitro* of sperm recovered from the oviduct to those in the ejaculate, examining specifically sperm number, concentration and collision rates with the egg.

In the human some attempts have been made to assess the fertilizing capacity of ejaculated sperm in relatively low sperm to egg ratios (500 per oocyte in 5 μl — Van der Ven *et al.*, 1989), but these studies need to be refined further and designed to compare the fertilizing capacity of sperm populations recovered from different parts of the female tract. As sperm can now be recovered confidently from mucus (Zinamen *et al.*, 1989) and the peritoneal cavity (Ramsewak *et al.*, 1990) these two populations could be compared to ejaculated sperm, which would provide an ideal starting point. Future studies can then concentrate on the factors governing the fertilizing capacity of these sperm.

Mechanisms controlling sperm storage and transport

The animal experiments illustrate clearly a dynamic co-ordinated interaction between the female tract and sperm. However, there is a paucity of studies on the mechanisms controlling this interaction, and as a consequence such mechanisms are poorly understood. Temperature differences between the isthmus and ampulla of the same oviduct (Hunter & Nichol, 1986b), the narrow lumen of the isthmus restricting sperm movement (Hunter, 1984), and tenacious mucus at the isthmus by

the time of ovulation (Jansen, 1980; 1984) have all been suggested to play a role in regulating sperm storage in the oviduct. Motility patterns of sperm within the oviduct and their regulation have received considerable attention. Suarez and colleagues described the suppression of sperm motility in some portions of the lower isthmus of rats (Suarez & Osmon, 1987) which is in agreement with observations in rabbits (Cooper *et al.*, 1979; Overstreet *et al.*, 1980a). In the latter, this inhibition of motility was reversible as sperm recovered from the isthmus and placed in media become motile (Burkman *et al.*, 1984). Suarez suggested the presence of a molecule in certain segments of the isthmus which suppressed sperm motility by increasing the viscoelasticity of oviductal fluid (Suarez, 1987).

Potassium may inhibit and pyruvate stimulate sperm motility in the isthmus (Burkman *et al.*, 1984), and low temperatures and oxygen tension may also act to reduce motility (Hunter & Nicol, 1986b).

Hyperactivated sperm motility

One important aspect of sperm movement within the oviduct is the expression of hyperactivation. Hyperactivated sperm have been observed directly in the ampulla of hamsters (Katz & Yanagimachi, 1980) and rats (Shalig & Philips, 1988), and it occurs in sperm recovered from the oviduct of rabbits (Cooper *et al.*, 1979; Suarez *et al.*, 1983), hamsters (Cummins & Yanagimachi, 1982), mice (Olds-Clarke, 1986; Suarez, 1987; Suarez & Osman, 1987) and sheep (Cummins, 1982). Although the biological importance and significance of hyperactivation is still a subject of intense debate (see Katz *et al.*, 1989, Mortimer & Mortimer, 1990) this form of sperm motility may facilitate migration of sperm by avoiding entrapment in the female tract and increase the probability of contact with the cumulus (Katz *et al.*, 1989). In hamsters, artificially capacitated sperm were unable to bind to the oviductal epithelium (Smith & Yanagimachi, 1991). Thus completion of capacitation in the oviduct may result in detachment from the epithelium — a process which may be concordant with the development of sperm hyperactivation. As yet there are no data on the expression of hyperactivated motility in human sperm recovered from any portion of the female tract. Using computerized motility analyses it would be relatively simple to determine the motility pattern of sperm which can be recovered readily from the human tract i.e. mucus and the peritoneal cavity (see later).

Whatever mechanisms are proposed, they need to explain the occurrence of motile and immotile sperm in adjacent sections of the isthmus (Suarez, 1987) and the species differences which exist. In hamsters for example, two thirds of sperm in the isthmus are dead, which is in contrast to rabbits in which sperm recovered from the isthmus can be stimulated to move by dilution of this fluid *in vitro* (Burkman *et al.*, 1984). Sperm attachment to the epithelium of the oviduct has been observed in hamsters (Smith & Yanagimachi, 1990), pigs (Hunter *et al.*, 1987), rabbits (Overstreet & Cooper, 1978) and bats (Racy, 1975), and may act to regulate sperm movement and function. In-vitro studies in cattle show that attachment to the oviductal epithelium alone improves the viability and fertilizing capacity of bovine sperm (Pollard *et al.*, 1991 see later human studies).

Sperm transport in humans

Background

The capacity of the human female reproductive tract to establish sperm reservoirs and the mechanisms controlling this have not been investigated. This is probably due to two factors. Firstly, studies on human sperm transport are fraught with ethical and logistic problems and, second, the concentration of research on assisted conception and the subsequent ability to achieve fertilization *in vitro* has undoubtedly detracted from the importance of sperm transport *in vivo*.

Sperm transport in the cervix

There have been a number of extensive studies on the interaction between sperm and human cervical mucus, an interaction which is highly complex. Sperm penetration is dependent not only on factors such as sperm concentration, motility and morphology (see Katz *et al.*, 1980, 1984; Mortimer *et al.*, 1982, 1986; Aitken *et al.*, 1986) but also on the capacitational status of sperm (Overstreet & Drobnis, 1991), seminal enzymes (Overstreet *et al.*, 1980b) and antisperm antibodies (Wang *et al.*, 1985). Interestingly, in-vitro studies of sperm mucus penetration have shown that movement characteristics account for 85% of the variability in penetration (Aitken *et al.*, 1986), with the concentration of motile sperm and linear velocity of progression being the two most important functions determining the number of sperm penetrating per unit time (Aitken *et al.*, 1986; Mortimer *et al.*, 1986).

Many morphologically abnormal sperm are excluded from penetration of cervical mucus which is probably due to the associated abnormality in flagellar activity (Katz *et al.*, 1990). The latter authors have studied specifically the relationship between sperm morphology and motility in both semen and cervical mucus (Katz *et al.*, 1990). Abnormal sperm swam more slowly in mucus than did normal sperm, and the heads of abnormal sperm experienced greater resistance from mucus than did those of normal sperm. Such studies emphasize the interrelationships between different functional aspects of the sperm. As such techniques can be adapted to investigate sperm movement in other regions of the tract (e.g. cumulus) they will act as a starting point for further investigations.

Although we know that sperm loss in the vagina is probably high in humans (R. Baker, personal communication) and animals (Gallagher & Senger, 1989), we do not know the proportion of ejaculated sperm which enter cervical mucus *in vivo*. Data from in-vitro studies in which a column of cervical mucus is placed directly into semen suggest that this is very variable, ranging from 3–46% in men with normal sperm counts (Katz *et al.*, 1980). It is difficult to extrapolate these data to the in-vivo situation, but simple in-vivo calculations comparing sperm numbers in the ejaculate and those recovered from cervical mucus suggest a ratio of 1:2000 sperm enter the cervical mucus (0.05%: Settlage *et al.*, 1973). Undoubtedly many factors will affect the passage of sperm into cervical mucus (e.g. hormonal status: Thompson *et al.*, 1990a), but this information is necessary if we are to begin to address confidently such questions as sperm wastage along the female tract. Bearing in mind the known enormous inter- and intra-individual variation in sperm counts in men, this factor also needs to be accounted for when designing experiments on sperm wastage.

Sperm transport in the oviduct

Bearing in mind that studies in animal species have shown a complex interaction between sperm and the female tract it is difficult to evaluate the data on sperm transport in the human reproductive tract, as factors which we now know to influence sperm transport have not been addressed. Such factors include:

(i) the time between recovery of the sperm and the precise timing of that ovulation;
(ii) the techniques used to recover the sperm, which have often been inadequate, for example Settlage *et al.* (1973) using a cytocentrifuge had a recovery rate of only 1%;
(iii) the results have usually been qualitative, and even when quantitive data are presented it is only possible to determine sperm migration at one time-point — the sequence of sperm transport cannot be determined;
(iv) some of the patients studied have had tubal or uterine pathology making comparison to the normal situation difficult. Nevertheless, a succinct summary of the previous data provides a starting point from which to develop an hypothesis.

Rubenstein *et al.* (1951) examined the reproductive tract of 51 women with fibroids or extensive endometriosis undergoing a hysterectomy. In 38 cases the oviducts (fallopian tubes) were dissected and washed and in 25 cases motile sperm were recovered from either or both tubes. As there was no synchronization with ovulation (3–64 days after menstration) it is impossible to ascertain if any synchrony of gamete transport existed. Alghren (1975) cannulated the oviducts in women at post-coital laparoscopy 'at the time of ovulation' 1–34 h after coitus. In the 70 oviducts examined (from 41 infertile patients) no sperm were observed in 21 women, and between 1 and 25 sperm were recovered in 46 women, whilst in only five women were more than 36 sperm found. Considerably more sperm (median = 750 range 2–23,000) were recovered from 12 women with laterally closed oviducts. Alghren concluded that the number of sperm in the oviducts was not in excess of 200. This figure is quoted regularly as the number of sperm in the human oviduct. However, bearing in mind that many of the factors which influence sperm transport in animals were not controlled for (see above), it should be stressed that this is a very preliminary observation.

Settlage *et al.* (1973) examined eight women who requested surgical sterilization and used a synchronized approach, with the oviduct being separated into fimbrial, ampullary and isthmic segments. All women were proved fertile, had abstained from sexual intercourse for 11–14 days before insemination and the operation was performed within 36 h of the pre-ovulatory oestradiol peak. Sperm were found in approximately 75% of either or both tubes 5–45 min post-insemination. Sperm were detected throughout the oviduct with the total number recovered (range 0–53) being related directly to the number in the inseminate. There was a greater number of sperm in the fimbria than in any other part of the oviduct, with almost equal numbers in the isthmus and ampulla. Settlage calculated that 1 in every 14 million sperm deposited in the vagina was present in the oviduct. In only one of eight subjects were sperm found in the endometrial or curettage washings.

Sperm transport in the uterus

Sperm recovery from the uterus has attracted limited attention. Moyer *et al.* (1970) examined sperm recovered at the time of ovulation from the uterus in 26 fertile women. At variable times after coitus, (25–41 h) a hysterectomy was performed and the uterus flushed. Sperm were recovered in only 6/26 women, and in these samples the total number of uterine sperm ranged from 1 to 4 and none of the sperm were motile. Control experiments, in which known numbers of sperm were placed in the uterus showed that over 90% of the sperm could be recovered after 30 min, although no control data were available for longer time intervals i.e. 25–41 h. There was a direct correlation between the number of sperm in cervical mucus and the presence of uterine sperm. In all the women in whom sperm were recovered, the endometrium was in the late proliferative phase. In 17 patients either one or both fallopian tubes were removed and flushed, yet in only one case were sperm recovered and in this instance only four sperm in total were counted.

Mortimer & Templeton (1982) attempted to recover sperm from the uterus at the peri-ovulatory period, but experienced considerable technical difficulty in flushing due to the inability to form a seal at the cervix. As a consequence, in only 37/64 cases could the technique be performed successfully; of these, sperm were recovered in 16 (64%) with the numbers recovered ranging from 68 to over 21,000.

The variable and often small number of sperm recovered from the uterus in the studies referred to above could be explained by three factors which are not necessarily mutually exclusive.

(1) The uterus may be a conduit to sperm transport thus not serving as a sperm reservoir.
(2) In both humans and animals there is now unequivocal evidence for an infiltration of leucocytes at the uterus and cervix (see review Barratt *et al.*, 1990). One of the prime functions of these cells is the phagocytosis of sperm, and this is probably the most important physiological mechanism for the disposal of sperm from the uterus in mice (Austin, 1957, 1960), rabbits (Howe, 1967; Soupart, 1970) and possibly humans (Pandya & Cohen, 1985; Thompson *et al.*, 1990b). Although the kinetics of this still remain to be established the numbers of sperm remaining in the uterus may only represent a small fraction of those present before active phagocytosis.
(3) The accurate estimation of sperm numbers in the uterus, as for other portions of the reproductive tract, is fraught with technical difficulties. Sperm attachment to the epithelium, if it occurs, may also lead to a gross underestimation of the number of sperm in the tract, as such sperm cannot be removed easily by simple flushing techniques (Smith & Yamagimachi, 1990).

Interestingly, a technique for direct aspiration of the contents of the uterus under ultrasound guidance has been developed in cynomologus macaques (Vande-Voort *et al.*, 1989). It remains to be seen if this method can be modified for use with humans. In order to ascertain the role of sperm transport within and through the human uterus, a reliable and accurate technique needs to be developed. Once this has been developed the functional status of recovered sperm can be examined easily using an array of sperm function tests (see below). This will also enable the

dynamics of sperm entry into the uterus to be established quickly and, with accurate techniques to detect the onset of ovulation, synchronization of gamete transport can be studied easily.

Sperm transport to the peritoneal cavity

One popular technique to determine successful sperm transport from the cervix through the oviduct to the peritoneal cavity has been the aspiration of the Pouch of Douglas using a laparoscope (see Ramsewak *et al.*, 1990).

Several clinical studies concentrating on couples with unexplained infertility proposed that sperm recovery 6–12 h after insemination could be used as a clinical test of sperm function (Mortimer & Templeton, 1982; Templeton *et al.*, 1982; Templeton & Mortimer, 1982). Sperm recovery rates ranged from 45–63% with significantly higher pregnancy rates in patients from whom sperm were isolated from the Pouch of Douglas, compared to those from whom no sperm were recovered. However, in some women, from whom no sperm could be recovered, pregnancy was achieved, thus casting doubt on the reliability of the test. However, Templeton and colleagues concluded that the technique could provide meaningful information on the interactions between sperm and the female tract and that impaired sperm transport was a significant factor in the infertility of couples with unexplained infertility (Mortimer & Templeton, 1982; Templeton *et al.*, 1982; Templeton & Mortimer, 1982).

We performed a similar study in which we analysed peritoneal fluid recovered 10–20 h after midcycle intra-cervical insemination in 15 women with unexplained infertility (Ramsewak *et al.*, 1990). Our results differed from those of Templeton and colleagues as we were consistently able to recover sperm in all appropriately timed cycles. We concluded that sperm transport studies should concentrate on investigation of the functional status of sperm which were resident within the cervix, uterus and oviduct, and that determination of the interaction between sperm and the female tract was an essential strategy to further our understanding of gamete physiology (Ramsewak *et al.*, 1990). As sperm can now be recovered from mucus (see below) and the peritoneal cavity we must develop our techniques further to recover sperm from the oviduct and uterus.

Procedures such as tubal transfer of embryos and GIFT are now routine and with a little ingenuity such techniques should be able to be adapted to recover sperm *in vivo*. This development is an absolute requirement, for although in-vitro studies provide very informative data they cannot replace the information given from in-vivo data. Specific attention must concentrate on sperm recovery from the oviduct at known time intervals in relation to ovulation.

In-vitro studies

Background. With the technical and ethical constraints of obtaining sperm from the human reproductive tract, an in-vitro approach for investigating the interaction of secretions from the tract and sperm represents a logical approach. Now, with the widespread use of in-vitro assays for sperm function e.g. hyperactivation (as a marker of capacitation, Mortimer & Mortimer, 1990; Robertson *et al.*, 1988),

induction of the acrosome reaction (Cross *et al.*, 1988; De Jonge *et al.*, 1988), and sperm–egg interaction (Liu & Baker, 1988, 1990; Aitken, 1990), this approach should produce exciting data if appropriate questions can be asked.

Physiological effects of cervical mucus. Overstreet and colleagues have investigated the biological function of cervical mucus in sperm transport using such an in-vitro approach (Gould *et al.*, 1984; Lambert *et al.*, 1985; Zinamen *et al.*, 1989; Katz *et al.*, 1990; Overstreet & Drobnis, 1991). Sperm recovered from cervical mucus into non-capacitating medium were successful in penetrating the human zona pellucida and zona-free hamster eggs, whilst sperm incubated in media for the same period were functionally inert, suggesting more rapid capacitation in cervical mucus compared to media (Lambert *et al.*, 1985). The capacitation status of sperm recovered from cervical mucus *in vivo* was examined further by challenging the sperm with a biological agonist for the acrosome reaction (human follicular fluid, solubilized human zonae pellucida). In such experiments, capacitation was defined functionally as the physiological prerequisite of the acrosome reaction (Zinamen *et al.*, 1989). One hour following donor insemination with fresh sperm, the cervical mucus was aspirated and sperm were allowed to swim out of cervical mucus into media. Such sperm did not acrosome react in response to follicular fluid after incubation for 1 h in media, but 15–20% acrosome reacted when the sperm were challenged with follicular fluid after incubation for 6 h in media. The capacitation time required to respond was independent of the time spent in the female tract as the response was similar if sperm were recovered 1 h, 1 day, 2 days or 3 days post-insemination. Concordant studies with sperm in media, but not exposed to cervical mucus, showed that a 24 h period was necessary before the sperm would respond to follicular fluid (Zinamen *et al.*, 1989). This suggested that the initiation of capacitation occurs quickly within the reproductive tract but that the function of the sperm is conserved. Similar results have been obtained with in-vitro penetration of human mucus, suggesting that in-vitro investigations, at least in this context, can be used to determine in-vivo action (see Overstreet & Drobnis, 1991).

Physiological effects of serum and follicular fluid. Such in-vitro studies are not restricted to cervical mucus as the effect of serum from different stages of the menstrual cycle, exogenous steroids or follicular fluid on sperm motion parameters during capacitation have been examined recently (Mbizvo *et al.*, 1990a,b). Surprisingly, it was early follicular phase serum (low oestradiol and progesterone) and not pre-ovulatory serum (high oestradiol and low progesterone) which provided optimal support in terms of longevity (Mbizvo *et al.*, 1990a). Progesterone increased the percentage of hyperactivated sperm three-fold, although oestradiol had no effect. The steroid rich fraction of follicular fluid stimulated a similar increase in hyperactivation (Mbizvo *et al.*, 1990b). Recent interest has focused on the role of follicular fluid and progesterone on sperm function, specifically on the acrosome reaction (Drahorad *et al.*, 1988; Thomas & Meizel, 1988; Mortimer & Camenzind, 1989; Stock *et al.*, 1989; Tesarik, 1989; Sullivan *et al.*, 1990; White *et al.*, 1990). Studies now suggest that progesterone can stimulate a rapid transient rise in Ca^{2+} (Thomas & Meizel, 1989) and that the similarities in response induced by human follicular fluid and progesterone suggest that the latter is the active factor in

follicular fluid which stimulates the acrosome reaction (Blackmore *et al.*, 1989). As follicular fluid has been shown to induce hyperactivation of sperm, even at low concentrations (1:40), this mechanism may operate *in vivo* and this specific hypothesis requires testing.

Physiological effects of the egg vestments: In-vitro experiments have also been designed to determine what interactions occur between the egg vestments and sperm. For example, human cumulus cells have been shown to induce the acrosome reaction *in vitro* (Tesarik, 1985; De Jonge *et al.*, 1988; Stock *et al.*, 1989). The active products are proteins from the intercellular matrix (apparent molecular weight 50,000 daltons [Siiteri *et al.*, 1988; Sullivan *et al.*, 1990]). Interestingly, glycoproteins of higher molecular weight, accelerate the activation of proacrosin to acrosin, demonstrating that different specific proteins, may exert distinct functions on the sperm. This strongly suggests a dynamic interaction (Tesarik *et al.*, 1988).

In-vitro experiments should not be confined to the biochemical level, e.g. examining the role of phosphoinositides on the acrosome reaction (Harrison & Roldan, 1990), as important physiological questions remain unanswered, such as the role of hyperactivated sperm motility in penetration of the human cumulus and zona. An interesting experiment of Tesarik and colleagues may provide an insight to this process in humans (Tesarik *et al.*, 1990). Motility patterns of sperm were examined under capacitating conditions and compared to the motility of sperm penetrating the human cumulus oophorus and solubilized fractions of the cumulus. Ninety per cent of the sperm within the cumulus exhibited a very linear motility, different from the hyperactivated motility seen in 10% of the sperm population incubated in media. This pattern of movement was induced by solubilized cumulus. The appearance of the 'cumulus motility pattern' was accompanied by a marked reduction in the subpopulation of sperm showing the hyperactivated form of motion in the media, implying that the sperm exhibiting the cumulus-related motility pattern are recruited from the hyperactivated sperm population. This hypothesis warrants further examination. An interesting experiment would be to artificially induce hyperactivation, possibly using progesterone, then examine sperm penetration of the cumulus.

Observations in hamsters suggest that during penetration of the cumulus, sperm do not display the type of movement characteristic of hyperactivation and that hyperactivated movement of sperm is not essential for penetration (Suarez *et al.*, 1984; Drobnis *et al.*, 1988). Also, in addition to sperm motility patterns, sperm surface characteristics are important for penetration. With the observation that plant cells (e.g. *Chlamydomonas*) can penetrate the hamster cumulus, these surface properties require further definition urgently (Talbot *et al.*, 1985).

Physiological effects of oviductal fluid: In humans, the ethical and logistic constraints of examining sperm transport *in vivo* necessitates further in-vitro experiments of the type described above to address some of the points raised. In-vitro studies in animals have shown that oviductal fluid contains a capacitating factor that exhibits properties similar to that of a heparin-like glycosaminoglycan (Parrish *et al.*, 1989). In addition, the release and selective absorption of substances during capacitation has been determined (see Volglmayr & Sawyer, 1986). As oviductal

fluid has been isolated successfully at different stages of the menstrual cycle (Lippes *et al.*, 1972), similar experiments in humans are feasible to determine the in-vitro interaction of oviductal fluid on sperm function. Such experiments can be taken one step further to attempt to isolate the active factors.

A unique human oviductal protein has been isolated recently from oviductal fluid (human oviductin-1) which is found in high concentrations in periovulatory fluid (Wagh & Lippes, 1989). This protein has been characterized and preliminary in-vitro data suggest that it binds selectively to the head region of sperm in the ejaculate, possibly acting to stabilize the acrosome (Lippes & Wagh, 1989). Further experiments are required to elucidate the effects of human oviductin-1 on sperm function. It would be relatively simple to answer such questions as 'can sperm which are bound with this protein acrosome react?' In this context, substances known to induce the acrosome reaction can be used (e.g. follicular fluid, A23187) and immunocytochemistry used to determine the proportion of sperm which bind oviductin-1.

If human oviductin-1 is a functionally active glycoprotein then sequencing and subsequent production of the glycoprotein would provide enough material to examine further questions e.g. the stage of the menstrual cycle at which the protein is expressed. It is however important with in-vitro studies on the oviductal microenvironment not to examine one product exclusively but to keep an open mind for the possible role of other products e.g. growth factors (Brenner *et al.*, 1990).

Development of in-vitro tissue/cell culture systems: One of the major problems with experiments involving the use of oviductal fluid is the difficulty in obtaining this fluid; in such cases, the development of a tissue culture system would appear appropriate. These systems provide excellent experimental approaches and have been used in animals to examine the *de novo* synthesis and release of specific glycoproteins secreted by the oviduct e.g. in baboons (Fazleabas & Verhage, 1986; Verhage & Fazleabas, 1988), pigs (Buhi *et al.*, 1989a) and cows (Boice *et al.*, 1990). Regional differences within the oviduct have also been examined successfully (Verhage *et al.*, 1988; Buhi *et al.*, 1989b). Several systems in the human have been described recently (Verhage *et al.*, 1988; Bongso *et al.*, 1989; Buhi *et al.*, 1989b) and structural features of human ampullary cell cultures appear similar in-vivo and in-vitro (Bongso *et al.*, 1989). Not only can such systems be used to determine the secretion of specific products and the factors controlling this secretion, but they can also be used to determine other factors which may be important for sperm survival e.g. epithelial attachment. In this context, a recent experiment in cattle compared the motility and fertilizing capacity of sperm cultured in media and incubated with endosalpingeal epithelial cells cultured on either tissue culture plasic (non polarizing) or matrigel-coated millicell (polarizing) substrata (Pollard *et al.*, 1991). Motility was maintained for 48 h in sperm bound to endosalpingeal cells and to a greater extent with polarized than non-polarized cells. Only sperm incubated with oviductal cells developed hyperactivated motility. Interestingly, sperm bound to the epithelial cells were observed to detach and fertilize oocytes. Fertilizing capacity was maintained for 30 h in sperm incubated with endosalpingeal cells, yet no fertilization occurred after 24 h in the media controls. These results demonstrate, firstly, the importance of the oviductal epithelium in maintaining sperm function

and, second, that such a system may be developed for use with human tissue.

Relevance to the in-vivo situation: The biochemical mechanisms and changes involved in the preparation of sperm for fertilization are now starting to be unfolded (see Fraser & Ahuja, 1988; Fraser & McIntyre, 1989; Roland & Harrison, 1989; Focarelli, 1990; Miller *et al.*, 1990; Thomas, 1991). However, in order to understand the mechanisms of sperm interaction with the female tract, we need to attempt to relate these biochemical events to the in-vivo situation. This does involve considerable speculation and our information needs to be extrapolated from animal species. For example, in animals, sperm movement from the isthmus to the ampulla may result from the completion of capacitation and possibly the expression of hyperactivation. Follicular fluid and progesterone, which have been shown to stimulate hyperactivation of sperm, may do so in the female tract. With the complex regional microenvironment in the oviduct, which is subject to local hormonal influences, progesterone may act at this level and not via the follicular fluid. It is known that the oviduct is able to concentrate oestradiol and progesterone to levels several times those found in circulating plasma (Hunter, 1988), leading to a concentration in the tubal environment which could possibly activate the sperm. The activation of sperm by progesterone is an attractive hypothesis which warrants further experimentation. However, many other factors are likely to be important (see animal studies) and studies should not concentrate on one factor which is presently 'in vogue'.

Future studies

The available data in animals suggest a highly synchronized movement of sperm through the female reproductive tract. Unfortunately, in humans, we know very little of this process. The fundamental interaction between the human female reproductive tract and the sperm now requires urgent investigation. This can be approached using three lines of investigation.

First, we must concentrate on developing accurate and reliable techniques to recover sperm from the female tract. These studies should concentrate on the oviduct and the uterus, and once these have been developed studies on sperm recovery must be co-ordinated with ovulation. Secondly, using the sophisticated sperm function tests now available (acrosome reaction, hyperactivation, zona binding) the interaction between the reproductive tract fluids (e.g. uterine and oviductal fluids) can be determined *in-vitro*. Third, a tissue culture system, as similar to the in-vivo situation as feasible (i.e. using polarized substrata) must be developed. The importance of epithelial attachment of sperm can then be addressed. Using a combination of these experimental approaches many of the important questions posed can be addresed confidently in the near future.

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Sperm numbers and distribution within the human Fallopian tube around ovulation

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This study aimed to determine the number and distribution of spermatozoa within the human Fallopian tubes around ovulation. Parous women, undergoing total abdominal hysterectomy for menorrhagia, were inseminated with either partner's semen (3/10) or donor semen (7/10). Approximately 18 h later both Fallopian tubes were ligatured into ampullary, isthmic and intramural regions. These were removed and assessed for sperm content by flushing, scanning electron microscopy (SEM) or homogenization. A median of 251 spermatozoa were recovered (range, 79–1386). The number of spermatozoa within each tube was not significantly different. The ovulatory ampulla contained a significantly ($P \leq 0.01$) larger percentage of spermatozoa than the non-ovulatory ampulla. The number of motile spermatozoa inseminated was not significantly correlated to the number of spermatozoa recovered, but a trend was identified. The time between the onset of the luteinizing hormone surge and hysterectomy was significantly correlated ($P \leq 0.01$) to the number of spermatozoa within the intramural regions, but not to the tubal sperm distribution. Spermatozoa were not observed, by SEM, bound to the tubal epithelium. These data suggest that, after artificial insemination at least, sperm access to the human Fallopian tube may be controlled, but that ovulation does not affect the redistribution of spermatozoa between tubal regions and that the isthmus does not appear to act specifically as a sperm reservoir.

Key words: Fallopian tubes/human sperm transport/ovulation/storage

Introduction

Sperm transport to and distribution within the Fallopian tube, around the time of ovulation, are processes which remain very poorly investigated in the human. This is primarily due to the ethical and logistic considerations involved in research using human subjects. Most published information concerning the processes of sperm transport and storage in the female reproductive tract come from extensive work in animal models (for review, see Barratt and Cooke, 1991).

Non-human mammalian species have demonstrated several consistent characteristics of sperm transport to and storage within the oviducts. Spermatozoa are present within the oviducts in numbers which are orders of magnitude less than those inseminated (Smith *et al.*, 1987; Hunter, 1988), highlighting a large degree of sperm loss. In the rabbit, spermatozoa are transported from the site of insemination to the site of fertilization under two régimes: rapid transport (Overstreet and Cooper, 1978) and sustained migration (Overstreet *et al.*, 1978). Spermatozoa supplied by rapid transport have been proposed to act as local messengers to prepare the oviduct for the supply of fertile spermatozoa during the sustained migration phase (Overstreet *et al.*, 1989). In many mammalian species, the isthmic region of the oviduct appears, in the period prior to fertilization, to behave as a distinct region for the sequestration of spermatozoa (Harper, 1973; Smith *et al.*, 1987; Suarez, 1987; Hunter, 1988), with this storage region being independent of the site of semen deposition (i.e. cervix or uterus). The rate of sperm transport to the site of sequestration from the site of insemination has been shown to increase as the time period between insemination and ovulation decreases (Hunter *et al.*, 1982; Ito *et al.*, 1991), indicating a level of control in the supply of spermatozoa to the site of fertilization. Spermatozoa remain within the isthmic region, for species-dependant lengths of time, until ovulation when they are released to the ampulla in very low numbers (Hunter, 1987; Smith *et al.*, 1987). In the hamster, the attachment of spermatozoa to the oviductal epithelium has been suggested to play a role in both storage and maintenance of sperm viability (Smith and Yanagimachi, 1990). Changes in the oviductal environment, such as temperature (Hunter and Nichol, 1986), potassium and pyruvate concentrations (Burkman *et al.*, 1984) or the presence of mucus (Fléchon and Hunter, 1981; Hunter *et al.*, 1991) may also affect the storage process. This work in mammals clearly demonstrates that sperm transport within the oviduct is a highly controlled process, regulated to synchronize the presence of both sets of gametes at the sites of fertilization.

There are few published studies that have investigated sperm numbers within the human Fallopian tube (Rubenstein *et al.*, 1951; Settlage *et al.*, 1973; Mortimer and Templeton, 1982). In general, these studies have lacked standardization of experimental protocol with inaccurate assessment of ovulatory status and varying time intervals between insemination/coitus and sperm recovery. Furthermore, the subject groups have included infertile patients and patients with pathologies likely to affect

sperm transport. In addition the efficiency of the techniques used to retrieve and quantify spermatozoa have often also not been determined. Although specific details vary, all studies have reported a large decrease in sperm numbers present within the Fallopian tubes, in comparison to the numbers inseminated (Settlage *et al.*, 1973; Ahlgren, 1975; Mortimer and Templeton, 1982; Williams *et al.*, 1992; for review, see Barratt and Cooke, 1991). For example, Williams *et al.* (1992) detailed the recovery of 815 spermatozoa, 2 days post-ovulation, from the Fallopian tubes of a histologically normal woman of proven fertility, 18 h after artificial insemination. Precise details on the distribution of spermatozoa in the peri-ovulatory human Fallopian tube, and any effect ovulation may have on the numbers present along with their redistribution, remains undocumented. Thus, the sequestration of spermatozoa within specific tubal regions, as seen in other mammals, has not been evaluated.

Spermatozoa have been shown, in a group of infertile women, to reside within the Fallopian tubes for up to 85 h *post coitum* (Ahlgren, 1975). In addition, *in vivo*, fertilization has been shown to occur when a single act of coitus has occurred from 6 days prior to and 3 days post-ovulation (France *et al.*, 1992). Thus, spermatozoa appear to be able to gain access to and remain functionally viable within the Fallopian tubes over a large number of days. Whether this involves specific storage regions within the Fallopian tubes needs to be determined.

The aim of this study was to utilize an experimental protocol with greater standardization, compared to previously published studies, to: (i) accurately determine the numbers of spermatozoa present within the Fallopian tubes of normal, proven fertile, women, around the time of ovulation, (ii) determine the distribution of spermatozoa within the Fallopian tubes, and (iii) determine any changes in sperm numbers and distribution in relation to the time of ovulation. From this information, our currently limited knowledge regarding the process of sperm transport and storage within the human Fallopian tubes will be improved and further, more specific, hypotheses can be developed and tested.

Materials and methods

Patient recruitment

Patients were recruited from March 1991 until November 1992. Women on the total abdominal hysterectomy waiting list at the Jessop Hospital for Women were approached by letter if they met the following criteria: (i) ≤ 41 years old, (ii) parous, (iii) no previous history of tubal or uterine pathology, (iv) regular menses, and (v) not previously sterilized. The age limit was set at less than 41 years old, as it has been demonstrated that hormonal function deteriorates to become abnormal beyond this age (Lenton *et al.*, 1988). Using the above criteria it was felt that the patients recruited for the study would have no particular problems that could influence sperm transport, and would be more standardized than recruited for previous studies. Ten women referred for total abdominal hysterectomy because of heavy menstrual bleeding elected to take part in the study. Patient recruitment and all treatment procedures had been passed by an ethical committee (University of Sheffield). All patients were counselled in detail regarding the need to accurately time the menstrual cycle, the technique to be employed for artificial insemination, any risks associated with artificial insemination (particularly the use of donor semen), and the minor variations to the hysterectomy technique necessary for the careful removal of the Fallopian tubes.

Cycle timing

All women were monitored daily for luteinizing hormone (LH) and oestradiol concentrations (Serozyme, Serono Diagnostics Ltd, Woking, UK) using either morning urine or serum samples, from day 10 of their cycle to the morning of the hysterectomy. This allowed the identification of the LH surge and determination of the time of ovulation (Lenton, 1993). The women were asked to refrain from sexual intercourse, unless using a sheath, from the start of the study cycle. This prevented contamination of the reproductive tract with spermatozoa prior to insemination. The women were admitted to the Jessop Hospital for Women at the closest convenient time-point after the onset of the LH surge (LH $\geq$ 10 mIU/ml).

Insemination

Cervical insemination was carried out on the evening of the woman's admission, approximately 18 h prior to the expected time of hysterectomy. It was hoped that this time period, from insemination to hysterectomy, would allow the study of sperm populations that had been established over a period of sustained migration, thus representing the fertilizing population (Hunter, 1987). Insemination was chosen in preference to coitus to allow for an assessment of semen parameters of the ejaculate, a determination of the number of spermatozoa inseminated, and the standardization of the timing from insemination to hysterectomy.

Of the group of women recruited to the study, seven had partners who had had a vasectomy and it was therefore necessary to use proven fertile frozen donor semen for insemination. The three partners who had not been vasectomized produced a semen sample by masturbation. This was allowed to liquefy at 37°C for 30 min before 0.5 ml was removed and used for intracervical insemination (mucus sampling syringe, Rocket, London, UK). The remainder of the sample was analysed for the characteristics of sperm motility (Hamilton Thorn motility analyser, Hamilton Thorn Research Inc., Danvers, MA, USA) (Zhu *et al.*, 1992). For those women whose partners had had a vasectomy, frozen donor semen was thawed and 0.5 ml was inseminated into the endocervix, with the remainder used for motility assessment. The median number of motile spermatozoa inseminated was 6.1×10^6 (range $2.85-38.7 \times 10^6$). This wide range was brought about by the use of either partner or donor semen.

Total abdominal hysterectomy

During total abdominal hysterectomy, the Fallopian tubes were identified and ligatured into ampullary, isthmic and intramural regions in order to prevent mixing of the luminal contents. During surgery, handling of the tubes was kept to a minimum. The tubes were dissected carefully from the ovaries and removed intact with the uterus. The tubes were then dissected from the body of the uterus. The presence of a follicle or corpus luteum on the ovary was recorded along with which tube came from the side of ovulation. Subsequent histological assessment of the

endometrium was used to confirm the ovulatory status of each woman and the normality of the endometrium.

Tubal processing

Each tube was processed as has been described previously (Williams *et al.*, 1992). The segments from the tube on the side of ovulation were flushed, with the aim of recovering spermatozoa to assess their numbers and functional status. The tube contralateral to the ovulatory ovary was processed either by scanning electron microscopy (SEM), or by homogenization. SEM of a section of each segment of each tube was carried out to enable observation of any sperm/epithelial interaction *in vivo*. Homogenization enabled complete segments rather than small proportions of each segment to be processed, thus quantifying the total numbers of spermatozoa present.

Treatment of the Fallopian tube from the side of ovulation

Flushing procedure. The tubal segments were flushed and the flush fluids treated as has been described previously (Williams *et al.*, 1992), with minor modifications. Briefly, each segment was flushed with 5–6 ml of Earle's balanced salt solution at 37°C (Gibco Ltd, Uxbridge, UK). A 10 μl of aliquot of each flush fluid was removed and observed (phase, ×400 magnification, Laborlux S, Leitz, Germany) for the presence of spermatozoa. The fluids were then incubated with a lysis buffer (10 mM $KHCO_3$, 0.155 mM NH_4Cl, 0.1 mM EDTA, Sigma Chemical Co., UK) for 15 min prior to centrifugation to pellet the contents at 3000 *g* for 30 min. Following centrifugation, the pellets were resuspended into a known volume of approximately 200–300 μl of supernatant. Two 10 μl aliquots of the pellet resuspension were removed and observed for the presence of spermatozoa using phase-contrast microscopy at ×400 magnification. The remainder of the suspension was spotted onto multispot glass slides (Hendley Ltd, Loughton, Essex, UK) in 10 μl aliquots and stained for sperm quantification, as described previously (Williams *et al.*, 1992).

SEM. The flushed segments were prepared for SEM to allow for the assessment of the number of spermatozoa that remained in the tube after flushing. The segments were fixed overnight at room temperature in 3% glutaraldehyde (Taab Laboratories, Aldermaston, UK) in 0.1 M sodium phosphate buffer (BDH, Poole, UK). The segments were then placed in 0.1 M sodium phosphate buffer (BDH) and stored at 4°C until processed. The fixed segments of Fallopian tube were washed well in 0.1 M sodium phosphate buffer, divided into three or four small sections and then post-fixed in 2% aqueous osmium tetroxide (Oxchem, Oxford, UK) for 2 h. Each section was then prepared for SEM, as has been described previously (Williams *et al.*, 1992).

Cannulation of the lumen. Prior to flushing in patients 6–10, the tubal segments were cannulated to recover native tubal fluid. A 20 μl capillary tube, which had been pulled to produce a fine tip (Microcaps, Drummond Scientific Co., USA), was passed carefully into the lumen of each segment and any fluid present, allowed to enter the tube by capillary action. This fluid was observed (×400 magnification, phase) for the presence of spermatozoa.

Control procedures. To assess the efficiency of the flushing technique, control segments of human Fallopian tube were inseminated with a known number of washed spermatozoa (median, 972; range, 474–40 976) and left for 15 min. Each segment was then flushed, as described above, and the numbers of spermatozoa recovered were recorded.

Treatment of the Fallopian tube contralateral to the side of ovulation

SEM. In patients 1–5, the segments from the tube contralateral to the ovulatory ovary were fixed and examined by SEM as described above. The number of spermatozoa present was quantified, as has been described previously (Williams *et al.*, 1992). Briefly, the surface area of the mucosa of each segment was calculated. Using this, together with the segment length, the area of segment scanned and the number of spermatozoa seen, the total number of sperm per segment could be calculated.

Homogenization. The tube segments from patients 6–10 were homogenized (modified from Johnson and Varner, 1988). The tubes were stored at −20°C prior to homogenization. Briefly, each tube segment was chopped into small pieces, added to 15 ml of homogenization fluid (0.5 M NaCl, 0.05% (v/v) Triton X-100, 3.8 mM NaN_3; Sigma) and then homogenized in a Waring blender until very fine. The homogenate was then filtered through 20 μm polyester mesh (Lockertex, Warrington, UK). This pore size allowed sperm passage, but removed the larger pieces of connective tissue which did not homogenize (unpublished observations). The filtrate was centrifuged at 1500 *g* for 30 min, and the pellet was resuspended in approximately 0.5 ml of supernatant. Aliquots of 5 μl were spotted onto multispot slides, air-dried and stained using the Feulgen DNA staining method (Drury and Wallington, 1967) to quantify the intact sperm heads within the homogenized tissue.

Control procedures. A control segment of Fallopian tube was inseminated with 230 washed sperm and left for 15 min. This was then processed for SEM as above and enabled assessment of the efficiency of the SEM technique. To determine the efficiency of the homogenization technique, control segments of Fallopian tube were inseminated with known numbers of washed sperm (median, 6533; range 509–112 333). These controls were then treated as described above.

Statistical analysis

All data analysis was performed using the SPSS.PC statistical package (Ashton Tate UK Ltd, UK). Non-parametric analyses were carried out due to the small numbers of patients involved and the limited nature of the data. The Pearson's rank correlation test was used to correlate the numbers of motile spermatozoa inseminated, and the time-period between the onset of the LH surge and the hysterectomy, to the number and percentage distribution of recovered spermatozoa. The Mann–Whitney *U*-test was used to analyse for differences between the numbers and distribution of spermatozoa between the different groups of patients. The Wilcoxon matched-pairs signed-ranks test was used to analyse for differences between sperm numbers and distribution within the ovulatory and non-ovulatory tubes. Because many comparisons were made on the same data, and the patient numbers were low, a correlation or difference was considered significant only at significance levels of $P \leq 0.01$. It was considered that with $P \leq 0.05$ a significance due to chance may arise.

Results

Selection of the required patient group was made difficult as a high proportion of those women on the waiting list for total abdominal hysterectomy were either over 40 years of age, previously sterilized, non-parous or had additional pathology and did not therefore fit the acceptance criteria. Over the study period of 21 months, 10 women replied to the recruitment letter and subsequently consented to take part in the trial. All ten patients complied well with the daily LH assessments and short notice admission prior to hysterectomy.

Patient group

The tubal pathology of those women recruited to the trial, the time between the onset of the LH surge and hysterectomy, the time interval between insemination and hysterectomy, semen type and number of motile spermatozoa inseminated are shown in Table I. In this table, the patients are numbered chronologically, with respect to their recruitment to the study, but they are ordered in sequence from pre-ovulation through to post-ovulation. The ovulatory status of each woman at the time of hysterectomy is also indicated. Five of the women recruited had their hysterectomies prior to ovulation, while five were carried out post-ovulation. At hysterectomy, patient 2 demonstrated a hydrosalpinx of the Fallopian tube on the side of ovulation, and a histological assessment of the endometrium indicated the presence of small fibroids. Patient 3 did not have an LH surge as expected, but was confirmed to be pre-ovulatory by the presence of a follicle. All patients, except patient 2 who had fibroids, were demonstrated to have a normal endometrial and uterine histology. When those patients inseminated with partner's semen were compared to those inseminated with donor semen, no significant differences were detected between the time from insemination to hysterectomy, the time between the onset of the LH surge and hysterectomy, the number of motile spermatozoa inseminated and the percentage sperm motility within the semen sample. Their data were therefore pooled for all subsequent analysis, as it was considered that adequate standardization between the two groups had been achieved.

Sperm recovery

The recovery efficiencies for the three techniques used to quantify spermatozoa recovered from the Fallopian tubes are shown in Table II. These results on the efficiency of recovery demonstrate that the experimentally determined values of sperm numbers within the Fallopian tubes were probably an underestimation of the true values, but were not inaccurate by orders of magnitude.

Using the flushing technique, spermatozoa were recovered from the Fallopian tube on the side of ovulation in all ten patients (Table III) (median of total spermatozoa, 136; range 23–1224). Spermatozoa were never observed within the aliquots removed from the flush prior to staining, due to their low numbers, and therefore motility assessment was not possible. Cannulation of the ampullary segments from patients 6–10, prior to flushing, produced approximately 20 μl of fluid in each case. Only four spermatozoa were observed after cannulation and all of them came from the ampullary native fluid of patient 7. Three of these four spermatozoa were motile, displaying the qualitative characteristics of hyperactivation (Burkman, 1984); the fourth spermatozoa showed a non-progressive weak movement. Cannulation of the isthmic and intramural regions of the Fallopian tube proved to be technically very difficult, due to the small diameter of the lumen and the very small volumes of fluid present. Spermatozoa were observed within the Fallopian tubes examined by SEM after flushing, but in patient number 7 only. These spermatozoa were lying on the surface of the mucosa and were not obviously attached to the epithelium.

The total numbers of spermatozoa observed by SEM within the non-ovulatory Fallopian tube are shown in Table III (median, 0; range, 0–673). Spermatozoa were never observed to be present within the folds of the tubal mucosa, and were not obviously attached to the epithelial cell surface on which they lay. After homogenization a median of 225 (range, 162–951) spermatozoa were recovered from the non-ovulatory tube. No significant difference existed between the number of spermatozoa recovered from the non-ovulatory tube by homogenization compared to the number calculated to be present by SEM. Thus, all data detailing the contralateral tube, obtained by the two

Table I. Pathology, timing of insemination and hysterectomy and semen characteristics for women undergoing total abdominal hysterectomy

Patient	Pathology	Interval from LH surge to hysterectomy (days)	Interval from insemination to hysterectomy (h)	Semen type	Number of motile spermatozoa inseminated ($\times 10^6$)
3[a]	Normal	?[c]	20½	Donor	3.00
10[a]	Normal	0	19½	Donor	11.04
5[a]	Normal	½	18½	Donor	3.35
9[a]	Normal	1½	19	Donor	6.60
4[a]	Normal	1½–2	20½	Partner	2.85
2[b]	Hydrosalpinx (ovulatory side)	2	20½	Donor	5.35
7[b]	Normal	2–2½	18½	Partner	38.70
6[b]	Normal	2½	19	Donor	6.10
8[b]	Normal	2½	17	Donor	11.50
1[b]	Normal	4	22	Partner	6.20

[a]Pre-ovulatory at hysterectomy.
[b]Post-ovulatory at hysterectomy.
[c]Unknown.
LH = luteinizing hormone.

methods, were pooled. The median of the total number of spermatozoa within the non-ovulatory tube for the whole group was therefore 162 (range 0–951). The total number of spermatozoa calculated to be present within both Fallopian tubes (median, 251; range 79–1386) represented approximately 1 in every 25 000 (median) of those motile sperm which had been inseminated (range, 1:8000 to 1:68 000), i.e. only 0.004% (0.013–0.001%) of those motile spermatozoa inseminated achieved access to the Fallopian tubes and were recovered.

No significant difference was detected between the numbers of spermatozoa present within the tubes of patients who were pre-ovulatory at hysterectomy compared to those who were post-ovulatory. Also, no significant difference was detected between the numbers of spermatozoa present within the non-ovulatory tube compared to the ovulatory tube. For patients 2 and 3, who demonstrated slight abnormalities from the initial acceptance criteria, no obvious deviations from the rest of the group in terms of sperm numbers and distribution within the Fallopian tubes (Table III) were observed. The data from these two patients have therefore been included in all the following analyses, as it appeared that there had been no obvious effect on sperm transport and distribution in these two women.

Effect of the numbers of motile spermatozoa inseminated, and the time-period from the onset of the LH surge to the hysterectomy, on sperm numbers and distribution within the Fallopian tubes

The numbers of motile spermatozoa inseminated and the time from the onset of the LH surge to hysterectomy were correlated with the numbers and percentage distribution of spermatozoa within the Fallopian tubes, to determine whether any effect of these factors could be detected on sperm transport. Correlations were determined for the group as a whole and individually for those patients whose hysterectomy occurred prior to ovulation and those patients whose hysterectomy occurred post-ovulation.

Numbers of spermatozoa within the Fallopian tubes

As no significant difference was detected in sperm numbers between the ovulatory and non-ovulatory tubes, correlations will be described for the combined data from like segments. In the whole group, a significant correlation ($r = 0.8258$, $P \leq 0.01$) existed between the number of motile spermatozoa inseminated and the total number of spermatozoa within the combined isthmic regions. This significance was not evident for the total number of spermatozoa recovered, although a trend was observed (Figure 1). In the pre- and post-ovulatory groups, no significant correlations were detected between the number of motile spermatozoa inseminated and the number recovered.

Table II. Percentage recovery efficiency of the techniques used to quantify spermatozoa collected from the Fallopian tubes

Technique	*n*	Percentage recovery	
		Median	Range
Flushing	5	61	32–100
SEM	1	83	—
Homogenization	4	25	20–45

N = number of experiments; SEM = scanning electron microscopy.

The time-period from the onset of the LH surge to the time of hysterectomy was significantly positively correlated to the number of spermatozoa within the intramural regions in the whole group ($r = 0.8681$, $P \leq 0.01$), and for the pre-ovulatory group ($r = 1.000$, $P \leq 0.001$). For the post-ovulatory group, no significance was detected.

Percentage distribution of spermatozoa

The percentage of spermatozoa within the segments of both Fallopian tubes are included in Table III. The percentages of spermatozoa within each segment of the Fallopian tubes were not significantly different between the pre- and post-ovulatory patients. In the whole group, there was a significantly ($P \leq 0.01$) greater percentage of spermatozoa within the ampullary region of the ovulatory tube compared to the percentage present within the ampullary region of the contralateral tube. No significant correlations were detected between the time-period from the onset of the LH surge to hysterectomy, and the percentage of spermatozoa within the combined regions for the whole group

Table III. Number (%) of spermatozoa recovered from the ampullary, isthmic and intramural regions of the Fallopian tubes

Patient	Ovulatory tube			Non-ovulatory tube		
	Ampulla	Isthmus	Intramural region	Ampulla	Isthmus	Intramural region
3[a]	99 (40)	28 (11)	124 (49)	0 (0)	0 (0)	0 (0)
10[b]	12 (53)	7 (30)	4 (17)	0 (0)	166 (100)	0 (0)
5[a]	64 (48)	58 (43)	12 (9)	0 (0)	0 (0)	0 (0)
9[b]	100 (53)	63 (33)	26 (14)	252 (47)	283 (53)	0 (0)
4[a]	15 (14)	65 (59)	30 (27)	0 (0)	0 (0)	0 (0)
2[a]	14 (18)	30 (38)	35 (44)	0 (0)	0 (0)	0 (0)
7[b]	1040 (85)	174 (14)	10 (1)	79 (49)	70 (43)	13 (8)
6[b]	0 (0)	114 (83)	24 (17)	ND	ND	ND
8[b]	87 (78)	24 (22)	0 (0)	561 (59)	344 (36)	46 (5)
1[a]	97 (68)	26 (18)	19 (14)	445 (66)	128 (19)	0 (15)

[a]Ovulatory tube flushed, non-ovulatory tube observed by scanning electron microscopy.
[b]Ovulatory tube flushed, non-ovulatory tube homogenized.
ND = no data.

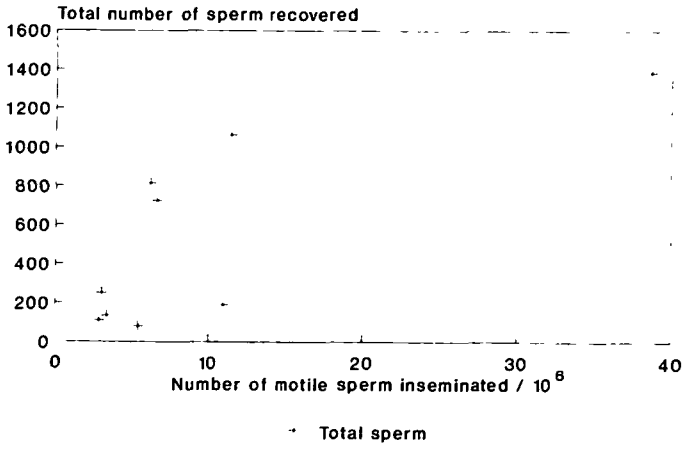

Fig. 1. Relationship between the number of motile spermatozoa inseminated and the total number of spermatozoa recovered from Fallopian tubes ($n = 9$).

or for the pre- and post-ovulatory groups individually. When the ampullary regions of the two tubes were treated separately, because of their difference in distribution in the whole group, there was still no significant correlation between timing and the percentage of spermatozoa present within either tube.

Discussion

The present study recruited women to enable a database to be determined, describing sperm numbers and their distribution within the Fallopian tubes around ovulation. The women who elected to take part in the study were as standardized and as 'normal' as could be recruited, and there was no reason to believe that their need for total abdominal hysterectomy would compromise sperm transport adversely. All but one of them had normal uterine histology, thus eliminating the possibility of uterine pathology affecting sperm transport. The strict inclusion criteria meant patient numbers were limited. Two patients recruited to the study after fulfilling the initial acceptance criteria presented with pathological or hormonal abnormalities at the time of hysterectomy. However, their sperm numbers and distribution within the Fallopian tubes were not obviously different when compared to the remaining patients. Sperm transport to the Fallopian tubes from the cervix had evidently not been significantly affected by these abnormalities.

The present study design enabled a standardized timing between insemination and ovulation, and a relatively constant timing from insemination to hysterectomy of 17–22 h. The timing of admission of the patients allowed the hysterectomies to be evenly spread within a small window of 4 days after the onset of the LH surge. Logistically, it was not possible to tighten this window, but the spread in timing of the hysterectomies meant that the influence of the time to ovulation on sperm numbers and distribution could be investigated. The constraints of a routine operating list did not permit any further decrease in the range in time between insemination and hysterectomy. If a sustained migration of spermatozoa to the Fallopian tubes had occurred, it could be assumed that by 17 h the fertile sperm population may have been established within the Fallopian tubes, so that the range in timing would have had little effect on the results achieved. It was not possible to detail the hour-by-hour changes that may have occurred when follicle rupture was imminent, but it was hoped that any changes that may have occurred over this short time-period would have been observed when the patients were divided into those whose hysterectomies occurred pre-ovulation or post-ovulation.

Spermatozoa were consistently recovered from the Fallopian tubes in small numbers. Sperm numbers decreased by an order of magnitude from the site of insemination to the site of fertilization; this observation accords with those made on other mammalian species (for review, see Barratt and Cooke, 1991). Very few spermatozoa were observed after SEM of the flushed tubes, demonstrating that the flush technique was efficient at removing those sperm present.

Semen type could not be standardized to partner only, since most of the women had partners who had been vasectomized. The protocol was not standardized to donor only, as the use of partner's semen allowed an investigation of the effect of a greater range in the number of spermatozoa inseminated.

The use of frozen donor spermatozoa did not appear to affect their transport to the Fallopian tubes. The number of spermatozoa recovered in this group were very similar to those recovered from the Fallopian tubes of two patients in whom a similar number of fresh spermatozoa were inseminated initially. The numbers of motile spermatozoa used for insemination in this study were similar to those regularly used for donor insemination (Barratt *et al.*, 1990), and which are known to lead to successful fertilization and pregnancy. Our results indicate that the previously frozen donor spermatozoa were transported and distributed within the Fallopian tubes in the same manner as those of fresh semen from the male partner.

The greatest number of spermatozoa were recovered from the patient with the highest number inseminated (patient 7) and whose hysterectomy was carried out 2–2½ days after the onset of the LH surge. It is not known whether larger numbers of spermatozoa were recovered because more were inseminated or whether the timing of hysterectomy was important. Since the recovery ratio (number of spermatozoa recovered/number of motile spermatozoa inseminated) for this patient (1:28 000) did not obviously differ from those for other patients in the group (median, 1:25 000; range 1:8000–1:68 000), it seems that there was a relationship between large number of spermatozoa recovered and the greater number inseminated. This conclusion was supported by the trend observed between the number of motile spermatozoa inseminated and the total number recovered; this concentration effect should be tested over a wider range of sperm concentrations in a similar patient group.

The fate of those spermatozoa not recovered from the Fallopian tubes was not apparent from the present study. Spermatozoa may have traversed the tubes to the peritoneal cavity (Ramsewak *et al.*, 1990), or have been lost from the vagina (Gallager and Senger, 1989; Baker and Bellis, 1993), or may have remained within the cervix.

To date, there have been no attempts to determine whether sperm transport between the two Fallopian tubes of the human female varies. In the present study, ovulatory and non-ovulatory Fallopian tubes were compared to identify any differences in the number of spermatozoa and their distribution. A significant difference was detected between the percentage of spermatozoa recovered from the ampullary region of the ovulatory tube as compared with the non-ovulatory tube. The greater proportion of sperm within the ovulatory ampulla may have been in preparation for fertilization. If subtle differences exist between the environment of the ovulatory and non-ovulatory tubes, these differences may exhibit some control over the internal distribution of spermatozoa. In the rhesus monkey, the oxygen tension within the ovulatory oviduct has been shown to differ from that of the non-ovulatory oviduct (Maas *et al.*, 1976). In the cow, the oviduct ipsilateral to ovulation has been demonstrated to exhibit a much greater muscular and secretory activity, as well as a decreased luminal sodium concentration compared to the oviduct contralateral to ovulation (for review, see Ellington, 1991). Thus, variations exist in the internal environment of the oviducts in other mammals, and they need to be investigated in

detail in the human, especially with reference to their effect on sperm transport.

The functional status of those spermatozoa recovered from the Fallopian tubes could only be assessed for the four spermatozoa within the native ampullary fluid of a single patient. Three out of four of these spermatozoa showed the characteristics of hyperactivation. While the numbers recovered were extremely low, the data are in agreement with the situation in other mammals, where spermatozoa present within the ampulla have been shown to be hyperactivated (Shalgi and Phillips, 1988; Demott and Suarez, 1992). Sperm hyperactivation in the ampulla is thought to facilitate detachment of spermatozoa from the mucosal walls and prevent entrapment within the isthmus, enabling fertilization to occur (Coddington *et al.*, 1991; Demott and Suarez, 1992). An assessment of the functional characteristics, such as motility, hyperactivation, capacitation and acrosomal status of those spermatozoa within the flush fluid was not feasible due to their small numbers. It was, therefore, not possible to determine whether the spermatozoa recovered from the Fallopian tubes were a selected functionally fertile population.

Future investigation of the process of sperm transport within the human female reproductive tract must aim to increase the numbers of spermatozoa within the Fallopian tubes and improve their recovery techniques, thus aiding the assessment of functional status. Increasing the numbers of spermatozoa present may be possible by the use of intracervical insemination of a very large number of spermatozoa in a small volume, inseminating spermatozoa directly into the uterine cavity or carrying out repeated pulsatile inseminations. If a physiological block exists to prevent large sperm numbers, such manipulations may be ineffectual in increasing sperm numbers within the Fallopian tubes to sufficiently high levels to assess their functional status. Flush techniques could be improved, but it must be remembered that the media may affect sperm function. The tubal lumen could be cannulated, if a sufficiently fine cannula could be produced to collect and examine the microlitre fluid volumes present within the isthmus and intramurine region. Transillumination of the human Fallopian tube is not possible, as used for the mouse oviduct (Suarez, 1987), and therefore human spermatozoa cannot be viewed *in situ* within excised tubes. It is important, therefore, that observation of sperm *in vivo* be developed, using techniques such as falloposcopy (Scudamore *et al.*, 1992).

Sperm/epithelial binding and sperm redistribution around the time of ovulation were investigated in relation to the existence of sperm storage reservoirs within the Fallopian tube. There was no evidence of binding of spermatozoa as observed by SEM in either the ovulatory or non-ovulatory tubes. Insufficient data existed to decide conclusively whether binding occurs between spermatozoa and the tubal epithelium, and more work is needed both *in vivo* and *in vitro*, to assess epithelial binding as a feature of human sperm transport, as observed in other mammals (Smith and Yanagimachi, 1990; Pollard *et al.*, 1991; Suarez *et al.*, 1991).

The percentage distribution of spermatozoa within each tube was not influenced by the interval from insemination to ovulation, within the time-span studied. No significant difference in percentage distribution arose between the pre- and post-ovulatory groups although this may occur with a larger time-span. Spermatozoa were apparently not redistributed from one region of the tube to another in relation to ovulation, which contrasts with observations in other mammals (Barratt and Cooke, 1991). It is possible that such a redistribution occurred earlier, e.g. when the LH surge began, and was overlooked, but this would seem to be illogical, since spermatozoa would be present at the site of insemination ~40 h prior to ovulation (Lenton, 1993), a time span much greater than in other mammals.

An increase in the number of sperm inseminated led to a greater number recovered from the isthmus, but not the ampulla or intramural region. The formation of an isthmic sperm 'reservoir', as in other mammals (Barratt and Cooke, 1991), could increase the number of sequestrated spermatozoa in this region as more were inseminated. Although spermatozoa may be sequestered by the isthmus, the lack of an obvious redistribution implies that a steady supply of spermatozoa to the ampulla is likely, rather than a sudden influx from a specific storage region.

Sperm numbers within the intramural region apparently rose with increasing time between the onset of the LH surge and the time of hysterectomy, i.e. as ovulation approached. Spermatozoa within the ampullary and isthmic regions showed no corresponding decrease, so it is unlikely that the increase in intramural spermatozoa arose through sperm migration from these regions. It is more feasible that spermatozoa were supplied from lower in the female tract. Perhaps sperm transport to the intramural region is enhanced as ovulation approaches as in other mammals (Hunter *et al.*, 1982). No corresponding increase existed beyond the intramural region, which may indicate a limitation on sperm numbers at the site of fertilization.

More patients must be recruited for well-timed, standardized studies to amplify the present analyses. Spermatozoa must be sampled from the Fallopian tubes at varying times to ovulation and larger numbers of spermatozoa must be recovered if their functional status is to be assessed. Other regions of the female reproductive tract must be examined, to identify any alternative sites of sperm storage.

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The Leukocytic Reaction of the Human Uterine Cervix

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PROBLEM: Sperm interaction with the immune system of the human cervix is poorly understood.
METHOD: The leukocytic response of the human cervix to sperm was examined in a closely monitored patient population (N = 10), using monoclonal antibody cell identification techniques. Baseline data were collected from both cervical mucus and smears sampled before treatment by donor insemination. Donor insemination was timed to coincide with ovulation by monitoring plasma LH concentrations twice daily. Following insemination the numbers of leukocytes were recorded in cervical mucus and smear samples taken over a 24-h period relative to the time of treatment. Controls treated with "pure sperm," seminal plasma, cryopreservative, and cervical smearing alone were also included in the study.
RESULTS: Only those women treated with sperm cells exhibited substantial elevations in leukocyte numbers following inseminations. Additionally, serial cervical smearing induced an inflammatory response of the cervix. In all the women, the neutrophil was the predominant leukocyte of the cervix both during the baseline and treatment periods (median values ranged from 77 to 86%). Macrophages, T-helper lymphocytes, and T-suppressor lymphocytes were also detected, but only in low numbers (2–10.6%). Two patients and one control ("pure sperm") became pregnant during their study cycle.
CONCLUSIONS: We conclude that the leukocytic reaction is a physiological response of the cervix to sperm, the function of which remains to be established. (Am J Reprod Immunol. 1992; 28:85–89.)

Key words: Sperm, cervical mucus, cervical smears, pre-and postinsemination, neutrophils

INTRODUCTION

Following the introduction of sperm into the mammalian reproductive tract a leukocytic influx is evoked across the uterine cervix,[1] the precise function of which remains unclear. However detailed studies in rabbits, ruminants, and cattle have enabled several hypotheses to be advanced, which include clearance by phagocytosis of the nonfertilizing sperm population and/or bacteria introduced during coitus,[2] sperm selection,[2,3] and supplementation of energy to the fertilizing sperm population.[4]

In humans very little is known of the leukocytic reaction (LR). However it is important to determine whether this response, which appears to be physiological,[5,6,7] is a prerequisite for unassisted fertilization in vivo. Human cervical mucus has recently been shown to be a "selective" filter, preventing penetration by morphologically abnormal sperm.[8] Such a process will substantially reduce the number of sperm entering the upper reproductive tract. Whether the LR forms part of this selective mechanism is yet to be established, but may be possible, as our previous studies have shown that the neutrophil, a phagocytic cell, is the major leukocyte involved in this response.[6,7]

There has been only one study to date[5] that has examined the human LR in any detail. As this study relied on the basic Papanicolaou stain it was not possible to identify the different leukocyte subsets involved in this response. In order to establish the role of the LR, accurate identification of the leukocytes involved is necessary. In the present study we expand on our initial observations of the LR, reported previously,[7] and examine the LR over a 24-h sampling period in a group of 10 women using monoclonal antibody cell identification techniques.

MATERIALS AND METHODS

Patient Population

The patient group comprised 10 couples attending the University Research Clinic, Jessop Hospital for Women, Sheffield U.K., for treatment by donor insemination (DI). The female partners comprised the same 10 patients reported previously.[7] Fertility investigations identified male factor as being the primary source of infertility. From hormonal, laparoscopic, and hysterosalpingogram investigations the female partners were found to have a normal hormonal profile, uterus, and fallopian tubes, and were therefore presumed to be potentially fertile. A prerequisite for commencing the DI program was that the women were free from genital tract infection, which was excluded by cervical culturing.

Experimental Design

The couples abstained from intercourse for at least 2 days prior to and during the study period. The women attended clinic from approximately 3 days prior to ovulation for twice daily LH blood and cervical mucus (CM) monitoring (see Table I). On the morning of the first visit a cervical smear (CS) was also obtained. This period was termed the "baseline period."

Starting on the day of the onset of the LH surge, follicular development and rupture was monitored by transvaginal ultrasound scanning (Kretz Combison 320, Zipf, Austria). Treatment by DI was given 24 h following the onset of the LH surge. Because ovulation had previously been shown to occur approximately 36–40 h following the onset of the LH surge[9] (Chauhan M., unpublished data), this allowed the sperm ample time to reach the site of fertilization. All treatment samples were stored at −196°C. Each semen sample had been screened for sexually transmitted diseases[10] and routinely analyzed before and after freezing. The samples were frozen in a 1:1 ratio with egg-yolk glycerol cryopreservative. Only post-thaw samples with > 10 million motile sperm/ml were accepted into

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TABLE I. Flow Chart of the Study Protocol

	Time	Sample
Baseline Period	0900	blood
		CM[a]
		CS[b] (baseline)
	1600	CM
	2100	blood
		↓
		Onset of LH surge
	0900	blood
		scan
		CM
	1600	CM
	2100	blood
		↓
		24 h following onset of LH surge
Treatment Period	t = 0	blood
		scan
		CM
		CS
		↓
		treatment
	t = 20 min	CM
	t = 4 h	CM
		CS
	t = 24 h	blood
		scan
		CM
		CS

[a]CM = cervical mucus
[b]CS = cervical smear

the donor insemination program. All the treatment samples were stored in insemination straws (Rocket, London, U.K.) and were thawed at room temperature, which took about 15 min. For treatment two straws were used, each straw inserted into an endocervical inseminator (Rocket, London, U.K.) and its contents expelled into the endocervix. CM and CS were sampled over a 24-h period relative to the time of insemination and this period was termed the "treatment period" (Table I). Scans and blood samples were also taken during this period to determine whether ovulation had occurred.

Five groups of controls were also included in the study, which fitted the criteria of the patient group and consisted of the following: (1) two nonsmear controls, (2) two "pure sperm" controls, (3) two seminal plasma controls, (4) two cryopreservative controls, and (5) two smear controls. Control groups 1–4 were treated identically to the patient group apart from omission of the cervical smears. To identify the exact stimuli of the LR the controls in groups 2–4 were each treated with a separate component of frozen semen samples, which were "pure sperm," seminal plasma, and cryopreservative, respectively. To assess the contribution of cervical smearing to the LR, the women in group 5 did not receive treatment.

"Pure Spermatozoal" Preparation

The pure spermatozoal populations were prepared on a discontinuous Percoll gradient according to the method of Kessopoulou et al.[11] The 80% fraction, containing the motile sperm population, was used for treatment following washing for 10 min at 500*g* in Earles/bovine serum albumin medium (3 mg/ml BSA).

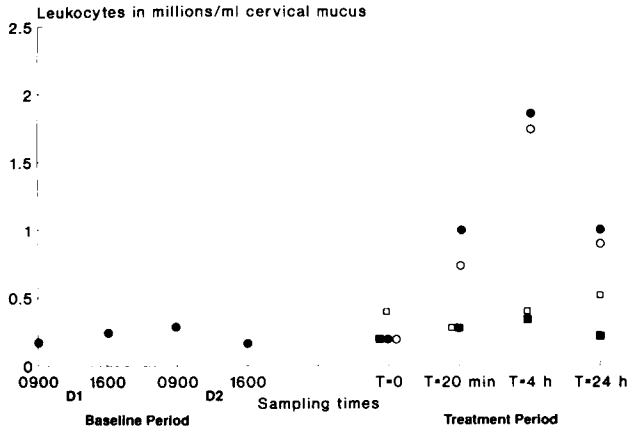

Fig. 2. The control group cervical mucus leukocyte data expressed as mean numbers of leukocytes per ml of cervical mucus. ● = nonsmear controls; □ = seminal plasma controls; ■ = egg-yolk glycerol controls; ○ = "pure sperm" controls.

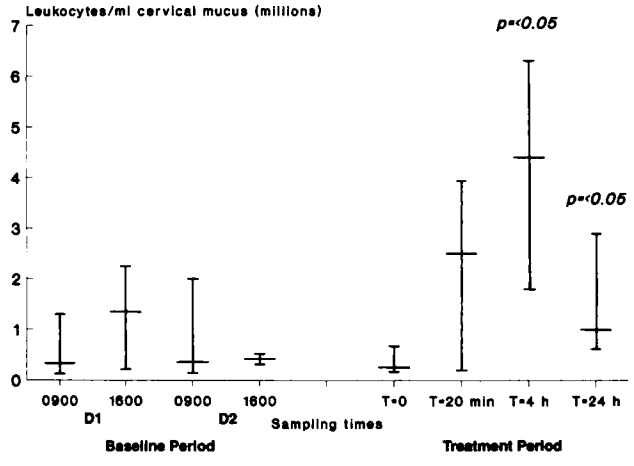

Fig. 1. The patient group cervical mucus leukocyte data expressed as median numbers of leukocytes with minimum and maximum values per ml of cervical mucus.

LH Measurement

Venous blood samples were obtained from each patient during the morning visit to the clinic, while the patients sampled their own blood in the evening using an autolet. The evening blood sample was returned to clinic at the next morning's visit. The two blood samples were assayed daily for LH concentration using a commercially available immunoradiometric assay (Maiaclone, Serono Diagnostics Ltd., Herts, U.K.; Assay sensitivity 0.15 U/L; inter- and intraassay coefficients of variation 3.9% and 1.6%, respectively).

TABLE II. The Individual Cervical Mucus Leukocyte Data of Three Women (Patients 1, 2, and 5) in the Patient Group Illustrating the Individual Variation in the Onset, Intensity, and Duration of the LR[a]

	Patient	T = 0	T = 20 min	T = 4 h	T = 24 h
Total Leukocytes	1	6.75	39.30	63.00	29.00
	2	1.75	22.00	44.00	7.00
	5	1.00	7.20	24.00	5.30
Neutrophils	1	3.75	31.00	42.50	21.00
	2	1.00	16.00	38.50	5.25
	5	1.25	6.50	22.00	4.50
Macrophages	1	0.75	1.95	4.50	2.0
	2	ND[b]	0.38	0.38	0.38
	5	ND	ND	0.75	0.25
T-helper cells	1	0.75	1.90	4.50	3.50
	2	0.25	0.75	1.52	1.25
	5	0.25	0.25	1.00	0.75
T-suppressor cytotoxic cells	1	0.75	1.10	4.00	1.50
	2	ND	ND	0.38	0.75
	5	ND	ND	0.25	ND

[a]Leukocytes are expressed × 10^5/ml of cervical mucus.
[b]ND = not detected.

Cervical Sampling

CM was sampled as outlined previously.[7] CS was performed following CM extraction by rotating an Ayres wooden spatula around the undocervix twice. The first rotation removed a dead cell layer, while the second rotation removed a living cell layer. Each CS was spread evenly and thinly over poly-l-lysine-coated slides using a coverslip. The slides were stored at −20°C for subsequent immunocytochemical staining.

Monoclonal Antibody Cell Identification Techniques

Immunobead cell selection assay

For reasons described previously immunocytochemical staining of the CM leukocyte populations proved an unsatisfactory method of leukocyte quantification.[7] Instead immunobead cell selection assays were performed on each CM sample; for details see Thompson et al.[7]

Immunocytochemical staining

Cervical smears were retrieved from storage at −20°C and left to thaw at room temperature. The slides were fixed and stained using the Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) procedure according to the method of Kessopoulou et al.[11] Each cervical smear was stained for total leukocytes, neutrophils, macrophages, and T lymphocytes. Positive and negative peripheral blood leukocyte controls were included in each staining procedure to control for MAb specificities and nonspecific binding/staining.

The slides were examined under bright field illumination (Leitz Laborlux, Wetslar, Germany) at ×400 magnification. Positively stained leukocytes were identified by red peripheral membranes and blue counterstained nuclei and these were quantitated per 100 squamous cells, according to the method of Pandya and Cohen.[5] As coefficients of variation of < 3% were achieved when reading the same area on a slide 10 times, the slides were subsequently read twice only, from which average values of leukocytes were calculated.

Statistical Analysis

The amount of time spent by each patient on the trial was dictated by menstrual cycle length. To enable standard statistical analyses to be performed on the data the study was split into two periods: (1) the baseline period, which incorporated the first two days of baseline data, and (2) the treatment period, which incorporated the t = 0–t = 24-h data. As the LR was subject to interpatient variation in terms of time of onset, intensity, and duration, it was only feasible to use descriptive statistics for most of these data, i.e., means, standard errors, medians, and minimum and maximum values. Where statistically appropriate, the data, which was nonparametric, was analyzed using the Mann-Whitney U test, the Wilcoxon signed-rank test, and the Spearmans rank correlation test.

RESULTS

The patients' CM leukocyte data are illustrated in Figure 1 as median numbers of leukocytes with minimum and maximum values. While each of the positive controls (both peripheral blood and CM leukocytes) run through the immunobead cell selection assay was antigen specific, none of the negative controls was specific to any of the primary MAb tested (data not shown). An average cell retrieval rate of 73.8% ± 1.59% (mean ± SE) was determined for the assay and, where statistically appropriate, coefficients of variation were calculated and were < 5%. Over the baseline period the neutrophil was the predominant leukocyte of the CM (median value 77%), while concentrations of macrophages, T-helper cells, and T-suppressor/cytotoxic cells were low (median values 8.25%, 10.5%, and 3.68%, respectively). A characteristic increase in leukocytes was observed in the day 1, 1600-h CM sample, which in some patients continued into the day 2, 0900-h sample (see maximum values at this sampling point). The increase corresponded to the CS given during the day 1, 0900-h visit and was not seen in the nonsmear control data (Fig. 2) where stable lev-

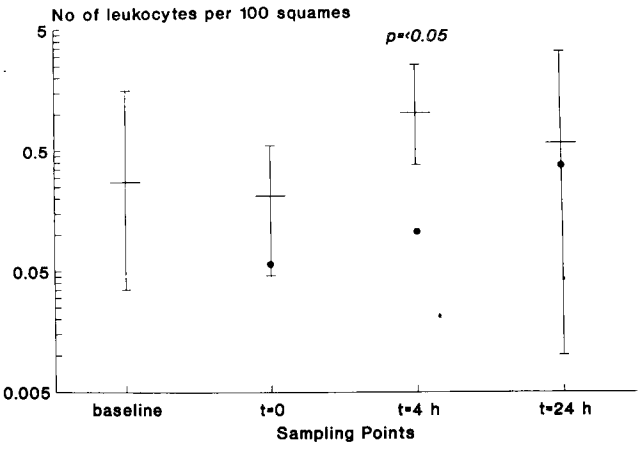

Fig. 3. Patient group cervical smear leukocyte data expressed as median ratios of leukocytes per 100 squamous cells with minimum and maximum values. • = smear control leukocyte data expressed as mean ratios of leukocytes per 100 squames. Squame = squamous cell.

els of leukocytes were seen throughout the baseline period. Like the patient group data, the neutrophil comprised the predominant cell type of this control group (mean value 83%).

Following insemination at t = 0 an elevation in leukocytes (the LR) was observed throughout the patient group, which occurred as rapidly as 20 min in some patients. Significantly more leukocytes ($P = <0.05$) were detected in the t = 4-h and t = 24-h patients' CM samples in comparison to their t = 0 samples. Concentrations of leukocyte either peaked at 4 h postinsemination or remained elevated at 24 h postinsemination (Fig. 1). Throughout the treatment period the neutrophil remained the predominant leukocyte of the patients' CM (median value 86%) while the numbers of macrophages, T-helper cells, and T-suppressor cells were low (median values 3.5%, 4.35%, and 3.4%, respectively). Table II shows the CM leukocyte data of three women in the patient group and demonstrates the individual variation in this response. A similar pattern was also observed in the nonsmear control group (Fig. 2), although the increase in leukocytes was not as marked as those of the patient group. Like the patient group the neutrophil was the principle leukocyte of the CM in all the control groups (median value 85%).

The "pure sperm" and seminal plasma control data confirm that the LR is initiated in response to sperm alone (Fig. 2). Neither the egg-yolk glycerol nor the seminal plasma controls elicited LRs to the different treatment regimes. A slight elevation in leukocytes was observed in the t = 24-h CM samples of the seminal plasma controls and the t = 4-h CM samples of the egg-yolk glycerol medium controls. However these elevations were probably due to cervical traumatization as a result of repeated CM sampling and did not follow the trend of the sperm-elicited LRs.

Figure 3 shows the patient group CS data as median ratios of leukocytes per 100 squamous cells. While each of the positive control slides run through the APAAP staining regime were highly specific, no positively stained cells were detected on the negative control slides. A high level of interpatient variation is seen in the patient CS data; for example, the baseline ratios of leukocytes ranged from 0.03 to 1.544. Whereas some patients had high baseline levels of leukocytes, others had low levels. The pattern of the LR varied according to the initial levels of leukocytes detected in the baseline smears. Those patients with high baseline levels of leukocytes showed little increase in leukocytes over the treatment period, while those patients with low baseline levels of leukocytes showed a marked increase in leukocytes, which either peaked at t = 4 h or remained elevated at t = 24 h postinsemination. As with the CM samples, the neutrophil comprised the predominant leukocyte of the CS both during the baseline and treatment periods (median value 79%). Again the levels of macrophages and T cells were low (median values 8.4% and 2%, respectively). Table III shows the individual CS leukocyte data of three women in the patient group, illustrating the huge variation of leukocytes in the pretreatment CSs and the individual variation of this response. The smear control data in Fig. 3 show how intense smearing of the cervix over a 24-h period causes local inflammation of the cervix.

Spermatozoa were rarely observed in the postinsemination cervical samples; however of those seen, the majority of sightings occurred in the t = 20 min cervical mucus samples. No correlation was found between the intensity of the LR and the density of spermatozoa in each inseminate sample ($P = 0.684$).

Three women became pregnant (two patients and one control) during their study cycles. Pregnancy outcome was not, however, correlated with the intensity of the LR ($P = 0.13$).

TABLE III. The Individual Cervical Smear Leukocyte Data of Three Women (Patients 1, 2, and 5) in the Patient Group Illustrating the Individual Variation in the Initial Levels of Leukocytes and the Onset, Duration, and Intensity of the LR[a]

		T = 0	T = 4 h	T = 24 h
Total leukocytes	1	0.10	0.37	0.01
	2	0.39	2.175	3.165
	5	0.535	0.53	0.22
Neutrophils	1	0.075	0.305	0.025
	2	0.26	2.229	3.155
	5	0.505	0.585	0.24
Macrophages	1	0.01	0.01	ND[b]
	2	0.02	0.01	0.055
	5	ND	0.005	0.005
T cells	1	0.015	0.01	0.005
	2	ND	ND	0.025
	5	ND	ND	0.02

[a]Leukocytes are expressed as ratios per 100 squamous cells.
[b]ND = not detected.

DISCUSSION

These data confirm that the LR is a physiological response of the human cervix, whose stimuli is specifically sperm.[5] This study accurately documents the leukocytic events occurring at the cervix before and after the introduction of sperm cells, seminal plasma, and cryopreservative medium. Only those women treated with sperm elicited LRs, which varied in terms of onset, duration, and intensity. Consistent with other studies the neutrophil was the predominant leukocyte at the cervix, both before and after the introduction of sperm cells[5,12] (median values 77% and 86%, respectively). These findings lead us to speculate a dual role exists for this cell at the cervix: (1) nonspecific protection of the upper reproductive tract against bacterial intrusion, and (2) phagocytosis of the nonviable/nonfertilizing sperm populations left at the cervix.

The levels of mononuclear cells (macrophages, T-helper cells, and T-suppressor cells) detected in this study were low, but were considerably higher than those previously reported by Pandya and Cohen.[5] The present data emphasize the need for conventional methods of cervical leukocyte staining to be reassessed.[7]

In our last study[7] we considered the contribution of seminal leukocytes to the LR, as it is well known that all semen samples contain leukocytes, predominantly neutrophils.[11] Seminal leukocytes were not found, however, to contribute to the LR, because significantly more leukocytes were isolated in the t = 24-h postinsemination

cervical mucus samples than were introduced at the cervix during insemination ($P = 0.000$). The present study further supports these findings. The control patients treated with sperm alone, i.e., samples not containing seminal leukocytes, elicited LRs comparable to the patient group, clearly demonstrating that seminal leukocytes are not significant contributors to the LR.

In an attempt to closely mimic the initial work of Pandya and Cohen on the human LR,[5] the leukocytes in our CSs were quantitated per 100 squamous cells. Although little to no variation in squamous cell numbers was observed throughout the sampling periods, we found this method of leukocyte sampling unsatisfactory. Two fundamental problems were encountered with this technique. First, as illustrated by our control data, intense smearing of the cervix over a short period of time induced an inflammatory response, which made interpretation of the LR difficult. Since CM sampling does not have the associated traumatization of cervical scraping (Fig. 2) we recommend that future studies on the LR employ this method of leukocyte sampling. Second, enumeration of the CS was highly subjective. Because many of the cell smears were two to three layers thick, areas of one cell layer thickness had to be selected for quantification. The CS data therefore only represents a proportion of the actual cell numbers involved in the LR.

Three women became pregnant in this study following CM aspirations 20 min postinsemination, which reiterates the fact that the fertilizing populations of sperm will penetrate through the cervical os within minutes of introduction. Presumably these spermatozoa bypass the LR. However, if the human uterine environment is similar to that of many other mammalian species, the LR will extend into it.[12,13] The fertilizing population of spermatozoa would require mechanisms of protection from the LR, particularly if spermatozoa storage occurs in the uterine cavity. Whether such sperm are equipped with antiphagocytic mechanisms needs to be addressed, as do sperm storage and leukocyte infiltration into the uterine cavity.

In conclusion, the human LR is a physiological phenomenon whose stimuli is specifically sperm. Neutrophils are the predominant leukocyte involved in this response. What now needs to be established is the function of the LR and whether it is a prerequisite for natural fertilization in vivo.

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Paper 5:

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Uterine flushing: a method to recover spermatozoa and leukocytes

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The process of sperm transport from the cervix, where a leukocytic reaction is initiated, through the uterus to gain access to the site of fertilization is very poorly understood. This preliminary study was designed to utilize a uterine flushing technique to determine firstly, the number of spermatozoa that can be recovered from the uterine cavity at 4 h post-insemination, around the time of ovulation, and secondly, to establish whether the spermatozoa initiate a leukocytic response while present. Uterine flushing was carried out in 10 potentially fertile women at 4 h post-insemination with donor semen, 24–36 h after the onset of the luteinizing hormone (LH) surge. The flush fluid was analysed for the numbers of spermatozoa and leukocytes present. In 8/10 women spermatozoa were retrieved from the uterus, in consistently low numbers (median 46, range 3–415). In 5/5 women leukocytes were recovered (median, 2.75×10^8/l, range $2.0 \times 10^8 - 12.7 \times 10^8$/l) from an origin other than peripheral blood contamination. These results suggest firstly that the flushing technique was a consistent method for retrieving spermatozoa and leukocytes from the uterine cavity, secondly that only low numbers of spermatozoa can be retrieved on flushing and thirdly that the leukocytic reaction to spermatozoa extends to the uterine cavity.

Key words: human uterine flushing/leukocytic reaction/spermatozoa

Introduction

Sperm transport through, and interaction with, the uterine cavity and its environment is poorly understood (Barratt and Cooke, 1991). This is primarily due to the ethical and logistical problems of working with humans, and the technical difficulties associated with retrieving the contents of the uterus (Mortimer and Templeton, 1982). In humans it is known that sperm progression from the vagina into cervical mucus elicits a leukocytic reaction (Thompson *et al.*, 1991). Subsequent progression of spermatozoa from the cervix to the oviducts (Williams *et al.*, 1992) and the peritoneal cavity (Ramsewak *et al.*, 1990) has been demonstrated, but in very low numbers. The role of the uterus in the overall process of sperm transport has largely been ignored and needs to be addressed.

From work in mammals it is known that sperm storage occurs within uterine glands (bats and guinea-pigs; Austin, 1960), synergistic interaction between the uterine and tubal environments prepare spermatozoa for fertilization (Hunter and Hall, 1974; Seki *et al.*, 1992), and following the passage of spermatozoa into the uterus, an influx of leukocytes is initiated (Soupart, 1970). Although phagocytosis of spermatozoa has been observed (goats, cattle and rabbits; Cohen, 1984), the exact function of the leukocytic reaction remains unclear.

Work in humans has shown that spermatozoa can be retrieved from the uterine cavity but the techniques used have been unreliable and information on the numbers present is variable (Moyer *et al.*, 1970; Mortimer and Templeton, 1982). No information is as yet available on the functional status of spermatozoa recovered from the uterus. At present it is not known whether the leukocytic reaction, as seen at the cervix (Thompson *et al.*, 1992), extends into the uterine cavity.

From the lack of information in humans and the limitations of the animal model, it is important that a reliable technique for flushing the uterus should be developed, to facilitate investigation of the spermatozoa and leukocytes present. In this study we utilized a flushing technique to quantify the numbers of spermatozoa and leukocytes that could be retrieved from the uterine cavity 4 h after cervical insemination, at around the time of ovulation. The study was designed to quantify the spermatozoa that could be recovered, not to assess their functional status, with a view to future development of the technique allowing such assessment.

Materials and methods

Patient recruitment

A group of 10 women routinely attending the University Research Clinic, Jessop Hospital for Women, Sheffield, UK, for donor insemination treatment were recruited to this study. Prior to recruitment, the study and its implications were explained to each patient and a consent form signed. The women had normal ovulatory cycles, as indicated by previously normal hormonal profiles, and had no pathological history, from which they were presumed to be potentially fertile (Chauhan *et al.*, 1989). The median number of previous donor insemination cycles per patient group was 3.5 (range 0–9). The women were asked to abstain from sexual intercourse during the study period.

Two additional women were recruited to the trial, as controls, to determine the numbers of leukocytes present in the uterus at the time of the onset of the luteinizing hormone (LH) surge, i.e. prior to insemination. Their recruitment, flushing and leukocyte assessment were carried out as described for the patient group. Donor insemination was performed on these women after flushing rather than before.

Three women attending the Jessop Hospital for Women radiology department for assessment of tubal patency by hysterosalpingogram (HSG) during the first 10 days of their cycle, were investigated for uterine volume; this was to determine whether, during flushing, any of the flush fluid was expelled into the Fallopian tubes. The HSGs were carried out using a Foley catheter, positioned as described for the uterine flushings (see below). The volume of the fluid required to fill the Foley catheter and uterine cavity without spillage to the Fallopian tubes and peritoneal cavity was measured. All three women were shown to have patent Fallopian tubes.

LH timing and insemination

Each patient attended clinic in the morning from day 10 of her cycle for daily LH assessment by either blood or urine sampling. The patient also collected blood (finger prick, Autolet) or urine samples in the evenings, to ensure accurate identification of the onset of the LH surge (Nulsen *et al.*, 1987). Inseminations were performed between 24–36 h of the onset of the LH surge. Patients were inseminated into the endocervix with 0.5 ml of donor semen (Barratt *et al.*, 1990) and were treated with a median number of 4.65×10^6 motile spermatozoa (range $0.18-13.10 \times 10^6$). Each patient has previously been assigned a suitable donor prior to recruitment to the trial and it was therefore not possible to standardize insemination using a single donor. The large range in number of motile spermatozoa inseminated was a result of standardizing the volume of semen inseminated and the variability of sperm concentration between the donors assigned to each patient. The volume of inseminate was standardized at 0.5 ml in keeping with the method used routinely within our donor insemination clinic. It was hoped that it would be possible to relate the variation in the number of motile spermatozoa inseminated to the variation in numbers of spermatozoa and leukocytes retrieved from the uterine cavity.

Uterine flushing

All uterine flushings were carried out at the University Research Clinic, on an out-patient basis. Flushing took place 4-h post-insemination. A period of 4 h ensured enough time for spermatozoa to penetrate through the cervical mucus, distribute throughout the tract and stimulate the leukocytic reaction (Thompson *et al.*, 1992). A bivalve speculum was inserted into the vagina to visualize the cervix. The cervical mucus was carefully and thoroughly removed from the os using a sampling syringe; in this way, any spermatozoa and leukocytes present in the cervical mucus were removed and contamination of the flush was thus prevented. The cervix was then cleaned with physiological saline. The flushing was carried out according to the method described by Li *et al.* (1992) with minor modifications. A size 8 Foley paediatric catheter (Rusch, UK) was passed through the cervix into the uterus, taking care to prevent uterine contamination with cervical contents. The Foley balloon was then inflated with 1 ml of physiological saline to hold the catheter in place. Two ml of physiological saline was slowly loaded through the Foley catheter and into the uterine cavity, left for ~30 s and withdrawn using gentle suction. The flushing procedure was repeated with the same fluid to ensure thorough washing of the uterine cavity. The volume of flush fluid retrieved was recorded, along with visual assessment of mucus contamination.

Sperm assessment

An aliquot of the cervical mucus sample was spread onto a glass slide, air-dried, fixed, stained (Papanicolaou, Department of Cytology, Jessop Hosptial for Women) and examined for the number of spermatozoa/10 high power fields (h.p.f.) (×400 magnification, bright field, Leitz Laborlux S). The flushing sample was centrifuged at 2869 *g* (4000 r.p.m.) for 30 min to recover as high a percentage of spermatozoa and leukocytes as possible, while maintaining cell morphology (M.Williams and L.A.Thompson, unpublished observations). This high spin speed may have affected sperm function but the primary aim of the study was to quantify spermatozoa and not to measure their functional status. A 2 ml control solution of known sperm concentration was treated identically to each flushing sample to enable determination of the sperm retrieval efficiency rate by centrifugation for each patient. The flush and control pellets were each resuspended into known volumes of phosphate-buffered saline/human serum albumin (PBS/HSA) medium. A 10 μl aliquot of the flush solution was examined (×400 magnification, phase contrast) for sperm content. A further eight 10 μl aliquots of the flush sample were stained using the modified alkaline phosphatase–anti alkaline phosphatase (APAAP) procedure (Tomlinson *et al.*, 1992). The number of spermatozoa present was recorded (×400 magnification, bright field microscopy). Four 10 μl aliquots of the control solution were stained as described for the flush sample. Since the total resuspension volume was known, the total number of spermatozoa present in the uterine flush fluid could be calculated.

Leukocyte assessment

The existence of minimal blood contamination within the flush meant it was necessary to determine whether or not the leukocytes in the flushing originated from peripheral blood contamination and/or another source. Prior to flushing the uterus a venous blood sample was taken from the patient. After the flushing procedure the flush and blood samples were analysed for whole blood cell count (Haematology Department, Jessop Hospital for Women; Coulter counter, Model S-Plus, Coulter Electronics Inc., FL, USA). Calculation of the ratio of the haemoglobin concentration in the two samples (blood haemoglobin/flush haemoglobin) enabled a dilution factor (DF) to be determined. Using this DF, along with the concentration of leukocytes in the peripheral blood ($[Lk]_{bl}$), the concentration of leukocytes in the flush ($[Lk]_{fl}$) and the background detection level of the counter (BG), the actual concentration of leukocytes in the flush from a source other than peripheral blood contamination ($[Lk]_{AS}$) was determined as follows:

$$[Lk]_{AS} = [Lk]_{fl} - ([Lk]_{bl}/DF) - BG$$

Statistics

The data were non-parametric and were therefore analysed using the Spearman's rank correlation coefficient on a PC with SPSS software. Correlations were determined between the number of spermatozoa flushed from the uterus and: (i) the number of spermatozoa inseminated, (ii) the number of leukocytes recovered, and (iii) the number of spermatozoa in the cervical mucus. *P*-values of ≤ 0.05 were considered to be statistically significant.

Results

The uterine flushing technique recovered spermatozoa from 8/10 of the women and leukocytes from 5/5 of the women (Table I). The fluid volume of 2 ml used during the flushing procedure was shown at HSG to fill the dead space of the Foley catheter with 1 ml and the uterus with a further 1 ml. Volumes of >1 ml were required to flush fluid from the uterus to the Fallopian tubes and peritoneal cavity during HSG. The median number of spermatozoa recovered, after correction for each individual centrifugation efficiency, was 43.5 (range 0–415) (prior to correction median 32.5, range 0–60). Spermatozoa were never observed within the wet film preparation. The numbers of spermatozoa recovered by flushing were not found to be significantly related to the number of motile spermatozoa inseminated. Spermatozoa were observed in the mucus of all 10 women (median 4/10 h.p.f., range 1–14/10 h.p.f.) but there was no significant relationship between the number present in the mucus and the number flushed from the uterine cavity. The median number of leukocytes recovered on flushing, originating from a source other than peripheral blood contamination, was 2.75×10^8/l (range $2.00-12.70 \times 10^8$/l). The control flushings contained numbers of leukocytes that were lower than the background detection levels of the Coulter counter. There was no significant correlation between the concentration of leukocytes recovered on flushing and the number of spermatozoa inseminated or recovered.

The flushing technique consistently recovered $\geq 85\%$ of the flush fluid (10/10 women) with minimal discomfort and inconvenience to the patient. Cervical mucus contamination was not observed on visual examination of each flush fluid.

Table I. Spermatozoa and leukocyte recovery from the uterus 4 h post-insemination

Patient	Motile spermatozoa[a] $\times 10^6$	Counted spermatozoa/ flush	Corrected spermatozoa/ flush	Leukocyte[b] concentration $\times 10^8$/l
1	0.46	2	3	ND
2	4.25	0	0	ND
3	1.93	25	42	ND
4	0.18	0	0	ND
5	4.80	4	7	ND
6	13.10	47	74	2.00
7	4.50	54	415	2.00
8	9.60	40	47	2.75
9	8.72	40	45	12.70
10	5.80	60	136	8.21

[a]Number of motile spermatozoa inseminated.
[b]Leukocytes from source other than blood contamination.
ND = not determined.

Discussion

This is the first study to describe a consistent technique for recovering spermatozoa and leukocytes from the human uterine cavity, timed to within 24 h of ovulation. Furthermore, these data are the first to suggest that the human leukocytic reaction to spermatozoa extends into the uterine cavity.

Previous attempts to retrieve spermatozoa from the uterine cavity have been inconsistent in their technical success (Moyer *et al.*, 1970; Mortimer and Templeton, 1982), with the number of spermatozoa recovered ranging from one to four (Moyer *et al.*, 1970) to 68–21 083 (Mortimer and Templeton, 1982). Problems were encountered with both mucus contamination and leakage through the cervix. These two studies are therefore inconclusive due to their inconsistencies. The present study employed a flushing technique which produced no mucus contamination of the flush and reliably recovered a high percentage of the flush volume, without leakage at the cervix, or loss to the Fallopian tubes and peritoneal cavity. Lack of mucus contamination within the flush suggested that our method of sampling the uterus by-passed the cervix, providing us with a uterine population of spermatozoa and leukocytes. This is substantiated by the very low numbers of spermatozoa recovered by flushing.

Retrospectively, no significant correlation existed between the number of motile spermatozoa inseminated and the number recovered from the uterine cavity, and thus the range of spermatozoa inseminated did not affect the study outcome. The range of sperm numbers recovered was much smaller than that shown by Mortimer and Templeton (1982), with greater similarity to the numbers recovered by Moyer *et al.* (1970). The low numbers of spermatozoa flushed from the uterus in this study may have been due to one or more of the following factors. Firstly, around the time of ovulation the uterus does not act as a storage site for spermatozoa and therefore very few were present. This is supported by the evidence that very low numbers of spermatozoa achieve access beyond the uterus to the Fallopian tubes (Williams *et al.*, 1992). Secondly, sperm epithelial attachment or storage in uterine crypts may have occurred, as seen in the oviducts of other animal species (Austin, 1960; Barratt and Cooke, 1991). Thus our flushing technique may not have been rigorous enough to dislodge those spermatozoa attached or stored. Most of the flush fluid was not displaced into the Fallopian tubes or peritoneal cavity, as $>85\%$ of the flushing fluid was recovered in all cases, and the HSGs showed the volume of fluid used did not enter the Fallopian tubes. Lastly, sperm numbers may have been considerably reduced by phagocytosis, in view of the large numbers of leukocytes retrieved. Indeed phagocytosis of spermatozoa has been shown to occur in the uteri of many other mammals (Howe, 1967).

Previous work has shown that the leukocytic reaction occurs at the cervix within 4 h of insemination (Thompson *et al.*, 1992). This enabled us to speculate that a similar time course may occur in the uterus. The results would suggest that the infiltration of leukocytes into the uterine lumen occurs within 4 h of insemina-

tion, and is not brought about by the flushing procedure itself. The leukocyte population recovered was almost certainly uterine in origin as the cervix was cleared prior to flushing and there was no evidence of cervical contamination both visually and from the number of spermatozoa retrieved. No significant correlation existed between the number of motile spermatozoa inseminated and the number of leukocytes retrieved, again providing evidence that our results were not affected by the range in number of motile spermatozoa inseminated.

The function of a leukocytic reaction within the uterus remains to be established but it may play a role in non-specific phagocytosis of non-viable or antigenically challenging spermatozoa and/or removal of bacterial contamination introduced during coitus (Moyer *et al.*, 1970). Other functions ascribed to the leukocytic reaction, such as induction of sperm capacitation (Soupart, 1970), should also be addressed.

This preliminary study indicates that our flushing technique was a quick and consistent method of sampling the uterine contents. Flushings were performed on an out-patient basis, were minimally invasive, and gave little inconvenience and discomfort to the patient. No pregnancies were achieved in this patient group, but little can be inferred from this due to the small size of the group. All patients were advised prior to recruitment that the procedure may reduce their chances of pregnancy during the study cycle. We are currently adapting our flushing technique to assess its efficiency of sperm recovery, and to utilize different timings and patient groups to enable determination of the role played by the uterus and the leukocytic reaction in the process of sperm transport. We are also aiming to develop the technique to allow concurrent assessment of sperm functional status, thus enabling determination of whether those spermatozoa recovered are a functional or dysfunctional population.

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Prognostic significance of computerized motility analysis for in vivo fertility*†

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Objective: To determine the predictive value of quantitative motility characteristics produced by the Hamilton Thorn Motility (HTM) Analyzer (Hamilton Thorn Research, Beverley, MA) for in vivo conception.

Design: A prospective analysis of 222 couples attending a regional infertility clinic. The measurements were made on a semen sample, and the presence or absence of a treatment-independent conception up to 22 months later was determined. The semen variables were then correlated to conception.

Setting: University based center for reproductive medicine.

Patients, Participants: The presence or absence of an in vivo conception was recorded in 222 couples in whom the influence on fertility of the female partner was minimized, i.e., normal in terms of history and examination, a regular menstrual cycle, ovulatory (midluteal serum P >18 nmol/L [5.6 ng/mL]), and the outcome of the hysterosalpingogram was normal. The median follow-up time was 13 months (range, 5 to 22 months).

Interventions: None.

Main Outcome Measure: Pregnancy.

Results: A number of variables were significantly related to time to conception. When a forward stepwise analysis was performed, the total number of spermatozoa was selected on the first step, and average path velocity was selected on the second step. No other variables were selected. The final variables consisted of the total number of spermatozoa and average path velocity.

Conclusion: The measurement of quantitative motility and sperm number using a HTM Analyser is of clinical value. Fertil Steril 1993;60:520–5

Key Words: Computer-aided sperm analysis, sperm motility, in vivo conception, Cox's regression

Recently, several large prospective studies have shown that the subjective measurement of sperm motility is of significant prognostic value for determining in vivo fertility (1, 2). Several other studies using intracervical donor insemination also emphasize the importance of subjective sperm motility for fertility (3). These results have encouraged the determination of the diagnostic potential of computer-aided sperm analysis (CASA) instruments because these provide rapid quantitative data with reduced technical error when using appropriate controls (4). However, despite the widespread use of CASA in many infertility laboratories, the predictive value of the sperm movement characteristics produced by such instruments for determining conception in vivo has not yet been determined in a

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prospective trial. A prospective study is the most appropriate method to determine the clinical value of such characteristics and the accumulation of this data is essential if the clinical value of these systems is to be realized (1, 5–7).

Interestingly, there are now clinical data showing that the measurement of sperm movement characteristics, using a variety of quantitative techniques, is of significant value in determining the fertilizing potential of a semen sample for IVF (8, 9). For example, Liu and colleagues (9) examined sperm movement characteristics using a Hamilton Thorn Motility (HTM) Analyzer (Hamilton Thorn, Beverley, MA) and related these characteristics to IVF-ET success in 108 patients. Using logistic regression analysis, linearity (LIN) in semen was positively correlated, and the percentage of sperm with an average path velocity (VAP) between 10 and 20 μm/s in the insemination medium negatively correlated to IVF rates (9). They concluded that the objective measurement of the motility characteristic by the HTM Analyzer was clearly superior to manual methods of motility analysis and that such measurements provided predictive information for IVF.

There are several studies that have determined the clinical value of quantitative motility measurements using a donor insemination population. These data suggest that the use of CASA techniques will be of clinical value. For example, Holt and colleagues (10) using a semiautomated computer system showed that the mean sperm velocity was significantly higher in donor ejaculates that achieved a conception compared with those that did not. This confirmed an earlier smaller study by Irvine and Aitken (11) who used time exposure photography to show that the concentration of motile cells inseminated and their mean velocity were positively correlated to pregnancy. Data from motion characteristics, analyzed using the Cell Soft system on frozen-thawed bull semen shortly after thawing demonstrated a significant correlation between percentage of motile spermatozoa, curvilinear velocity (VCL), lateral head displacement (ALH), straight line velocity (VSL), and fertility (12). A combination of these motion characteristics accounted for >88% of the variation in the competitive fertility index.

A very recent retrospective analysis of 1,147 intrauterine cycles of insemination with frozen-thawed donor spermatozoa in 191 women examined the predictive value for fertility of the Cell Trak/s system (Motion Analysis Corporation, Santa Rosa, CA) (13). Sperm concentration and percent motility were analyzed by a manual method. Using multiple hazards regression, the total number of motile sperm inseminated demonstrated the best association with fertile outcome ($P < 0.003$). Only the addition of VCL of the inseminating sperm added to the predictive value of the model. This study is the only one on a large group of patients that addressed the diagnostic value of CASA systems for in vivo conception. The relevance of these measurements to the examination of semen samples in couples attending for infertility investigations is difficult to assess because the semen examined was from highly selected donors, and all inseminations were intrauterine. To determine the predictive value of quantitative motility measurements for determining time to conception, we examined the semen characteristics of men attending our infertility clinic using a HTM Analyzer and followed up the patients to determine whether they achieved a spontaneous conception.

MATERIALS AND METHODS

Patients

A prospective study was undertaken of subjects referred for the investigation of subfertility between April 1990 and January 1992. Couples were investigated according to the World Health Organisation protocol for the management of the infertile couple (unpublished data). Couples with a clinical diagnosis of sexual dysfunction in either partner or ejaculatory dysfunction in the male partner were excluded from this study because the relative contribution of semen parameters in these couples would have been impossible to estimate. Men with azoospermia were excluded from the analysis. To assess the predictive value of semen parameters, the influence of the female partner on fertility needs to be minimized (5). Therefore, in this study we only used data from men with whom the female partner had the following characteristics: normal in terms of history and examination, a regular menstrual cycle, ovulatory (midluteal serum P >18 nmol/L [5.6 ng/mL]), and the outcome of the hysterosalpingogram (HSG) was normal. All patients had a normal plasma PRL and thyroid function tests. The median follow-up time was 13 months (range, 5 to 22 months). Two hundred thirty-eight couples were recruited to the study, and follow-up information was available for 222 couples (93% follow-up). If the subject indicated that a conception had occurred and the pregnancy had not been

managed at the Jessop Hospital for Women, confirmation of the pregnancy was sought. If the subject indicated that a spontaneous abortion had occurred, this was only accepted as a conception if evidence was available from histology, ultrasound examination, visualization of a fetus or gestational sac by a reliable witness, or an elevated serum β-hCG (1, 6). The median female age was 31 years (range, 19 to 44 years); the median male age was 30 years (range, 21 to 54). Eighty-three percent of women had primary infertility, and 17% had secondary infertility.

Measurement of Sperm Motility and Concentration

Semen samples were obtained by masturbation after a period of 48 to 72 hours of sexual abstinence from men attending the University Research Clinic at the Jessop Hospital for Women, Sheffield, United Kingdom. Without exception, all samples were collected on the premises to allow for the quick examination of the samples. The semen samples were allowed to liquefy at 37°C (approximately 30 minutes) and were examined within a maximum of 1 hour after production. Each sample was mixed gently before analysis to ensure an even distribution of spermatozoa. Spermatozoa were loaded into prewarmed 20-μm disposable microslides (Launch Diagnostics, Dartford, Kent, United Kingdom). These chambers provide consistent and accurate data on sperm concentration and motion kinetics (14). Sperm motility and concentration were analyzed using the HTM Analyzer (2030, version 7; Hamilton Thorn Research Inc.). The pertinent settings used during the HTM analysis were the following: recording time 0.8 s; frame rate of 20 at 25 frames/s; minimum contrast of 6; minimum size of 4; lo/hi size gates of 0.3 and 1.7; lo/hi intensity gates of 0.3 and 1.5; magnification of 3.31 (15). Low VAP value was set at 10 μm/s, medium VAP value at 25 μm/s, slow motile cells were counted as not motile, and the threshold for straightness was 80%. The playback function of the HTM Analyzer was used to accurately identify motile cells as a red dot and immotile cells as a blue dot and was used to check the threshold settings on every occasion. The microslides were loaded onto a heated stage (37°C) on an Olympus BH-2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with negative phase-contrast objective (×20) at an approximate total magnification of ×200. The internal optical system of the HTM Analyzer was not used. An external video camera (WV-BL200/B; Panasonic, Tokyo, Japan) was connected to a (VHS) video recorder (NV-870; Panasonic) and to the HTM Analyzer. All samples were videotaped and stored so that it was possible to repeat the analysis if at all necessary.

For each semen sample at least three fields were examined and at least 50 spermatozoa per semen sample were analyzed. The precision of measurements of the HTM have been previously reported to be very good with coefficients of variations (CVs) <11% for motility parameters (9). Our data that compared motion characteristic at different sperm concentrations (>3 to 20 × 10^6/mL; 21 to 150 × 10^6/mL) confirmed these observations.

We measured VCL, VAP, VSL, ALH, and linearity (LIN). We also reported percentage of rapid (>25 μm/s), moderate (≥10 to ≤25 μm/s), slow (<10 μm/s), and static spermatozoa. The following parameters were calculated: progressive motility (% rapid + % moderate), concentration of motile spermatozoa (% rapid + % moderate × concentration/100), concentration of rapid spermatozoa (% rapid × concentration/100), total number of progressively motile spermatozoa (concentration of motile sperm × volume), total number of spermatozoa (concentration × volume). Men with severe oligozoospermia (sperm concentration <5 × 10^6/mL) were not analyzed with the HTM Analyzer because of difficulties in the analysis of sufficient number of spermatozoa (4); also excluded from the analysis were men with high numbers of spermatozoa (>150 × 10^6/mL) because the measurement of sperm motion characteristics in such men was subject to error. Men with antisperm antibodies were not included in this study.

Statistical Analysis

In this type of study in which the objective is to determine the predictive value of measurements, it is necessary to use statistical tests that will determine if parameters are predictive of future conception and also take into account time to achieve a conception (1, 2, 5, 6). To determine which variables or combination of variables were independently and significantly related to conception, life-table analysis was performed using Cox's proportional hazards model, which was implemented by the SPSS system for windows (version 5.03; SPSS Inc., Chicago, IL) on a personal computer (16). This model enabled the relationship between semen variables and cumulative conception rates to be studied both individually and in various combi-

Table 1 Semen Characteristics of 222 Men Whose Partners Had Normal Menstrual History, Normal HSG, and Were Ovulatory*

Variable	Pregnant (n = 54)	Not pregnant (n = 168)
Volume	4 (0.8 to 11.0)	3 (0.8 to 8.0)
Sperm concentration ($\times 10^6$/ML)	51 (8 to 144)	28 (6 to 147)
Rapid progressive motility (>25 μm/s) (%)	28 (0 to 76)	14 (0 to 71)
Moderate progressive motility (≤25 μm/s to ≤10 μm/s)	9 (2 to 45)	7 (0 to 32)
Slow motility (<10 μm/s) (%)	8 (0 to 25)	4 (0 to 42)
Static (%)	54 (10 to 87)	71 (0 to 98)
Progressive motility (>10 μm/s) (%)	38 (9 to 86)	24 (0 to 86)
Concentration of rapid progressive spermatozoa	11 (0 to 80)	4 (0 to 77)
Concentration of progressively motile sperm cells ($\times 10^6$)	16 (1 to 95)	6 (0 to 93)
Total number of spermatozoa	160 (30 to 700)	80 (6 to 620)
Total number of progressively motile sperm ($\times 10^6$)	58 (3 to 382)	16 (0 to 372)
Ideal forms (%)	42 (11 to 88)	31 (0 to 75)
Head (%)	40 (2 to 82)	47 (1 to 98)
Midpiece (%)	16 (0 to 39)	19 (0 to 60)
Tail (%)	10 (0 to 82)	14 (0 to 69)
VAP (μm/s)	45 (23 to 72)	39 (0 to 60)
VSL (μm/s)	37 (19 to 65)	31 (0 to 50)
VCL (μm/s)	57 (28 to 81)	48 (0 to 91)
ALH (μm)	3 (2 to 5)	3 (0 to 9)
LIN (%)	65 (48 to 78)	64 (0 to 82)
Straightness (%)	79 (61 to 87)	78 (0 to 98)
Beat cross frequency (Hz)	8.0 (2 to 15)	8.0(0 to 13)

* Values are medians and with ranges in parentheses.

nations. This analysis expresses the chance of a particular male to conceive within a certain period of time (1, 2, 5, 6, 16). For the purpose of statistical analysis, follow-up commenced at the point of recruitment to the study. Variables were analyzed as continuous variables.

RESULTS

This study was based on a prospective analysis of the incidence of spontaneous pregnancy in 222 couples in whom the influence of the female factors on conception was minimized. Fifty-four patients conceived within the duration of the study. The median and ranges of the semen variables are presented in Table 1. Many variables were noticeably higher in the conception group compared with the nonconception group. When analyzing the results using Cox proportional hazards model, a number of variables were significantly related to conception rate (Table 2). The percentage of static spermatozoa was negatively associated with time to conception (Table 2). The most significant variable was the total number of spermatozoa ($P = 0.0002$) followed by the total number of progressively motile spermatozoa ($P = 0.003$) (Table 2).

A forward stepwise analysis was performed (see Table 2 for significant variables). The total number of spermatozoa was selected on the first step, and VAP was selected on the second step. No other variables were significant. The final equation was $h(t;z) = \lambda\alpha t^{\alpha-1}e^{(b1z1+b2z2)}$, where $\alpha = 3.136$, $\lambda = 1.5 \times 10^{-5}$, b1 = 0.0241, b2 = 0.0025 (17). The proportion of variation accounted for by our model was 72%. Using this model, we can estimate baseline survival function, and then we can estimate the probability of conceiving at time t for a particular patient with independent variable values z which is given by $\hat{P}(t;z) = 1 - \hat{S}(t;z) = 1 - [\hat{S}_o(t)]^{e^{b'z}}$. In our model the baseline survival function is obtained by $\hat{S}_o(t) = \exp[-\int_o^t \lambda\alpha u_{du}^{\alpha-1}]$. The estimated set of parameters **b** are then applied to a particular set of independent variables using the following equation: b'z = (0.0241 × VAP) + (0.0025 × total number of spermatozoa). The conversion factor to convert the baseline survival function estimate $\hat{S}_o(t)$ to the survival probability for a particular covariate pattern $\hat{S}(t;z)$ are 0.87 and 0.68 for 12 and 18 months, respectively. For example, a man who has a total number of spermatozoa of 150 and a VAP of 40: b'z = (0.0241 × 40) + (0.0025 × 150) = 1.339. As probability of achieving a conception by 18 months = $1 - [0.68]^{e^{1.339}} = 1 - 0.23 = 0.77$ (i.e., a 77% chance of conceiving at 18 months).

Table 2 Significant Variables Using Cox's Regression in 222 Ovulatory Women

Variable	χ^2	Probability
Total number of spermatozoa ($\times 10^6$)*	13.8565	0.0002
Total number of progressively motile spermatozoa ($\times 10^6$)	8.6327	0.0033
Static spermatozoa (%)	6.3875	0.0115
Concentration of spermatozoa ($\times 10^6$/mL)	6.1910	0.0128
VAP (μm/s)†	5.4947	0.0191
Concentration of progressively motile spermatozoa ($\times 10^6$/mL)	5.3413	0.0208
Progressive motility (%)	4.7303	0.0296
VCL (μm/s)	4.6389	0.0313

* First variable selected using stepwise method.
† Second variable selected using stepwise method.

DISCUSSION

This is the first prospective study to examine the predictive value of quantitative motility measurements made using a CASA system to determine time to in vivo conception. This study clearly shows that such measurements are of clinical significance. Many of the motility measurements were statistically significantly related to time to conception. The most predictive parameter was the total number of spermatozoa followed closely by the total number of progressively motile spermatozoa. Future studies must concentrate on improving our diagnosis by including other complementary measurements of sperm function (7, 15).

In this study we used the HTM Analyzer to measure quantitative sperm movement. We used appropriate quality control procedures for the effective use of such apparatus, e.g., analyzed a minimum of 50 cells in three random fields, determined our limits of detection by analyzing CV at various sperm concentrations, used a videotape system as a back up to prevent the loss of data, and used chambers of a fixed depth (20-μm microslides) that allowed for accurate determination of motility kinetics and concentration (14). We used the playback system to identify motile and nonmotile cells and to determine if any adjustment of the system was necessary. Such important aspects of quality control are necessary because in our laboratory considerable experience in the operation of the HTM Analyzer was necessary to ensure the acquisition of accurate data. We also used appropriate statistical techniques, i.e., Cox's regression, to determine the influence of movement characteristics on time to conception. The influence of the female factor on fertility was minimized because we only performed the analysis on patients in whom the female partner was thought to be normal. We have previously emphasized the importance of using appropriate statistical analysis and minimizing the effect of the female factor.

In view of the indirect evidence supporting the use of quantitative motility measurements in the investigation of male infertility and the known importance of motility to ascend the female tract (18), it was not surprising that such measurements made using the HTM Analyzer were significantly related to time to conception (Table 2). Our finding that the total number of spermatozoa independent of sperm motion was the most predictive factor of time to conception is different from that concluded by Holt et al. (10) and Marshburn et al. (13) who have examined the fertility of donor semen. However, this difference is probably due to the different subject selection because in the present study men were attending an infertility clinic, thus their semen characteristics are very different from those of semen donors.

The total number of progressively motile spermatozoa showed a highly significant correlation to conception (Table 2), which is concurrent with other studies, e.g., Marshburn and colleagues (13) who, using the Cell Trak/S system in a donor insemination population, demonstrated the total number of motile sperm inseminated showed the best association with fertile outcome ($P < 0.003$). It is also interesting that the addition of a specific motility parameter, e.g., VAP in our study and VCL in that of Marshburn and colleagues (13) added significant value to the models. This is very encouraging and strongly suggests that the measurement of sperm concentration and motility characteristics using CASA systems are appropriate and of clinical value. This should encourage the use of these systems in andrology laboratories for the assessment of sperm function.

Our finding that the speed of sperm movement was significantly related to time to in vivo conception is also concurrent with previous in vivo studies that have determined quantitative sperm movement (10, 11). However, because of the high degree of correlation between many of the motion characteristics, after selection of the most significant variables, other less significant variables were no longer of significance.

Using the data presented in this study, it is possible to calculate the probability of conception over time using the regression coefficients. For example, for a man who has a total sperm number of 20×10^6 and VAP of 10 μm/s, there is a 40% chance of a conception at 18 months after production of the semen specimen. If a man had total sperm count of 250×10^6 and VAP of 60 μm/s, there would be a 95% chance of a conception at 18 months. These calculations should only be used as approximated and only apply to couples when the female has characteristics of the patient group described in Materials and Methods. As a result of our experimental design and standard methodology using a large number of patients, these results should be applicable to other laboratories wishing to estimate the chances of conception in vivo from CASA systems.

In the future, other complementary measurements of sperm function that are not dependent on motility or concentration should be used to predict

in vivo fertility, e.g., measurement of reactive oxygen species (7, 15) and the presence and induction of the acrosome reaction. It is not realistic to expect that such tests of sperm fertilizing ability can predict fertility in all cases, but we should be optimistic and aim to improve our predictive value to approximately 90%.

It will also be important to examine whether we can improve on our existing analysis of sperm motility. This could be performed using several methods, e.g., measuring more spermatozoa and using multivariate cluster analysis to identify subpopulations of spermatozoa that may further refine the movement measurements that predict sperm fertility (8, 19, 20). Also, in addition to mean values that were analyzed in this study, the clinical value of frequency distributions of sperm movement characteristics requires examination because these measurements may be of more diagnostic value and more robust (Sherrins RJ, unpublished observation). It may also be appropriate to measure the kinetics of capacitated spermatozoa, specifically hyperactivated populations, because the ability to undergo hyperactivated motility is an important aspect of sperm function in the oviduct in animals and may also be in humans (18).

In conclusion, the measurement of quantitative motility and concentration using a HTM Analyzer is of clinical value. Clinicians and scientists can now be confident about using such information as a guideline for diagnostic significance. However, this study should be seen as a starting point only, and future studies must concentrate on improving our diagnosis by assessing other complementary measurements of sperm function.

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Paper 7:

Tomlinson MJ, Barratt CL, Cooke ID. Prospective study of leukocytes and leukocyte subpopulations in semen suggests they are not a cause of male infertility. *Fertil Steril.* 1993 **60**:1069-75.

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Prospective study of leukocytes and leukocyte subpopulations in semen suggests they are not a cause of male infertility*

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Objective: To determine the effects of leukocytes in semen on sperm quality and the ability to achieve conception.

Design: A prospective analysis of 512 couples attending a regional infertility clinic. Leukocyte subsets were quantified using a monoclonal antibody-based staining procedure. In addition to basic seminal parameters (density, motility, morphology, and antisperm antibodies), reactive oxygen species and immature germ cells were also quantified in the semen of each patient. The presence or absence of a treatment-independent conception was determined 22 months after the start of the study. Semen parameters were then related to the ability to conceive.

Setting: University-based center for reproductive medicine.

Participants: Success or failure to conceive was recorded from 512 couples. Couples were then selected to minimize the influence of any pathology of the female on outcome. A final study group of 229 couples, in which the women had regular menstrual cycles, ovulatory midluteal serum P levels of >18 nmol/L, and patent fallopian tubes was finally selected for analysis.

Main Outcome Measure: Pregnancy.

Results: Leukocyte concentration (total or individual subsets) was not associated with either reduced semen quality or conception rates. Similarly, neither reactive oxygen species or antisperm antibody (immunobead) concentration had any bearing on the outcome. Of all semen parameters measured, only the level of immature germ cells was found to be negatively associated with the rate of conception.

Conclusion: Measurement of seminal leukocytes in routine semen analysis appears to be of little prognostic value with regard to male fertilizing potential. As reactive oxygen species and antisperm measurement were of similar predictive value, the term "immunologic male infertility" should be redefined. Fertil Steril 1993;60:1069–75

Key Words: Seminal leukocytes, prospective follow-up, in vivo conception, immature germ cells, reactive oxygen species, immunologic infertility

The relationship between leukocyte populations of the ejaculate and male fertility status remains controversial. Many studies have implicated seminal leukocytes in male factor infertility but generally indirectly, merely demonstrating associations between leukocyte concentration and reduced sperm function (1, 2). Conflicting results obtained from numerous studies, possibly related to differing cytological techniques used by individual centers have only confused the issue. Indeed, two studies comparing leukocyte prevalence in the ejaculates of fertile men with those of subfertile men reached opposite conclusions (3, 4). In separate investigations, Cohen et al. (5) and Van der Ven et al. (6)

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found correlations between increased "round cell" concentration (assumed to be leukocytes) and reduced success with their IVF procedures. When this work was repeated but with the advantage of more modern cell identification methods that enable differentiation between leukocytes and other round cell elements also present in semen, we found no such relationship (7). Instead, the concentration of those other round cell elements, principally immature germ cells, were found to be significantly higher in the group of semen samples that failed to fertilize an egg in a natural IVF cycle. The logical step forward from these types of investigations would therefore be to determine whether round cells (leukocytes and immature germ cells) are similarly associated with a reduction in the chances of a natural conception.

For some time a concentration of $>1 \times 10^6$ leukocytes/mL of semen has been used as a clinical marker for pathologically significant leukocytospermia (8), not only because this number is associated with infection of the genital tract (9) but also because more recent evidence has suggested that there is an increased chance that sperm quality is significantly lowered (2). However, as yet, no study has considered whether this 1×10^6 threshold is actually associated with a significant reduction in an individual's chance of achieving a pregnancy or indeed the prevalence of leukocyte-related infertility in a large patient population.

For the present study, semen data (including leukocyte profiles) on 512 men attending our infertility clinic was collected over a 2-year period. These patients were subsequently followed-up to determine success/failure of conception, which was then in turn related to the various seminal characteristics, including leukocyte profile, immature germ cell content, quantitative motility measurements, morphology, reactive oxygen species concentration, and antisperm antibodies. From these data we were able to determine the full effect of both leukocytes and the germ cell elements on male fertility status.

MATERIALS AND METHODS

Study Subjects

All study patients were attending the University Department of Obstetrics and Gynaecology, Jessop Hospital for Women, Sheffield, United Kingdom between April 1990 and February 1992 (median duration of infertility, 38 months, range, 7 to 120 months) for infertility investigation/semen analysis. All couples were investigated according to World Health Organization (WHO) guidelines (10). Females with any reproductive dysfunction/pathology and men with sexual dysfunction or azoospermia were excluded from the study. The criteria for normality of the female were confirmed by hysterosalpingogram and/or diagnostic laparoscopy, regular menstrual cycles, one or two midluteal serum P measurements of >18 mmol/L (5.6 ng/mL), plasma PRL concentration, and normal thyroid function. Median female age was 31 years (range, 19 to 44 years). Primary infertility was found in 83% and 17% had secondary infertility.

Follow-up was performed by a standard questionnaire. This provided information on both the couple's success/failure to conceive and whether they had or were about to receive any form of treatment. The median time to follow-up was 13 months (range, 4 to 22 months). Conception was defined either by evidence of ultrasound examination or elevated beta-hCG levels.

From a total of 512, follow-up information was available for 464 (91%) couples, of which 229 met the criteria for normality in the female and had not received any form of therapeutic intervention. Data from this group of 229 were analyzed to determine the most powerful predictive semen variables with regard to successful conception. Semen parameters were correlated to individual leukocyte subsets in the entire population of 512 patients.

Semen Analysis

The following semen parameters were measured on each patient: sperm concentration and motility, sperm morphology, immature germ cells, antisperm antibodies, seminal reactive oxygen species, and seminal leukocyte profile. Basic semen analysis was performed in accordance with WHO guidelines (8). Antisperm antibodies were tested using either the direct or indirect (where appropriate) immunobead test described elsewhere (11).

Seminal leukocytes were stained using the monoclonal alkaline phosphatase antialkaline phosphatase staining procedure (Dakopatts, High Wycombe, United Kingdom) described by Tomlinson and co-workers (12). Monoclonal antibodies directed at individual leukocytic subsets are described as follows: HLe-1 (pan-leukocyte; Becton-Dickinson, Oxford, United Kingdom); FMC-10 (neutrophils; Serotec, Oxford, United Kingdom); Dako-Macrophage, Dako-B cell, T8 (suppressor/cytotoxic), and T4 (helper/inducer)

(Dakopatts). Those round cells that remained unstained when using the HLe-1 antibody were determined to be immature germ cells. These were quantified per 100 sperm. Reactive oxygen species were measured by luminometry according to the methods of Kessopoulou and co-workers (13).

Statistical Analysis

Logistic regression, a nonparametric multivariable statistical method, was used to assess the basic predictive value (whether positive or negative) of the measured seminal parameters (alone or in combination) with regard to future conception. To account for the time taken to conceive, Cox's (proportional hazards) regression was also used for increased sensitivity (14).

The logistic (1) and Cox's (2) regression equation for more than one independent variable are shown below:

$$\text{Probability (pregnancy)} = \frac{1}{1 + e^{-z}} \qquad (1)$$

$$\text{Pregnancy (time t)} = 1 - \exp(\exp[B1 \times VAR1 + B2 \times VAR2 + \cdots Bnx\ VARn] \times \ln [1 - P_{0t}]), \qquad (2)$$

Where P_{0t} is the underlying pregnancy rate before time t is taken into account.

$$Z = \text{constant} - (B1 \times VAR1) + (B2 \times VAR2) + \cdots (BX \times VARX)$$

and B is the estimated regression coefficient for each named variable from Bostofte et al. (14).

Logistic regression was performed using SPSS-PC on a Vig 2 (Viglen Computers, United Kingdom) IBM compatible personal computer licensed to Sheffield University. Analysis using Cox's regression model was performed using the statistical analysis system package for the personal computer. Correlations between either leukocytes or immature germ cells and semen parameters were made using Spearman's ranked correlation, performed on SPSS-PC (SPSS Inc., Chicago, IL).

RESULTS

From the total of 512 couples selected for analysis, 229 satisfied the criteria for normality of the female partner. In this latter group, spontaneous conception occurred in 54 (23.5%) within the duration of the study. Of the "traditional" semen variables, sperm density, progressive motility, and the percentage of morphologically ideal sperm forms were all significantly higher in the conceiving group, whereas the number of immature germ cells (per 100 sperm) were significantly lower in this group (Table 1). In contrast, no such difference was observed in either reactive oxygen species concentration or concentration of any leukocytic subset.

Table 1 Semen Parameters in 229 Conceiving and Nonconceiving Couples in Whom Influence of Pathology in the Female was Minimized*

Semen parameter	Conceiving (n = 54)	Nonconceiving (n = 175)
Motile density ($\times 10^6$/mL)	22 (3, 68)	4.5 (0, 58)‡
Sperm density ($\times 10^6$/mL)	55 (13, 117)	23.5 (5, 117)‡
Progressive motility (%)	25 (7, 51)	12 (3, 40)‡
Ideal forms (%)	41 (20, 64)	27 (15, 60)‡
Total leukocytes ($\times 10^3$/mL)	14 (ND,§ 262)	13 (1, 244)
Neutrophils ($\times 10^3$/mL)	7.5 (ND, 234)	4.5 (ND, 149)
Macrophages ($\times 10^3$/mL)	3 (ND, 48)	4 (ND, 69)
HLA-DR-positive cells ($\times 10^3$/mL)	2 (ND, 24)	2 (ND, 20)
T4 lymphocytes ($\times 10^3$/mL)	ND (ND, 3.8)	ND (ND, 2.4)
T8 lymphocytes ($\times 10^3$/mL)	ND (ND, 3.4)	ND (ND, 3.0)
B lymphocytes ($\times 10^3$/mL)	ND (ND, 4.0)	ND (ND, 2.0)
Interleukin-2-positive cells ($\times 10^3$/mL)	ND (ND, 0.8)	ND (ND, 2.0)
Reactive oxygen species (mVs)	18 (1, 719)	19 (1, 406)
Immature germ cells (cells/100 sperm)	11 (3, 30)	14 (4, 69)†

* Values are medians with 10% and 90% confidence limits in parenthesis.
† Groups are significantly different, $P < 0.05$.
‡ Groups are significantly different, $P < 0.0001$.
§ ND, not detected.

From logistic regression and Cox's analysis of the 229 normal couples, the concentration of motile sperm, progressive motility, and sperm density were the most significant predictive parameters of pregnancy as shown by Table 2. The percentage of ideal forms and concentration of seminal neutrophils were also positive predictors. The level of seminal infiltration by immature germ cells was negatively associated with pregnancy outcome. Other immunologic seminal parameters, i.e., reactive oxygen species measurements (peak value or timed integral) and antisperm antibodies (immunoglobulin

Table 2 Semen Parameters Predictive of Pregnancy in the 229 Selected Couples

Semen parameter	Logistic regression χ^2	Logistic regression P	Cox's regression χ^2	Cox's regression P
Motile concentration	18.962	0.0000*	6.24	0.013
Progressive motility	24.07	0.0000*	5.16	0.017
Sperm concentration	15.455	0.0001*	5.117	0.024
Percent ideal forms	8.349	0.0039*	4.35	0.037
Total neutrophils	6.269	0.012*	4.262	0.039
Total leukocytes	5.832	0.016*	2.25	0.093
Immature germ cells	5.04	0.025†	3.29	0.06

* Positively correlated with pregnancy.
† Negatively correlated with pregnancy.

[Ig]G or IgA), had no bearing on pregnancy outcome.

Relationship of Leukocytes to Semen Quality

Seminal parameters were correlated with the concentration of leukocyte subsets/activation markers in a total of 512 infertility patients. Leukocytes were detected in 90% of patients, with neutrophils and macrophages the most abundant phenotypes. T or B lymphocytes were rarely detected (see Table 3).

No leukocyte phenotype was associated with a reduction in semen quality. As Table 4 shows, only the appearance of activation markers human leukocyte antigen (locus) DR (HLA-DR) on the surface of macrophages had any significant detrimental effect on sperm function, being correlated with reductions in progressive motility ($P < 0.01$).

This apart, leukocytes were associated with improved semen quality, which concurs with their positive association with pregnancy. Concentration of neutrophils and macrophages were highly correlated with ideal sperm morphology. Sperm head defects were correspondingly negatively correlated with these cell types. In addition, macrophage concentration was associated with an increased sperm density (Table 4).

Reactive oxygen species were strongly correlated with neutrophils, macrophages, and HLA-DR expression but not with other semen variables. Neither T (CD4+/CD8+) or B lymphocytes, or the appearance of the interleukin-2 (TAC) receptor on their surface were associated with any semen variable.

Leukocytospermia

Leukocytospermia, defined as a concentration of $>1 \times 10^6$ leukocytes/mL of semen occurred in 14 of 512 patients (2.7%). Comparison of these with non-leukocytospermics revealed a significant difference only in the levels of reactive oxygen species ($P < 0.0001$). All other semen parameters were similar in both groups. Within the 229 normal couples only seven males were leukocytospermic, three of whom achieved a pregnancy (43%). In the remaining non-leukocytospermic patients, the pregnancy rate was 24%.

Oligozoospermia

Significantly fewer leukocytes in total ($P < 0.0002$), neutrophils ($P < 0.005$), and macrophages ($P < 0.00001$) were found in oligozoospermic semen. Macrophage activation (HLA-DR) was not similarly reduced. All major motility variables were also significantly poorer in this group, e.g., progressive motility ($P < 0.00001$).Sperm morphology was significantly poorer in oligozoospermics with a reduction in percent of ideal forms ($P < 0.00001$) and corresponding increases in head ($P < 0.0001$) and midpiece defects ($P < 0.0005$).

Leukocytes, Antisperm Antibodies, and Vasovasostomies

Of the 512 patients, 11% were antisperm antibody positive and 48% of these were associated with vasectomy reversal operations (vasovasostomy). Neither the presence or titre of antisperm antibodies were associated with any particular leukocyte phenotype. Similarly, antisperm antibodies were

Table 3 Leukocyte Subsets in the Semen of 512 (Unselected) Infertility Patients

Leukocyte phenotype	Detected in %	Mean ± SE	Median	Range
Total count ($\times 10^3$/mL)	90	132.8 ± 26.9	15	ND* to 5,340
Neutrophils ($\times 10^3$/mL)	70	67.0 ± 14.6	5	ND to 4,300
Macrophages ($\times 10^3$/mL)	76	23.9 ± 3.2	4	ND to 902
T4 lymphocytes ($\times 10^3$/mL)	25	0.94 ± 0.16	ND	ND to 57.6
T8 lymphocytes ($\times 10^3$/mL)	25	1.17 ± 0.18	ND	ND to 37
B lymphocytes ($\times 10^3$/mL)	20	1.62 ± 0.46	ND	ND to 164
HLA-DR positive ($\times 10^3$/mL)	65	10.5 ± 1.71	1.1	ND to 450
Interleukin-2 positive ($\times 10^3$/mL)	15	0.86 ± 0.19	ND	ND to 50

* Not detected.

Table 4 Correlation Matrix for Leukocytes with Seminal Parameters

Leukocyte phenotype	Total count		Neutrophil		Macrophage		HLA-DR +ve	
	r	*P**	*r*	*P**	*r*	*P**	*r*	*P**
Percent ideal forms	+0.18	0.001	+0.19	0.001	+0.15	0.01	NS	
Percent head defects	+0.17	0.001	−0.17	0.001	−0.15	0.01	NS	
Progressive motility	NS†		NS		NS		−0.21	0.001
Sperm density	NS†		NS		NS		NS	
Reactive oxygen species	+0.55	0.001	+0.45	0.001	+0.44	0.001	+0.40	0.001

* $P <$ either 0.01 or 0.001.

† NS, not significant.

not associated with reductions in semen quality or indeed reduced chance of pregnancy. Both classes of antisperm antibodies tested (IgG and IgA) were highly correlated with agglutination ($P < 0.001$) but again this has no apparent bearing on semen quality or conception rates.

A significantly higher concentration of macrophages ($P < 0.001$) and HLA-DR-positive cells were found in vasovasostomized individuals with a concomitant increase in the levels of reactive oxygen species. These latter two parameters (HLA-DR and reactive oxygen species) were both strongly associated with the poorer motility parameters observed in this group ($P < 0.001$). Immature germ cells were significantly correlated with low sperm density ($r = -0.54$, $P < 0.001$), poor progressive motility ($r = 0.28$, $P < 0.001$), and poor morphology ($r = -0.383$, $P < 0.001$).

DISCUSSION

This prospective study presented the first detailed examination of the influence of the round cell content of semen, i.e., leukocyte subsets, immature germ cells on pregnancy outcome, and their relationship to other seminal parameters. From a total of 229 couples selected for absence of pathology in the female partner, the predictive value of each of these variables with regard to conception success was also calculated using logistic and the slightly more sensitive Cox's regression analyses.

Contrary to previous reports (1, 2, 5, 6), reductions in either sperm fertilizing capacity or semen quality were not found to be leukocyte related. Neither the leukocyte total nor any of the individual leukocyte phenotypes were associated with reduced natural conception rates or a loss of semen quality (as indicated by progressive motility and sperm morphology). Similarly, levels of reactive oxygen species, which correlated very strongly with leukocyte concentration, did not adversely affect semen quality or pregnancy outcome.

More interestingly was the finding that immature germ cells were more prevalent in those men who failed to achieve a conception. This was concordant with our earlier study, which showed that ejaculates from men with unexplained infertility that failed to fertilize at IVF contained a significantly higher concentration of immature germ cells when compared with fertilizing ejaculates (7). Because semen quality was similar between groups, we concluded that rather than germinal cells having any direct effect on sperm function, their increased presence was a reflection of hidden abnormality/immaturity of the sperm population. However, the present study, which was based on a much larger patient population, did reveal strong associations between levels of immature germ cells/germinal cells and poorer semen quality. Germinal cells were more prevalent in oligozoospermic/asthenozoospermic and teratozoospermic samples and may as previously indicated (8) suggest a serious problem during spermatogenesis.

Our results appear to largely disagree with earlier findings, suggesting that an increased leukocyte presence is associated with reduced semen quality (2, 15), sperm-oocyte fusion (1, 16), and fertilization at IVF (5, 6). In addition, others have demonstrated that levels of seminal reactive oxygen species are associated with reduced oocyte fusion and pregnancy rates (17).

In contrast to this previously published work, all the evidence of this present study suggests that the fertilizing capacity of an ejaculate can actually be enhanced by the presence of certain leukocyte phenotypes, including those that are the principal reactive oxygen species releasers, namely neutrophils. Neutrophils alone were positively predictive of pregnancy outcome (and significantly so) using

both logistic and Cox's regression methods. Along with the macrophages, these phagocytic cells were highly correlated with an increased percentage of morphologically ideal sperm, in agreement with our earlier findings (12) suggesting that in certain individuals both neutrophils and to a lesser extent macrophages may "cleanse" and increase the quality of an ejaculate possibly by removal of abnormal and degenerate forms. Work by Bize and colleagues (18) suggested that reactive oxygen species, namely hydrogen peroxide, were in contrast to earlier findings (17, 19) vital for sperm capacitation, acrosome reaction, and sperm-oocyte fusion. Our present study would appear to support such a hypothesis, with a major hydrogen peroxide producer, i.e., neutrophils, correlating with improved sperm morphology and an increased pregnancy rate. Macrophages were positively associated with sperm density, supporting the hypothesis that both have a common source, i.e., the epididymis and not the major accessory glands (Tomlinson MJ, unpublished data).

Of the 512 ejaculates initially entered into the study, only 2.7% contained $>1 \times 10^6$/mL leukocytes, i.e., were leukocytospermic. Semen parameters for these individuals were no poorer and in fact the pregnancy rate in this group was slightly higher than in the nonleukocytospermic patients. The only parameter to be significantly different in leukocytospermics was the reactive oxygen species level, which may be expected, because the correlation between leukocytes and reactive oxygen species was highly significant.

Only one leukocyte parameter showed any negative correlation with semen quality. HLA-DR expression was associated with reduced progressive motility and an increase in immotile sperm, two of the most important predictive parameters with regard to fertility. This relationship may well be associated with the release of soluble mediators that are dependent on macrophage stimulation, e.g., reactive nitrogen intermediates (20), into the local milieu. Although this relationship appeared to be highly significant, it was not sufficient to directly affect the pregnancy rate. Therefore, perhaps only in exceptional circumstances where the introduction of a high concentration of activated macrophages occurs, e.g., subclinical chronic infection of the epididymis, orchitis, and others, subfertility due to the release of factors such as toxic nitrogen intermediates may result. It is interesting that in vasovasostomized individuals HLA-DR expression is significantly increased and this is accompanied by a highly significant reduction in motility parameters. Incomplete healing, subclinical infection, or residual granuloma from the original vasectomy are all potential sources of the inflammatory macrophages in such cases. Therefore local release of inflammatory mediators could reduce sperm motility in an "innocent bystander" situation in these individuals. This would be a more likely reason for their subfertility than would, for example, the presence of antisperm antibodies often associated with vasectomy reversal. This hypothesis is supported by the present data in that neither the presence of IgA nor of IgG antibodies were related to semen quality or pregnancy outcome. This latter was most surprising, considering the amount of literature implicating antisperm antibodies as a major male factor infertility problem (21, 22). However, some studies do support our data. Critser and co-workers (23) questioned the clinical significance of antisperm antibody results from the immunobead test after circulating antisperm antibody titres in fertile individuals were found to be no different than those in infertility patients. Using the tray agglutination test (TAT), Aitken et al. (24) found no correlation between antisperm antibody titres and sperm function and showed that antisperm antibodies could either promote or suppress sperm/oocyte binding. Poor correlation between different antisperm antibody testing methods has also been reported (25). Perhaps future efforts should concentrate on standardization of antisperm antibody testing and selection of the most appropriate method for establishing their true effects on sperm fertilizing capacity. The numbers of neutrophils and macrophages were significantly reduced in those ejaculates with $<20 \times 10^6$ sperm/mL (oligozoospermics) but, interestingly, HLA-DR expression did not alter. This therefore effectively leaves them with an increased proportion of HLA-DR expressive macrophages and suggests that the poorer motility observed in oligozoospermics may also have a related etiology.

In conclusion, round cells in semen are two discrete cell populations and should not (as they have in the past) be treated as a single entity. It is clear from our patient data that seminal leukocytes do not significantly affect semen quality or male fertility status, whether present in concentrations of either greater than or less than 1×10^6/mL. Routine measurement of leukocytes in the diagnostic andrology laboratory, which has been suggested by some to be a necessary integral part of semen analysis, (8) therefore appears to be of little value. In addition, because neither reactive oxygen species or

antisperm antibodies had any influence on semen quality or pregnancy outcome, perhaps the term immunologic male infertility should be re-evaluated. With regard to round cells in general, perhaps greater understanding of the significance of immature germ cells, which have been largely overlooked by many, is required, as these are most certainly associated with poorer semen quality and a lowered chance of conception.

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Paper 8:

Barratt CL, Dunphy BC, McLeod I, Cooke ID. The poor prognostic value of low to moderate levels of sperm surface-bound antibodies. *Hum Reprod*. 1992 **7**:95-8.

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The poor prognostic value of low to moderate levels of sperm surface-bound antibodies

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The clinical significance of antisperm antibodies for fertility remains controversial. In this study, we determined whether the presence, isotype, region and/or amount of sperm-bound antibody was of any predictive value for future fertility in 534 men using Cox's proportional hazards model. Significant correlations between the presence of antibodies and semen parameters were recorded, such as sperm mucus penetration and sperm motility. However, low (<10%) negative binding and moderate (<50%) binding had no significant effect on the probability of conception or the time to conception. This study confirms in-vitro data suggesting that sperm function is not impaired unless the degree of antibody binding to spermatozoa is very high.

Key words: antisperm antibodies/cervical mucus penetration/conception/prognostic factors

Introduction

The clinical significance of antisperm antibodies for fertility is still the subject of some debate (Critser *et al.*, 1989; Barratt *et al.*, 1989a, 1990a; Alexander, 1990; Meinertz *et al.*, 1990). The consensus of opinion is that the importance of circulating antisperm antibodies, as determined with currently available techniques, is low (Eggert-Kruse *et al.*, 1989; Critser *et al.*, 1989) and the detection of the amount, isotype (IgA, IgG) and location of antibodies on spermatozoa should provide an accurate diagnosis of the fertility status of the male (Matson *et al.*, 1988; Adeghe *et al.*, 1988; Hammitt *et al.*, 1988; Meinertz *et al.*, 1990). However, there is a paucity of studies examining the *in vivo* prognostic value of the amount, isotype and location of sperm surface antibodies.

Numerous studies have documented the effect of sperm surface antibodies on cervical mucus penetration and sperm–oocyte interaction (Clarke, 1988; Matson *et al.*, 1988). Sperm-bound IgA antibodies are associated with poor cervical mucus penetration but sperm-bound antibodies of the IgG class do not appear to be associated with poor cervical mucus penetration (Wang *et al.*, 1985; Parslow *et al.*, 1985; Clarke, 1988). The regional specificity of the IgA class affecting penetration is not yet determined. The work of Wang *et al.* (1988) suggested that head-directed antisperm antibodies were of primary importance. In contrast, Clarke (1988) suggested that antisperm antibodies directed to the tail were of primary importance, confirming earlier observations (Kremer and Jager, 1980). The fertilization rates of human oocytes *in vitro* are not significantly impaired, even when the levels of antisperm antibody binding are high (>70% of motile spermatozoa bound; see Alexander, 1990; De Almedia *et al.*, 1989). For example, Clarke *et al.* (1985) observed a significant decrease in the fertilization rate only when >80% of the motile spermatozoa were bound with both IgA and IgG. A 72% fertilization rate was obtained when <80% of the spermatozoa were bound with IgA and IgG; high levels of IgG (>90%) did not affect the fertilization rates.

The type of antibody on the spermatozoa and its clinical relevance may vary according to the stimulus for its production (Parslow *et al.*, 1985). Vasovasostomized men with antisperm antibodies have a higher conception rate than men with antisperm antibodies where the stimulus for their production is unknown (Parslow *et al.*, 1985). Meinertz *et al.* (1990) examined the effect of sperm surface IgG, IgA and secretory IgA antisperm antibodies on conception *in vivo* in 216 vasovasostomized men. In a sub-group of men with a pure IgG response, the conception rate reached 85.7%, yet when all the spermatozoa were bound by IgA, the conception rate was reduced to 21.7%. The combination of IgA on all spermatozoa and a strong immune response (a titre ≥256) was associated with a conception rate of zero (Meinertz *et al.*, 1990). There are no comparable data for men with antisperm antibodies attending an infertility clinic, the majority of whom (~85%) have not had a vasovasectomy and are classed as having idiopathic infertility (Barratt *et al.*, 1989a).

The aim of our study was to examine whether the presence, isotype, region and/or amount of antisperm antibody attached to the sperm surface was of any predictive value for future fertility in men attending an infertility clinic.

Materials and methods

Experimental design

The presence of antisperm antibodies on spermatozoa or in seminal plasma was measured in 534 men, excluding those with azoospermia, and the results correlated to standard semen parameters, including two putative sperm function tests. The prognostic significance of antisperm antibodies was initially determined by examining the incidence of pregnancy in the partners of men with and without antisperm antibodies and then the possible influence of antisperm antibodies on time to conception was examined using Cox's proportional hazards model (Dunphy *et al.*, 1989a).

Subject recruitment and follow-up

A prospective study was undertaken of 534 subjects referred for investigation of fertility between October 1988 and January 1990. Couples were investigated according to the World Health Organization (WHO) protocol for the management of the infertile couple. A follow-up programme was instituted by questionnaire and telephone. If a subject indicated that a conception had occurred and the pregnancy had not been managed at the Jessop Hospital for Women, Sheffield, confirmation of the pregnancy details was obtained. If the subject indicated that a spontaneous abortion had occurred, this was only accepted as a conception if evidence was available from histology, ultrasound examination, visualization of the fetus or gestational sac by a reliable witness, or an elevated level of serum beta-human chorionic gonadotrophin.

Semen analysis

The semen samples were allowed to liquefy completely (~30 min) and the following semen characteristics were measured using standard techniques as previously described (Barratt *et al.*, 1988): semen volume, pH, sperm concentration and sperm motility. Motility was graded as follows: Grade 1, fast forward progresson; Grade 2, moderate forward progression; Grade 3, motile but no forward progression; Grade 4, immotile spermatozoa (see Barratt *et al.*, 1988). Sperm motility and the presence of sperm agglutination was determined at ×400 magnification using phase contrast microscopy. Sperm morphology was determined using bright field illumination at ×1000 magnification, after preparing air-dried Papanicolaou-stained smears (Barratt *et al.*, 1988, 1989b).

Hypo-osmotic swelling (HOS) test

This test was performed and the results interpreted as described previously (Barratt *et al.*, 1989b).

Bovine mucus penetration test (BMPT)

This test was performed using commercially available preparations of mucus (Penetrak Serono Diagnostics, Massachusetts, USA) stored at −20°C until use. One flat capillary tube containing mucus was allowed to reach room temperature (~25 min) and was placed vertically in a BEEM OO capsule (Agar AIDS EM Supplies, Stansted, UK) containing 200 μl of liquefied semen. After 90 min of incubation, the capillary tubes were placed on a calibrated microscope slide and the number of spermatozoa per high power field (×400 magnification, phase contrast microscopy) at 10, 20, 30, 50 and 65 mm counted. The distance travelled by the vanguard spermatozoon was recorded to the nearest millimetre. All procedures were performed at a constant temperature (25°C). This was important as fluctuations in the ambient temperature can produce significant variation in BMPT results (Stumpf *et al.*, 1984).

Immunobead test

The immunobead test was chosen as a test for autoimmunity as it is simple, quick, enables the class and region of antibody binding to be determined, is independent of sperm motility and has repeatedly been shown to be a reliable test for the detection of antisperm antibodies (Adeghe *et al.*, 1988; Barratt *et al.*, 1989a). A direct immunobead test was performed on all semen samples except where not suitable, e.g. in men with low sperm motility or a low sperm concentration. In such cases, the indirect immunobead test was performed on seminal plasma samples. The seminal plasma samples were obtained by centrifugation of aliquots of fresh semen at 700 *g* for 10 min and the samples were stored at −20°C (see Adeghe *et al.*, 1988; Barrett *et al.*, 1989a for further details). Strict quality control procedures were adopted for the direct (IBT) and the indirect immunobead tests (IIBT) with simultaneous positive and negative control samples used for the latter. In an attempt to avoid false positive results, a 1:4 dilution of seminal plasma was used for the IIBT. For both tests, 100 spermatozoa were counted and the results were expressed as motile spermatozoa with one or more immunobeads attached to the surface. These analyses were performed using phase contrast microscopy, at ×400 magnification. The region of binding was noted (head, middle, tail) and the percentage binding recorded in each case (see Barratt *et al.*, 1989a). Anti-IgA and anti-IgG immunobeads were obtained from Biorad Laboratories (Biorad, Richmond, CA, USA). The immunobeads were stored at 4°C at a concentration of 2 mg/ml. When required, 1 ml was washed to remove sodium azide and any free antibody and was then resuspended in 1 ml Earle's medium (37°C).

Statistical analysis

A Spearman's correlation was calculated to examine the relationship between individual semen parameters and the presence, type and region of binding of antisperm antibodies. Only those relationships which were significant at the 1% level were tabulated. In order to determine whether the incidence of spontaneous pregnancy was significantly higher in women whose partners had antisperm antibodies compared to those without, the Mann−Whitney U non-parametric test was performed. The above analyses were performed using SPSS/PC at the computing services at the University of Sheffield.

Life Table Analysis was performed using Cox's proportional hazards model, which was implemented by the statistical analysis system. For the purpose of the statistical analysis, follow-up commenced at the point of recruitment of the study, with the variables analysed as continuous variables. Relative risks were calculated as outlined by Polansky and Lamb (1988). In all the above analyses, the data were examined with regard to percentage binding, region of binding, total amount of binding and isotype. Total sperm-bound IgA, -IgG, seminal plasma IgA and seminal plasma IgG were also calculated. The relevance of a putative

Table I. Incidence of IgG and IgA antibodies

	Percentage of motile spermatozoa bound				
Isotype	0	1–9	10–20	21–50	≥51
Sperm bound antibodies:					
IgG	357	63	9	11	2
IgA	348	68	23	11	1
Seminal plasma antibodies.					
IgG	64	14	0	2	1
IgA	58	13	3	3	2

threshold point was also evaluated at $<10\%$ binding, $10-20\%$ binding, $21-50\%$ binding and $\geq 51\%$ binding with either IgA and/or IgG.

Results

The incidence of antisperm antibodies on spermatozoa and in seminal plasma is shown in Table I. Using a threshold level of $\geq 10\%$ binding, in 58% of the cases both IgA and IgG were present on the spermatozoa, in one case IgG was present in the absence of IgA and in 14 cases IgA was present in the absence of IgG. In men with seminal plasma antibodies (using $<10\%$ binding as a threshold level), all men who had IgG also had IgA antibodies.

Of a total of 534 couples who were recruited to the study, 75 achieved a spontaneous conception. Of 251 couples of whom the female partner was demonstrated to have regular spontaneous ovulation and patent Fallopian tubes at either hysterosalpingography or laparoscopy, 42 achieved a spontaneous conception. The mean female age was 29 years and the mean duration of infertility prior to investigation was 56 months. First the data from all couples were examined. Correlations between the antibodies and semen parameters are outlined in Table II.

There was a highly significant negative correlation between the presence of antisperm antibodies and sperm cervical mucus penetration (Table II). The presence of antibodies was also positively associated with the concentration of round cells, and the number of peroxidase positive cells. The proportion of slow/moderate progressive spermatozoa was significantly reduced in the presence of antisperm antibodies (Table II).

Examining the data for all 534 couples, there was no significant difference between the pregnant and non-pregnant groups for any of the antisperm antibody categories alone or in combination. In addition, no significant difference was detected when the analysis was confined to those with regular spontaneous ovulation and patent Fallopian tubes (the 5% level of significance was used as a cut-off point). No antibody parameter had a significant effect on time to conception when analysing the data using Cox's regression.

Table II. Correlations between spermatozoal antisperm antibodies and semen parameters

Region of binding and isotype		Parameter	*P*-value
IgA head	0.1726	concentration of round cells	<0.001
	0.1651	[a]percentage of peroxidase positive cells	<0.001
	−0.1457	pH	<0.01
	0.1197	agglutination in semen	<0.01
IgA tail	−0.1491	pH	<0.01
	−0.1432	vanguard	<0.01
	−0.1230	concentration of spermatozoa in mucus	<0.01
	0.1478	agglutination in semen	<0.001
IgG tail	−0.1281	moderate/slow progressive motility	<0.001
	−0.1205	vanguard	<0.01
IgG head	0.2314	agglutination in semen	<0.001
	0.1617	agglutination in semen	<0.001
	−0.1250	concentration of spermatozoa in mucus	<0.01
IgG mixed	−0.1251	concentration of spermatozoa in mucus	<0.01

[a] = mainly neutrophils (Barratt *et al.*, 1990c).

Discussion

The results of this study clearly indicate that the presence of sperm-bound antisperm antibodies, at least at low (10–20% binding) and moderate (<50% binding) levels, has no significant effect on the probability of conception or the time interval to conception. The incidence of antibodies in this study using <10% binding as a cut-off point (WHO, 1987) was 6.5% which agrees with our previous studies (Barratt *et al.*, 1989a) and those of others (De Almeida *et al.*, 1989). Our strict quality control procedures in semen analysis and the immunobead antisperm antibody testing, as previously outlined (Barratt *et al.*, 1989a; Dunphy *et al.*, 1989b), ensured that the reliability and reproducibility of the results were satisfactory.

Concordant with other studies, the presence of antisperm antibodies significantly affected penetration into cervical mucus and the degree (distance) of penetration. Both IgA and IgG tail antibodies affected this penetration, which is in agreement with previous studies (Clarke, 1988; Kremer and Jager, 1980). In contrast to the studies of Wang *et al.* (1985), we did not observe an effect of head-directed antibodies on cervical mucus penetration, although in this study we used bovine cervical mucus and not human cervical mucus. More significant results may be obtained if human cervical mucus is used. Sperm motility was significantly affected by the presence of IgG on the tail ($P < 0.01$), confirming our previous observations of the effects of sperm antibodies on sperm motility (Barratt *et al.*, 1989a). In agreement with other studies, we noted an association between antisperm antibodies and sperm agglutination in the semen (De Almeida *et al.*, 1989) and the presence of round cells and peroxidase-positive cells (WHO, 1987). It is interesting to speculate on the association between round cells and antisperm antibodies. In previous studies, an increase in antisperm antibodies has been documented in men with a genital infection (Witkin and Sonnabend, 1983). We have categorized a higher median number of round cells and peroxidase positive cells in men with such infections compared to controls (Barratt *et al.*, 1990b,c). It is therefore feasible that this association reflects a possible development of the antibodies from a genital infection. The association between antisperm antibodies, genital infection and leukocytes warrants further investigation.

The finding that there was no significant effect of antisperm antibodies on conception is not surprising in view of the fact that only five out of 535 men in this study had high levels of antibody binding ($\geq 50\%$). The low incidence of high levels of antibodies in this study enables us to examine realistically the effects of low/moderate levels of antibodies. In this context, the results are in accordance with Meinertz *et al.* (1990) who demonstrated no significant effect on conception except at high levels of antisperm antibody binding. Interestingly, a retrospective analysis of conception rates in men with <50% antibody binding and >50% antibody binding clearly shows that the latter group had a significantly lower conception rate ($P < 0.005$) (Ayvaliotis *et al.*, 1985). It is also clear from *in vitro* studies (using cervical mucus and human eggs) that a high level of antisperm antibody binding is necessary before significant effects on sperm function are observed (Clarke *et al.*, 1985; Clarke, 1988). These results raise important questions for the treatment of such men. The use of

corticosteroids has recently been demonstrated to be of significant benefit in men with antisperm antibodies (Hendry *et al.*, 1990). This treatment does have side-effects and such treatment should be restricted to appropriate cases. From our data we would only recommend treatment using corticosteroids to men who have high levels of antisperm antibodies, i.e. >50%, and where there is a clear impediment of sperm function, e.g. on sperm cervical mucus testing, as recommended by Hendry *et al.* (1990).

In conclusion, our study demonstrates that the region, type and/or percentage of sperm bound antibodies have no effect on conception rates, at least when the regional antibody binding is <50%. We therefore recommend that treatment should not be implemented in men unless specific defects in sperm function can be observed.

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Paper 9:

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On the accuracy and clinical value of semen laboratory tests

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In their article (this issue, pp.247–248), Seibel and Zilberstein make several obvious but important points. The World Health Organization (WHO, 1993) and the Human Fertilisation and Embryology Authority (1991) strongly suggest that patients are dealt with as a couple rather than as individuals. Consequently, the role of the female factor in the interpretation of semen analysis results is continually emphasized, e.g. many long-term follow-up studies where the in-vivo prognostic significance of semen variables has been addressed confine observations to apparently normal female partners, thus minimizing the female factor. In addition, the negative influence of female age is now well documented. A second obvious point is that one semen variable examined in isolation will not reveal the whole picture; other semen variables (concentration of spermatozoa, presence of antisperm antibodies etc.) must be taken into consideration when providing the couple with diagnostic and prognostic information. Thirdly, prognostic information is often a question of probability of conception (and live birth) with or without various forms of treatment. Using the example of donor-inseminated patients (Seibel and Zilberstein, 1995), it is very likely that the use of donor semen would significantly increase the chances of conception for these couples compared to the chances of a natural conception. In the final analysis, the clinician, using reliable and accurate data, makes recommendations to the couple so they can make an informed decision. The vast majority of professionals are well aware of the above points; however, several other points may not be so obvious.

How standard, and accurate, are semen laboratory tests?

This is a critical question. Time after time, studies emphasize that technicians in different laboratories examining the same sample (and sometimes the same slide) produce results which are quite disturbing with respect to the high degree of variation. This degree of variation is generally regarded as unacceptable but, paradoxically, very little is done about correcting it. As it is critical that the andrology laboratory performing the semen assessment provides accurate results, why do we have such poor standards of quality in andrology laboratories?

Two factors compound the problem: (i) The vast majority of andrology laboratories do not have standard training procedures for the technicians performing semen assessments. Moreover, they do not regard quality as an important aspect of laboratory practice. Many excuses are offered, e.g. no standards are available for andrology, quality control is difficult using live gametes and the results of semen analysis provide poor prognostic information so why bother fine-tuning the standard laboratory tests? These points are easily addressed. For example, standard material, although not perfect, is available (WHO, 1992), quality control can be performed on live gametes and, if the technical variation of a procedure is high, then it is unlikely to produce accurate data which have any meaning (the obvious often requires emphasizing). In addition to this, training methods for technicians in semen assessment are available, so are numerous courses [run by the British Andrology Society and the European Society of Human Reproduction and Embryology (ESHRE)]; thus reliable and accurate data can be produced in the andrology laboratory, providing appropriate procedures are used.

(ii) The methods used to perform standard semen assessments vary considerably. The question becomes, What is current practice? In this context, What is a normal spermatozoon? Opinions vary as to the answer. The WHO, whose published laboratory procedure is often regarded as the standard reference method (WHO, 1992), has recently changed its definition of normal (see Table I) and, amazingly, this change was not based on any biological data. As a consequence, the implementation of these new standards has resulted in some controversy (to put it mildly). It is clear that the examination of sperm morphology has moved to using more strict criteria, e.g. Tygerberg criteria, where a biological endpoint for the definition of normal has been developed (examination of spermatozoa that had penetrated cervical mucus). Perhaps as a result, there is a considerable body of clinical data [specifically from in-vitro fertilization (IVF)] to support the use of strict criteria. However, even using strict criteria it is difficult to determine if results between studies are really comparable, e.g. the Tygerberg dimensions of a normal spermatozoon have been redefined over time (Table I). In addition, other authors using so-called strict criteria actually use different definitions of normal, e.g. Kobayashi *et al.* (1991). Therefore it probably comes as no surprise that problems in interpretation occur and differences in clinical results between centres appear to be commonplace. External quality control schemes operating amid such 'confusion' show this point clearly. For example, in the British Andrology Society external quality control scheme, the same sample examined for percentage normal morphology showed a 12–80% range (median 50%, 18 laboratories; Matson, 1995). What is the answer? Automated methods may help, and there are some encouraging data to support their use (Kruger *et al.*, 1993), but let us hope we don't follow the same tortuous path as has been the case for sperm motility, where we had to wait a number of years before clinical data on the usefulness of these measurements became available. Interestingly, there remains a paucity of biological data to support the use of computer-aided semen analysis in a clinical setting (Barratt *et al.*, 1993). In general, the trend is to wait for improved software, e.g. for imaging morphology, but I would plead with anybody who is developing these systems to remember the importance of biological data to support the measurements made. This is the strength of strict criteria for morphology assessment (Kruger *et al.*, 1988). What should we do while we are waiting for computerized morphology to appear in a cost-effective form? An alternative may be to define limits of normality within each laboratory, as suggested by the WHO (1992). This can be done for morphology (using

Table I. Strict criteria: head dimensions of a normal spermatozoon

Study	Kruger *et al.* (1986)	Kruger *et al.* (1988)	Menkveld *et al.* (1990)	Kobayashi *et al.* (1991)	WHO (1987)	WHO (1992)
Length (μm)	—[a]	5–6	3–5	4–6	3–5	4–5.5
Width (μm)	—[a]	2.5–3.5	2–3	2.4–3.5	2–3	2.5–3.5
Length/width ratio	ND	ND	1.5–1.67	ND	1.5–2.0	1.5–1.75
Stain	Pap	Diff-Quik	Pap	Diff-Quik	Pap	Pap
Cut-off point	>14% normal fertilization rate	<4% very poor fertilization rate; >14% normal rate	ND	<12% lower pregnancy rate/cycle	ND ≥50% normal	ND ≥30% normal

[a]Quantitative data not supplied in reported study.
WHO = World Health Organization; Pap = Papanicolaou stain; ND = no data provided in reported study.

an eyepiece graticule, and/or a drawing tube attached to the microscope to estimate sperm sizes). The problem comes in what should be measured, i.e. defining normal. Using the Tygerberg data may help, but it is important to consider that different preparation methods and staining regimes will alter cell size. In addition, spermatozoa from very fertile men need to be examined. As these are often difficult to obtain in anything like the numbers required, it is important to locate other sources of control material, e.g. the regional semen bank. In the near future, I envisage computerized morphology systems will still only be found in elite laboratories, awaiting further development. It is therefore important to use the data available in the literature and develop your own system using high standards of technician training. If an external quality control scheme is available, join it.

Are sperm function tests of any clinical value?

In view of the apparent poor prognostic value of basic semen parameters, in the andrology laboratory there is a niche for sperm function testing, e.g. examination of the acrosome reaction, zona binding and reactive oxygen species (ROS) to name but a few. An increasing body of evidence supports the use of such data in many aspects of clinical management. Two examples will suffice to illustrate this point: (i) several independent studies have indicated no fertilization or very poor fertilization rates in human IVF when spermatozoa are unable to acrosome react (usually in response to A23187). This is a defined cause of sperm dysfunction. Interestingly, Liu and Baker (1994) have developed such observations by defining a subgroup of these men whose spermatozoa were able to bind to the zona (and thus presumably have the egg sperm recognition process intact) but did not acrosome react. Single spermatozoa bound to the zona (acrosome intact) have been used for intracytoplasmic sperm injection and pregnancies achieved. (ii) Another example of the usefulness of sperm function testing is the detection of ROS, which are known to damage spermatozoa. Men with high levels of ROS production show highly significant improvements in human sperm zona binding after treatment with vitamin E (600 mg per day for 3 months). Trials involving placebo and active drug at IVF are now being planned. These two examples illustrate the use of sperm function tests, not as a primary line of investigation, but as a secondary line where a problem may be suspected. Accurate diagnostic information can be provided and possible treatment options discussed. Andrology laboratories should be encouraged to use selected sperm function testing in an appropriate and effective manner.

What progress has been made in our understanding of how spermatozoa work?

I infer from the Seibel and Zilberstein's article that they share the view of many andrologists, namely, what real progress in our understanding of male infertility has been made? The answer I believe is very little. A concentrated effort now needs to be applied at a basic research level so that improvements in our understanding of male fertility/infertility can be made. There are many studies, with I suspect, many more in the near future, examining the role of traditional semen variables in infertility, but I think we should now concentrate on determining the cellular/molecular basis of how spermatozoa work, and on an understanding of spermatogenesis.

One well-known example highlights our ignorance. To date, over 1000000 cycles of IVF have been performed in the world, yet we know practically nothing about how a human spermatozoon interacts with a human egg. This is astounding, as the interaction is observed by hundreds of scientists all over the world every day of the week! Animal studies don't really provide much information. For example, although the consensus of opinion suggests that ZP3 is the ligand on the zona responsible for sperm binding and the acrosome reaction (despite an almost total lack of information in the human), a plethora of complementary receptors on the spermatozoon are suggested. Why don't we know more? The simple answer is that not enough high-quality basic research is being performed using human gametes. With rapid advancements in cellular and molecular biology we should be able to draw on experiences in other disciplines to answer basic questions in reproductive biology, e.g. how a spermatozoon interacts with an egg. Being optimistic, we may even manage to address the enigma of capacitation!

In conclusion, a major reason why the basic assessments of semen have variable predictive value is due to a high degree

of error in the techniques used to assess the samples. In addition, different laboratories may not use the same techniques (although they may think they do; see Table I). It is clear that a considerable amount of technician training is required. Also, external quality control programmes are necessary. It is vital for satisfactory patient management that high standards in semen assessment are achieved. In this context, it is particularly welcome that ESHRE has identified these areas and taken affirmative action by instigating basic semen assessment training courses throughout Europe. It is critical that each andrology laboratory supports these efforts. Finally, sperm function tests are appropriate and clinically useful in some cases; however, real progress will only come if we attempt to understand more about how a spermatozoon is made and how it works.

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Implementing comprehensive quality control in the andrology laboratory

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Comprehensive quality control procedures were integrated into the routine semen analysis workload of a large university-based andrology laboratory. Methods were chosen to match as far as possible those which have been used successfully for many years in disciplines such as clinical chemistry. Levey–Jennings and cusum charts were plotted in order to monitor the immunobead-binding test for antisperm antibodies and a video-taped control sample for computerized semen analysis. A cryopreserved semen control was also charted. Daily manual sperm counts were plotted against the corresponding computer-assisted semen analysis (CASA) value. Multiple readings of 30 slides were used to monitor morphology assessments. Monthly means for morphology were also calculated regularly. Coefficients of variation were calculated for all variables and were found to be more appropriate for some aspects, such as CASA, than for others, such as morphology, when difference from the previous reading of the same slide was found to be more useful. These integrated quality control procedures had a direct influence on the production of results from the laboratory. Together with a high standard of technician training, comprehensive routine quality control based on repeated analyses of control samples is an effective way of assuring the validity of semen analysis results.

Key words: computer-assisted semen analysis/controls/cusum plots/quality control/semen analysis

Introduction

Almost 30 years have passed since Freund (1966) showed that basic semen analysis could be subject to gross error. From a multicentre study on morphology assessment he concluded that the methods used were 'subjective, qualitative, non-repeatable and difficult to teach'. This potential for gross analytical error in semen analysis has been revealed by a number of subsequent studies (Jequier and Ukombe, 1983; Dunphy *et al.*, 1989; Neuwinger *et al.*, 1990). In addition, concern has been expressed at the lack of operational standards in computer-assisted semen analysis (CASA; Davis and Katz, 1993a,b). A comparison of the measurements obtained by computer-assisted image analysis with those obtained by conventional means has shown the potential for discrepancy due to the methodology used (Macleod *et al.*, 1994). The need for suitable quality control within the andrology laboratory in order to reduce the level of imprecision in semen analysis was emphasized by Mortimer *et al.* (1986). The requirement for adequate internal quality control for accreditation purposes has provided added impetus in this field. However, despite the urgency of the problem, there remains relatively little to guide the andrologist in the practical application of routine quality control.

Descriptions of quality control systems have tended to focus almost exclusively on the training of technicians to a high standard in order to assure the quality of semen analysis data. Mortimer *et al.* (1986) showed how improvements to basic manual techniques could reduce the differences between technicians analysing the same sample, to levels which were considered unlikely to influence clinical decisions. Dunphy *et al.* (1989) assessed inter- and intra-technician variability in manual semen analysis and noted that large errors could arise in the determination of motility and morphology. They pointed out that the interpretation of semen analysis data must involve an appreciation of this analytical error, and stressed that technicians should be selected on their natural abilities as observers. A comprehensive scheme for monitoring, on a regular basis, inter- and intra-technician performance of motility, concentration and morphology assessment was described by Cooper *et al.* (1992). Mortimer (1994) has stated the need for goal-orientated, intensive technician-training, not only as a prerequisite for new technicians but as an on-going process in the quality control of technical skills in the laboratory. In 1992, the World Health Organization (WHO) introduced the subject of quality control into the *Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction*, and again, tended to concentrate on the training of technicians (WHO, 1992). This emphasis is understandable when the complex nature of semen analysis is considered, with its reliance on manual techniques. Technician training has a central role in the quality assurance programme of the andrology laboratory and has been integral to our laboratory. However, operator error is only one component of the total analytical error in any method. Adequate training is essential if better precision and accuracy are to be introduced into semen analysis, but should not be seen as a panacea. The best-trained technician can never produce results of high accuracy and precision using a method of analysis which is essentially prone to error, just as a poorly trained technician will be unlikely to assure optimum results from the most reliable of assays.

There is an urgent need for quality control procedures which operate together with technician training and which, by their

integration into the routine workload of the laboratory, can be used more directly to influence the quality of the results produced (see Barratt, 1995). In 1981, the WHO report on the external assessment of health laboratories described internal quality control as having 'an immediate effect on the information emerging from the laboratory at the time of checking... closely analogous to the quality control of randomly selected samples of a product emerging from an industrial process'. The system of internal quality control which has been implemented in our laboratory is based on this model. Some attention has already been paid to the provision of the control samples which are essential for the continuous monitoring of assays. The WHO (1992) Laboratory Manual suggests the use of stored aliquots of diluted semen for concentration determinations at periodic intervals and recommends the re-reading of morphology slides to assess consistency of scoring. Also suggested is the use of cryopreserved semen and of videotaped samples to provide longer-term control. The Manual also notes that positive and negative controls for antisperm antibody tests may be produced in-house from pooled and frozen seminal plasma or serum samples (WHO, 1992). In addition, Mortimer (1994) has addressed the problem of control samples for vitality and motility assessment. In the IVF laboratory, Muller (1992) has described the use of frozen aliquots of semen to control assays of sperm penetration of oocytes. Davis and Katz (1993b) have discussed methods to achieve quality control of computerized semen analysis by the use of beads, videotapes and computer simulations of sperm samples. The pilot scheme for external quality assessment organized by the British Andrology Society, in which samples were distributed to 20 laboratories throughout the UK, has demonstrated the successful use of control samples (Matson, 1995). In addition, Knuth *et al.* (1989) and Cooper *et al.* (1992) have illustrated the effectiveness of long-term sampling of the monthly means of semen analysis data in revealing systematic changes.

The present study shows that continuous process control can be successfully introduced into the andrology laboratory, using simple methods based on Levey–Jennings and cusum charts, which have long been employed in disciplines such as clinical chemistry, in order to assure and hopefully, improve the quality of semen analysis. Quality control was first introduced into this laboratory almost exclusively in the form of inter- and intra-technician comparison exercises. These are now seen as belonging to an overall strategy of quality assurance which also includes, for example, education, specimen reception and handling, reagent quality, instrument checks, and the reporting and checking of results (see Muller, 1992).

Materials and methods

The andrology laboratory at the Jessop Hospital for Women, Sheffield, UK has performed exercises in quality control for many years (see Dunphy *et al.*, 1989; Barratt *et al.*, 1993). However, a more comprehensive system of routine internal quality control was introduced from September 1992 (morphology), May 1993 (antisperm antibodies) and August 1993 (CASA). Emphasis was placed on the quality control of CASA rather than on manual methods for determining sperm concentration and motility, as we have used computerized methods almost exclusively for analysis of donor and patient samples for a period of several years (see Barratt *et al.*, 1993; Zhu *et al.*, 1994). This university-based centre performed over 1000 patient semen analyses in 1993 in addition to ~4000 analyses on fresh and post-thaw samples from semen donors. Throughout the period of the study, semen analysis was performed by the same three full-time and three part-time technicians on both fresh and cryopreserved samples. Most of the facilities used in routine analysis were also shared by scientists engaged in research. Procedures for quality control were introduced gradually and incorporated into written protocols. Results were entered onto the appropriate forms in a loose-leaf quality control binder. Data analysis of each month's results was performed promptly, and relevant charts were displayed in the laboratory. Quality control was reviewed at monthly laboratory meetings.

Antisperm antibodies

Immunoglobulin (Ig)A and IgG antisperm antibodies were assessed by an indirect immunobead test based on the WHO method (Barratt *et al.*, 1992). Ideally, controls should match patient samples as closely as possible. Over 95% of the patient samples tested were seminal plasma, so this was the material of choice. Pooled seminal plasma which tested antibody negative (i.e. 0% for both IgA and IgG) by the immunobead test was used for the negative control. To obtain a positive control for the test, a large pool (20 ml) of highly positive seminal plasma (immunobead binding of either or both classes of at least 50% by either or both direct and indirect methods) was prepared from patient samples previously tested and stored frozen at –20°C. High levels of binding were required in the control sample as only these levels were considered to be of clinical significance (Barratt *et al.*, 1992; WHO, 1992). Both controls were heat-inactivated at 56°C for 30 min. The pools were divided into over 100 aliquots of 125 µl and stored frozen at –20°C. A large number of aliquots was required in order to maintain long-term quality control. Batches of indirect antibodies were run once weekly, with one aliquot of positive and negative control being thawed and incorporated into each run. Total percentage binding was plotted on a Levey–Jennings control chart for IgA and IgG. Negative controls were not plotted.

Computer-assisted semen analysis (CASA)

A videotape was used to monitor the performance of the Hamilton–Thorn Motility (HTM) analyser (2030, version 7; Hamilton–Thorn Research Inc, Beverly, MA, USA) under optimal conditions, i.e. the tape was of a single field, and thus the variation due to operator, set-up of the optical system, sampling error, field selection and chamber depth was eliminated (Pedigo *et al.*, 1989). The videotape was used to assess instrument reliability over an extended period in order to provide assurance to its users. The same section of tape was located for each analysis by means of a digital counter. However, no attempt was made to capture exactly the same spermatozoa on each analysis. The tape was used as the closest approximation to a live semen sample which could be used from week to week, allowing us to estimate the error involved in the analysis of various populations of ~200 cells belonging to the same sample. The error involved was not related simply to the starting point, but also to the random error in the routine analysis of a relatively small number of cells. The concentration of the sample recorded was at the lower range of suitability for CASA, i.e. 20×10^6 spermatozoa/ml. The motility was ~50% (rapid + medium + slow grades as defined below), allowing assessment of the performance of immotile analysis. The sample also included occasional large round cells, which allowed assessment of the ability of the instrument to discriminate within a mixed population of immotile objects. This sample therefore provided results which

represent the limits for normality as suggested by the WHO (1992). The tape was run weekly and at least 200 cells were analysed. Parameters recorded were total concentration and four grades of motility. These parameters were chosen as they form part of the routine semen analysis report. However, percentage medium (10–25 μm/s) and slow (>0<10 μm/s) motilities were very low and were not plotted on control charts. Percentage rapid motility (>25 μm/s) and percentage immotile (<0 μm/s) were recorded. Levey–Jennings charts based on the mean and two standard deviations were established (n = 12), upon which results were plotted weekly (n = 22). A cusum plot was made at intervals of several months in order to detect long-term trends in instrument performance.

A single cryopreserved ejaculate comprising 50 straws, obtained from a donor who produced a large sample volume, was also used as a routine quality control sample to monitor concentration, motile concentration and percentage total motility in post-thaw samples from patients and donors. These samples formed ~40% of the total workload of the HTM analyser. The control sample was diluted 1:1 in a glycerol–egg yolk–citrate buffer with a final glycerol concentration of 7.5% by volume, packaged into 0.25 ml straws (Rocket, London, UK) and frozen in a programmable freezer (Planer Products, Middlesex, UK) using the following cooling rates: 20°C to 5°C at –1°C/min, then –10°C/min to –110°C, before storage in liquid nitrogen. One straw was thawed at room temperature and allowed to equilibriate for several minutes before CASA. Values obtained from analysis of at least 200 cells were plotted on a Levey–Jennings control chart weekly (n = 30). The sample chosen had a motile (i.e. mean average path velocity (VAP) >0 μm/s) concentration of ~10×10^6/ml, as this was the lower limit of acceptability for post-thaw samples from semen donors attending our centre. A limited number of straws (six) was used to set up tentative limits for this control on a Levey–Jennings chart. This was in order to retain a sufficient number of straws to allow the ejaculate to be used over the long term. Limits were recalculated after 18 measurements. However, owing to the wide range of values obtained over a period of 6 months, this method was discontinued.

In order to validate the semen analysis data obtained from the HTM analyser, a manual concentration was carried out on the first sample of each day's run. This represented ~10% of patient specimens. Only samples suitable for CASA were included, i.e. <10% agglutination and <10% concentration of round cells and debris (Davis and Katz, 1992). CASA was performed as previously described (Barratt *et al.*, 1993). All samples with a concentration >70×10^6/ml were diluted to an appropriate level with Dulbecco's phosphate buffer supplemented with bovine serum albumin (0.3 g/l) and glucose (1 g/l) (WHO, 1992). Homologous seminal plasma was not used because of practical difficulties, including frequent cases of high viscosity and low volume in our patient samples. Manual concentrations were assessed by the use of an improved Neubauer chamber (WHO, 1992). The set-up of the HTM analyser was adjusted to give a concentration measurement to match the manual count. This was usually achieved by adjusting the illumination intensity and the focus on the external optical system to change the size and brightness of the digitized image of the spermatozoa heads. Results of these checks were recorded on a daily basis and analysed at the end of each month for display as a Bland and Altman type chart (see Statistics). Over time, there seemed to be an increased awareness of which samples were unsuitable for HTM analysis and also an improvement in operator skill in capturing accurate video images by use of the external optics.

Morphology

Smears were prepared for morphology by spreading either 5, 10 or 20 μl of semen on clean glass slides, using a coverslip to drag the sample gently across the slide. The volume of sample used depended on the concentration of spermatozoa (Menkveld *et al.*, 1990). In the case of viscous samples, an aliquot of semen was placed on one slide and then covered by a second. After allowing the sample to spread between the slides, the two slides were then gently pulled apart (WHO, 1992). If the sample was oligo- or azoospermic, a smear was prepared from the pellet obtained by centrifugation at 600 *g* for 20 min. The slides were fixed in methanol after being allowed to air-dry, and then sent for Papanicolaou staining at a routine histology laboratory. Scoring of normal (ideal) forms only was by WHO (1987) criteria from September 1992 until November 1992, when WHO (1992) criteria were adopted. Continuous monitoring of the method was carried out using two approaches. The first involved periodically selecting sets of five slides from a variety of patients or donors. A wide range of slides was selected, including those showing a high proportion of very poor morphology and others with borderline forms. These were assigned a code-number, and were included in the routine runs of morphology slides on a regular basis. The procedure finally adopted was to include one set of five quality control slides with every 50 routine slides. A check was made on the previous reading obtained for each slide, allowing continuous monitoring of intra- and inter-technician variability. Estimation of the precision of the method over a long period was also possible after a suitable number of results had been accrued.

The second method of quality control was that of calculating the monthly mean. The mean of all the patient morphology results was calculated immediately at the end of the month; it was only done occasionally initially (March, September and December 1992, May, July and September 1993), and then for every month thereafter, and trends in the overall level of results were examined. This value was used to aid interpretation of trends which were apparent in the reading of the quality control slides when changing from WHO (1987) to WHO (1992) criteria.

Statistical analysis

To establish Levey–Jennings and cusum control charts it was necessary to accumulate sufficient data from repeated analysis of the control samples in a reasonably short period in order to set confidence limits. Normally, at least 20 samples are required to set limits for precision in clinical chemistry (Broughton, 1978). The relatively small number of semen analyses generally performed may preclude the rapid collection of large volumes of data. Mortimer (1994) has suggested that at least 10 values are required when defining precision in semen analysis. In addition, more valid limits are set by analysis of control samples under routine conditions when the precision is likely to be less than that of repeated analysis under optimal conditions, such as by a single operator on one occasion. These constraints mean that frequently small numbers of data showing a wide range of values are available. Some approaches, such as calculating the correlation coefficient and coefficient of variation (CV), may be unsuitable in this case. (Bland and Altman, 1986; Davis and Gravance, 1993). Data in the form of percentages were not transformed before analysis as it was felt that procedures should be kept as simple as possible, and that the benefit of such manipulation was debatable (Broughton, 1978).

The emphasis in our laboratory was on implementing, as quickly as possible, a comprehensive system of simple quality control procedures which could be used by technical staff to monitor routine analyses. As a crude check on the normality of the data we compared the mean with the median value; in all cases this was similar, e.g. for immunobead binding (see Table I). Levey–Jennings charts were established by drawing limits of 2 SD each side of the mean and plotting control values as obtained. Cusum plots involved plotting

Table I. Change in value of percentage binding to IgA immunobeads shown by an antisperm antibody positive control

	18/05/93–27/10/93		03/11/93–29/03/94	
	IgA	IgG	IgA	IgG
Mean	74.7	82.8	61.9	72.7
SD	12.1	8.5	14.5	18.8
CV	16.2	10.3	23.5	25.1
Min	53.0	67	28.0	33
Max	90.0	97	84.0	97
n	18	18	17	17

consecutively the cumulative sum of the differences from the original mean value. In the immunobead-binding test, limits were based on the first 18 results obtained under routine conditions. The coefficients of variation for both IgA and IgG positive controls for the first 18 and the subsequent 17 plotted values were calculated in order to detect any trend. Based on the mean value of the first 18 samples, a cusum plot was also made for IgA and IgG positive controls. This technique was used to amplify any systematic error which was not immediately obvious from the Levey–Jennings chart. Similar methods were used to analyse data from the videotape, using concentration, percentage rapid motility, and percentage of static spermatozoa as variables. Limits were set on the first 12 values obtained under routine conditions. To set limits for the cryopreserved control, six straws from a single ejaculate of 50 were analysed by the HTM analyser for motility and concentration measurement. A Levey–Jennings control chart was based on the CASA values.

Initially, a scatter-plot was made to display CASA concentration values versus the manual check. The correlation coefficient was also calculated. This was done on a monthly basis, but was replaced by a method similar to that of Bland and Altman (1986). (Emphasis was placed on the manual concentration as a reference value rather than following the original procedure of Bland and Altman, which was based on the comparison of two equivalent methods.) This involved plotting the difference between the CASA and the manual values, as a percentage of the manual count (Mortimer, 1994), against the manual count. The manual count was preferred to the mean of the two methods as being the best approximation of the true value, to which the CASA system should be calibrated (Davis and Katz, 1992). The mean, median and range of the percentage differences was also calculated monthly to allow assessment of bias (mean) and potential for discrepancy (range) (Mortimer, 1994).

For morphology, coefficients of variation, means and ranges based on small numbers of readings (minimum of four, maximum of eight) were calculated at intervals. A 'previous value check' (Statland and Westgard, 1991) was also applied to successive re-readings of the same slide. This was expressed as difference in score from the previous value. The monthly mean of normal spermatozoa per patient was plotted as a bar chart, with all patient results being included except those from azoospermic cases, as the only data available for azoospermic samples was for the incidence of round cells etc.

Interpretation of the Levey–Jennings charts for antisperm antibody positive controls, CASA videotaped control and cryopreserved control involved observation of increased dispersion of recorded points due to a decrease in precision, systematic drift due to a slowly developing problem, and an abrupt change due to a sudden shift in the method (Statland and Westgard, 1991). The cusum charts helped to amplify any systematic changes in values. A horizontal gradient indicated that the method was in control with values obtained similar to the established mean. A downward or upward slope showed that values obtained were systematically lower or higher than those expected. The cusum plots were inspected for sharp changes in gradient, which indicated problems as they arose.

Results

Antisperm antibodies

A wide range of values was obtained for the positive immunobead controls over the 12 month period (Figure 1a). The mean and median values for 39 assays of IgA were 69.2 and 70% respectively, range 28–90%. Slightly higher values were obtained for IgG, which produced a mean of 77.5% and a median of 80%, range 33–97%. The overall CVs were 20.4 (IgA) and 19.1 (IgG). Weekly values were plotted on the control charts (Figure 1b and c). Limits (2 SD) set were 51–99% (IgA) and 66–100% (IgG). Three controls for IgA and six controls for IgG lay outside the limits, representing a total of 26% of the plotted values. The cusum chart (Figure 1d) showed a systematic tendency to give lower values than expected (downward gradient), although this bias was not in evidence towards the end of the study (horizontal gradient) when the method appeared to be in control. The data used to establish the control charts and those which were plotted are summarized in Table I. Binding was rarely observed in the negative control and was never >10%. For this reason, negative control values were not plotted.

Computer-assisted semen analysis

Control charts for the videotaped sample are shown in Figure 2. The limits set were 18.2–22.2 (concentration), 27.6–44.8 (rapid motility) and 39.7–65.3 (static). Eleven of the 66 plotted values (16.7%) were outside the 2 SD limits (six concentration, one rapid motility and four static measurements). This is somewhat higher than the expected 5%. The cusum plot for the videotaped control sample (Figure 2d) shows little change in the value of concentration over time, but a systematic decline in the percentage of cells classified as rapid, accompanied by a reciprocal increase in the percentage of cells classified as static. However, summaries of the data used to set the limits and that used to plot the cusum chart (Table II) show that these changes are relatively small.

Control charts for the cryopreserved sample are shown in Figure 3, with the plotted values summarized in Table III. The range of values provided by this particular sample was found to be too wide to facilitate the effective use of this material as a control, with a large number of results lying outside the 95% limits. This method was rejected as part of the routine quality control in the laboratory.

Correlation coefficients with values close to 1.0 were regularly attained when comparing paired CASA and manual concentration values (e.g. August–November 1993, $r = 0.96$, $n = 68$). Analysis of the percentage difference of CASA results from the manual control showed a potential for discrepancy more effectively. Figure 3c shows a plot of percentage differences obtained in March 1994 against the manual values. The magnitude of discrepancy did not appear to be related to the concentration value. The range of differences was on occasion very wide, but the mean differences were relatively small, showing little bias of the HTM values from the manual

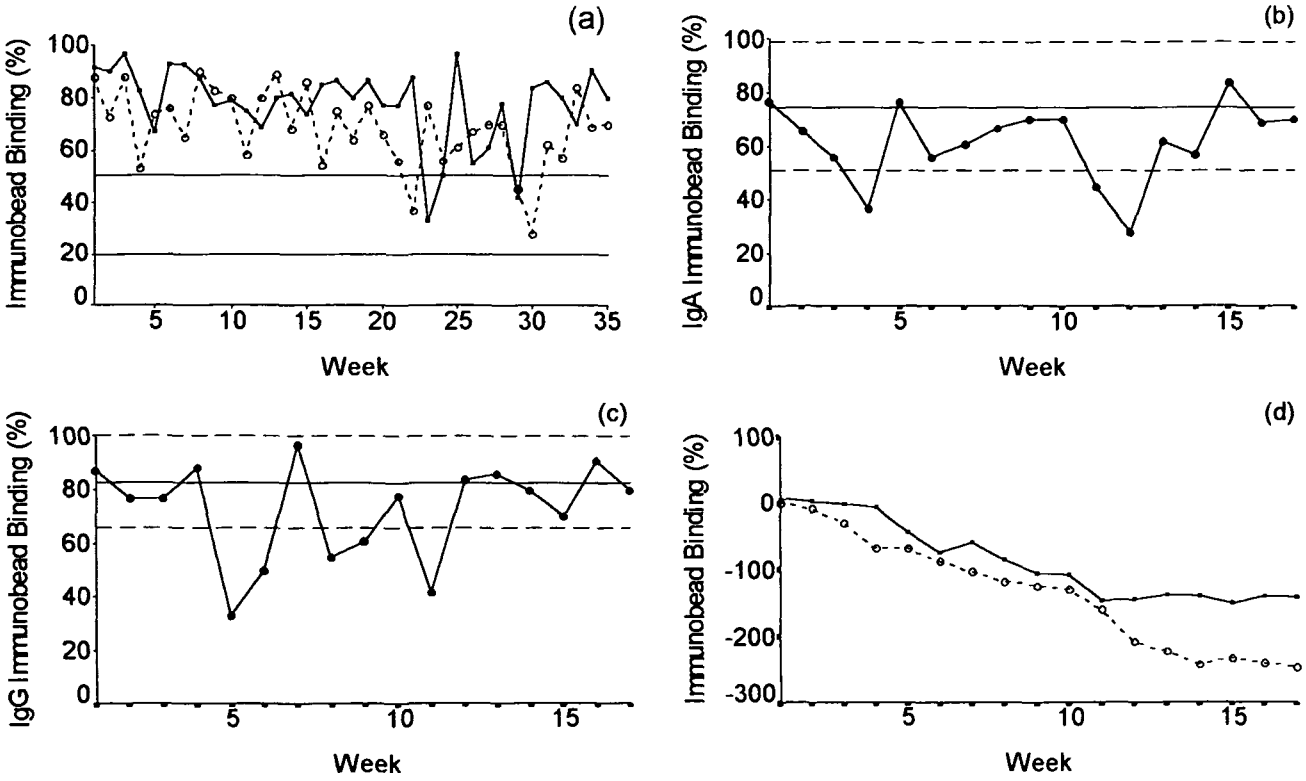

Figure 1. Routine quality control for antisperm antibodies. (**a**) Variation in percentage binding to IgA and IgG immunobeads in a pooled seminal plasma positive control for an indirect antisperm antibody assay over a period of 12 months. Binding to IgG immunobeads is shown by the solid line, and to IgA by the broken line. Horizontal lines show lower limits suggested by WHO for positive (20%) and clinically significant (50%) levels of immunobead binding. (**b**) and (**c**) Levey–Jennings control charts for percentage binding to IgA and IgG immunobeads in a pooled seminal plasma positive control. Results were plotted weekly. Horizontal broken lines denote upper and lower limits of 2 SD from the mean (solid horizontal line). Limits were set on the previous 18 values. (**d**) Cusum plot of percentage binding to IgA and IgG immunobeads in a pooled seminal plasma positive control. Binding to IgG immunobeads is shown by the solid line, and to IgA by the broken line. Lines represent the cumulative sum of differences from the mean value of 18 previous measurements.

control. The agreement between the two methods steadily improved over a 6-month period, with a reduction in the range of differences (Figure 3d).

Morphology

Results for routine morphology are summarized in Table IV. A mean of 6.4 readings was obtained per slide (median = 7). A wide range of CVs was obtained, with a mean value of 24.0% (median 18.6%). Slides with lower mean values had higher CVs. Analysis of the absolute difference in score from the previous reading is provided by Figure 4a. Of the 145 repeat assessments (25 slides) by WHO (1992) criteria, 72 (49.7%) differed by a score of $\leq 3\%$, 13 repeats (9.0%) were identical, and nine (5.5%) varied by a score of $>10\%$. The magnitude of the differences was not related to the number of times that the slide had been read. Monthly mean values are shown in Figure 4b and reveal a large reduction in the average score, from 50% to just over 17% during the period of study. This shift appears to be related to the change in criteria applied, as the same technician read all the slides during the period March 1992–November 1993, and comparable monthly means for concentration and motility did not show trends to indicate a significant change in the population (data not shown).

Discussion

To our knowledge, this is the first description of the successful introduction of a comprehensive system of routine, integrated internal quality control procedures into a laboratory dedicated to semen analysis that is not centred on technician assessment. We consider that our system was successful for the following reasons. Firstly, because it was sustainable and non-disruptive in the context of a busy service andrology laboratory, being integrated into the normal work-flow. Secondly, it was not misconstrued as a 'checking-up' exercise on technicians' abilities/performances. Thirdly, it improved our awareness of the overall error of our methods and helped prevent the release of potentially erroneous results. For example, manual checks on the HTM analyser have helped us to improve our use of CASA. Fourthly, through our systematic use of internal quality control, we have begun to establish a 'quality culture' in our laboratory.

Other systems have been reported which have addressed the fundamental requirement for a high level of technician training, the absence of which would undermine the value of routine quality control procedures. Very high levels of precision in analyses have been obtained by a commitment to goal-oriented technician training and an emphasis on the adherence to

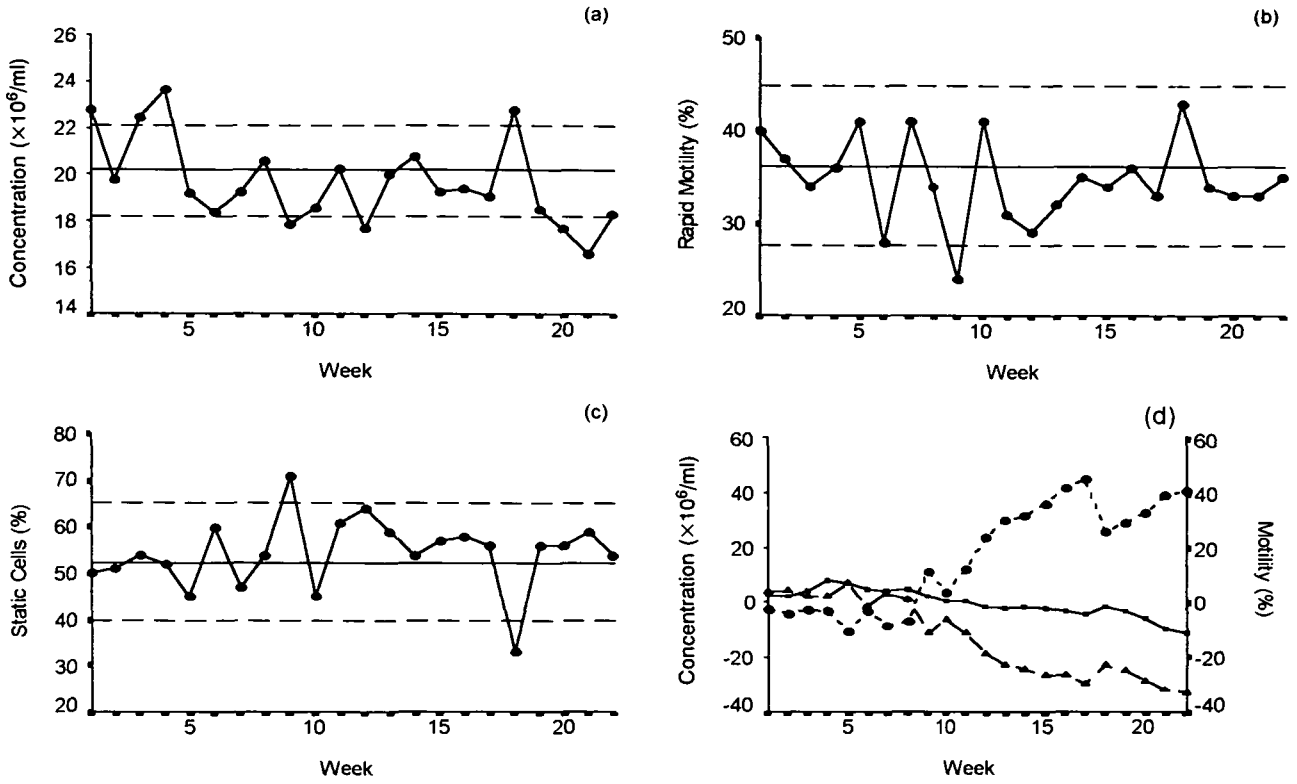

Figure 2. Routine quality control for CASA based on a videotaped semen control sample. Results were plotted weekly on Levey–Jennings control charts for sperm concentration (**a**), percentage rapid sperm motility (**b**) and percentage static cells (**c**). Horizontal broken lines denote upper and lower limits of 2 SD from the mean (solid horizontal line). Limits were set on the previous 12 values. (**d**) Cusum plot. Concentration, percentage rapid motility and percentage static spermatozoa are denoted by solid, broken and dotted lines respectively. Lines represent the cumulative sum of differences from the mean value of 12 previous measurements.

Table II. Videotaped control. The same section of tape was analysed weekly by the Hamilton–Thorn motility analyser. a = values used to establish control chart; b = values plotted on control chart

	Concentration (×10⁶/ml)		Rapid motile (%)		Medium motile (%)		Slow motile (%)		Static (%)	
	a	b	a	b	a	b	a	b	a	b
Mean	20.2	19.7	36.2	34.7	8.9	8.4	2.7	2.4	52.5	54.4
SD	1.0	1.9	4.3	4.6	3.0	2.7	1.5	1.6	6.4	7.7
CV	4.9	9.5	11.8	13.3	33.6	32.6	56.1	67.3	12.2	14.1
Min	18.5	16.6	27.0	24	5.0	5.0	1.0	0.0	39.0	33
Max	21.9	23.7	45.0	43	16.0	17.0	6.0	7.0	63.0	71
n	12	22	12	22	12	22	12	22	12	22

rigorous standards at the bench (Cooper *et al.*, 1992; Mortimer, 1994). There may, however, be some disadvantages in applying a technician-orientated approach as a tool for the continuous monitoring of analytical performance. It may not be appropriate to subject fully trained technicians, who have obtained certificates of competence at courses held by relevant professional bodies such as the British Andrology Society, to repeated testing of their performance in this way. The 'quality control' of technicians may be a sensitive issue (Dharan, 1977). Inter-technician quality control exercises may also be time-consuming and disruptive to the work-flow in the laboratory, and may be seen as somewhat artificial in that they do not reflect routine working practice. Although the scheme described by Cooper *et al.* (1992), based largely on such exercises, is very comprehensive, it could prove expensive to implement. A disadvantage of this system, in which small numbers of control samples are assessed repeatedly over time, is that it is perhaps unsuitable for monitoring techniques which are highly subjective and obviously prone to bias through familiarity. It is notable that the CVs reported in that study, such as for morphology, are generally lower than those obtained in our laboratory. Although this may be a consequence of the transformation of data by Cooper *et al.*, it may also be explained by the way in which we have attempted to integrate control samples into the workload — this approach would be expected to give higher CVs which reflect the overall analytical variation of the method. Subjectivity and familiarity are, however, features of the quality control used for the

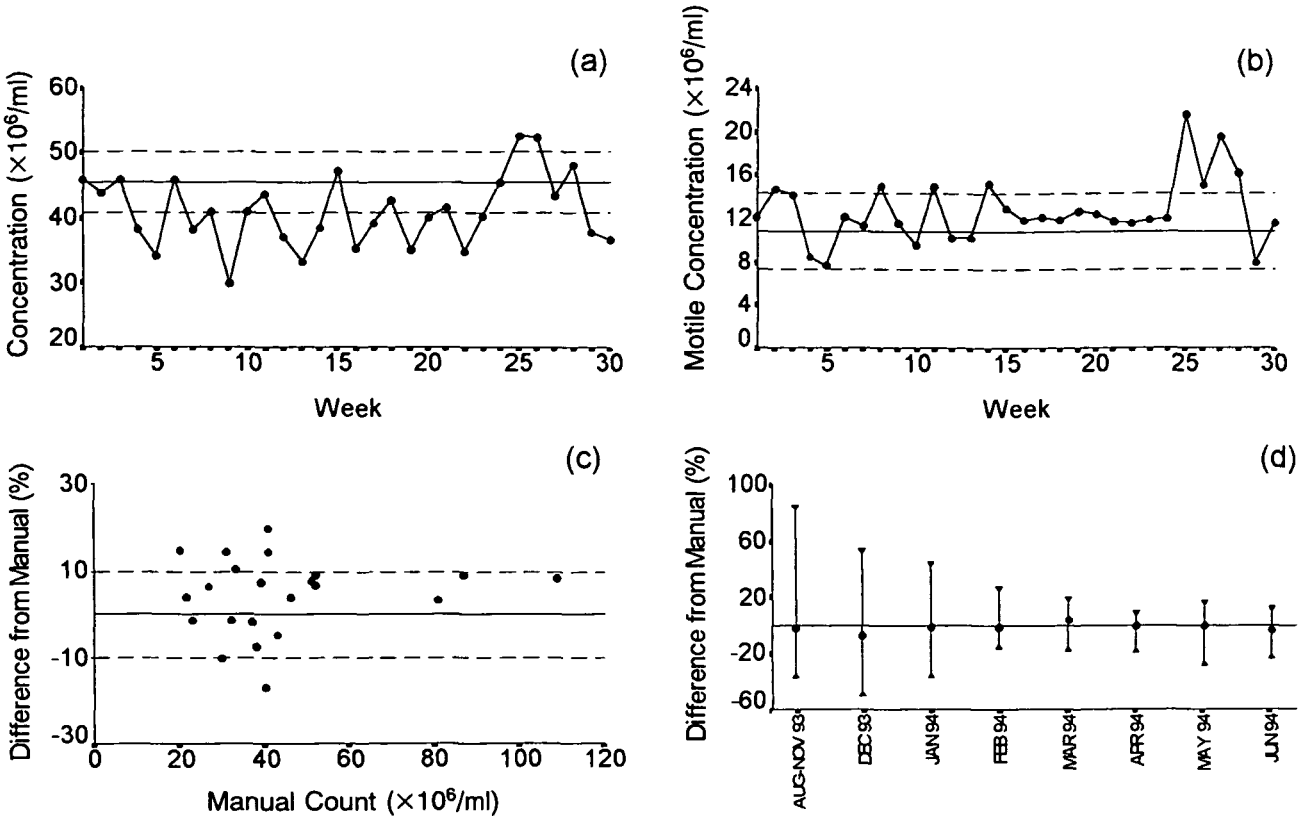

Figure 3. Routine quality control of CASA based on a cryopreserved semen control sample and a 'first sample' manual check. Results were plotted weekly. Levey–Jennings control charts are shown for the cryopreserved sample: (**a**) concentration and (**b**) motile concentration. Solid horizontal lines denote mean and upper and lower limits of 2 SD based on analysis of six samples. (**c**) Monthly plot of differences between CASA and manual measurements of a 'first sample' check sperm concentration, expressed as a percentage of the manual value. Values are plotted against the manual value. Points within the broken lines denote CASA values which are within 10% of the manual value. Samples from patients and semen donors were assessed at the commencement of each day's workload. (**d**) Mean and range of differences between CASA and manual measurements of sperm concentration, expressed as a percentage of the manual value, plotted monthly. Triangles represent the maximum and minimum values, circles represent the means.

Table III. Cryopreserved control sample assessed by the Hamilton–Thorn motility analyser. One straw from the same ejaculate was analysed at approximately weekly intervals

	Concentration (×10⁶/ml)	Motile concentration (×10⁶/ml)	Motility (%)
Mean	41.1	12.7	31.1
Median	40.7	12.2	31.0
SD	5.5	3.0	5.7
CV	13.3	23.7	18.4
Min	30.0	7.7	21.0
Max	52.7	21.6	45.0
n	30	30	30

immunobead-binding test in this study. To help overcome personal bias in this test, all positive antisperm antibody results are now checked by another technician.

Other systems have been described which, although based on the analysis of data which has been obtained under routine conditions, suffer from being retrospective (Macleod *et al.*, 1994). However, the technique of sampling the monthly mean of semen variables described by Knuth *et al.* (1989) is easily adapted as an on-going quality control measure for morphology by prompt calculation of the statistic at the completion of the month's work (Cooper *et al.*, 1992). This study showed how batch means were used to monitor the large change in values obtained when applying stricter criteria to patient morphology.

The assessment of error in semen analysis has been the focus of attention of other studies. From the results of a 1-day workshop on semen analysis held by the British Andrology Society in 1981, it was concluded by Jequier and Ukombe (1983) that such errors could be very large. Dunphy *et al.* (1989) undertook a detailed investigation into the performance of standard semen analysis by three technicians and noted the potential for discrepancy in manual measurements of motility and also in morphology assessments performed by a single, highly experienced observer. In order to reduce such widely reported levels of error, our laboratory has concentrated on replacing manual with automated methods for analysis of sperm motility and on the strict application of WHO criteria in the classification of sperm morphology.

Much attention has been given to the quality of CASA in clinical practice. In a study of the quality of semen data from HTM analysis, Pedigo *et al.* (1989) determined instrument precision by means of replicate analyses of the same section of videotape. This technique was adapted in our laboratory to provide long-term control over the performance of the HTM analyser. (Currently the tape has been analysed 54 times over a period of 15 months.) In general, operational standards for CASA, which were suggested by Davis and Katz (1992,

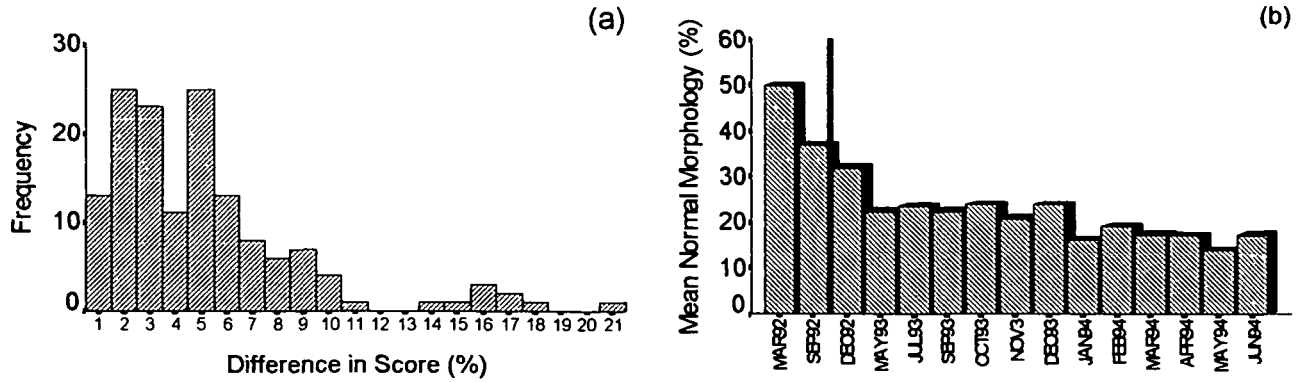

Figure 4. Routine quality control of morphology. (**a**) Histogram of difference in score from the previous reading based on 25 quality control slides for assessment of morphologically normal spermatozoa. Slides were read according to WHO (1992) criteria. Semen smears were from patients and donors and were stained by a Papanicolaou method. (**b**) Bar chart of monthly mean values for percentage morphologically normal spermatozoa from patients undergoing routine infertility investigations. Mean values are given for the following months: March, September, and December 1992, May and July 1993 and then each month from September 1993 to June 1994. WHO (1987) criteria were applied until November 1992, whereafter WHO (1992) criteria were applied. The vertical reference line denotes the change in methodology.

Table IV. Values represent percentage morphologically normal forms obtained by repeated assessment of 30 quality control slides. Slides 1–5 were read according to WHO (1987) criteria, slides 6–30 were read according to WHO (1992) criteria

Slide	Mean	SD	CV	Min	Max	*n*
1	43.0	4.1	9.5	38	48	4
2	54.5	6.7	12.0	46	62	4
3	22.0	2.2	9.8	20	25	4
4	48.5	13.7	28.3	40	69	4
5	26.0	2.7	10.4	22	28	4
6	57.0	8.8	15.5	44	68	5
7	20.6	6.6	31.9	15	31	5
8	10.6	4.3	40.4	7	18	5
9	15.4	5.6	36.0	7	22	5
10	22.8	3.3	14.4	20	28	5
11	21.7	6.6	30.3	13	32	6
12	37.5	4.6	12.4	30	42	6
13	15.0	3.5	23.1	9	18	6
14	46.9	2.6	5.4	45	52	7
15	34.8	4.4	12.5	28	40	7
16	34.7	6.0	17.3	28	46	7
17	31.9	5.8	18.2	22	38	7
18	43.3	2.6	5.9	40	47	7
19	42.7	4.6	10.8	37	49	6
20	44.3	2.7	6.1	41	48	7
21	8.5	3.7	43.6	4	12	8
22	20.6	7.1	34.4	11	34	8
23	13.8	6.2	45.3	7	23	8
24	38.5	4.2	10.9	33	46	8
25	5.3	1.6	30.1	4	8	8
26	2.8	1.4	50.5	1	5	8
27	31.5	6.0	19.1	20	40	8
28	5.4	2.7	50.6	0	9	8
29	34.6	6.8	19.6	19	40	8
30	8.6	5.5	64.1	0	13	8

1993b), have been introduced into this laboratory. However, it has not been considered necessary to perform a manual control of concentration for each sample, as the results from the 'first sample' check which we have implemented suggest that, provided that the operational standards concerning concentration limits, agglutination and round cell interference are adhered to, large discrepancy is unlikely.

It is of interest that a retrospective study, based in a regional andrology service laboratory (Aberdeen), of the comparative assessment of 1435 routine patient semen samples using both the HTM analyser and manual methods did not provide comparable data (Macleod *et al.*, 1994) for either concentration or motility measurements. Reasons for this may be that unsuitable samples were analysed on the HTM analyser, as all patient samples were analysed. (Our laboratory rejects ~25% of patient samples as being unsuitable for CASA.) The use of an external microscope has also been found by this laboratory to be a considerable improvement on the internal optical system used in the study of MacLeod *et al.* We have also found the use of homologous seminal plasma as a diluent, as described in the Aberdeen study, to be unsatisfactory in many cases. In addition to frequent disorders of low specimen volume and high viscosity affecting samples, there is the problem of ensuring the homogeneity of the diluted specimen. Similarly to Macleod *et al.* (1994), emphasis was placed in our study on the analysis of differences between manual and CASA concentration values. However, our approach differed in three ways. Firstly, we calculated the percentage rather than the absolute difference between the two values as being a more useful measure of discrepancy (see Mortimer, 1994). Secondly, we plotted these differences against the manual value rather than the mean of the two methods, as the manual method is usually considered to be less subject to error (WHO, 1992). Thirdly, we used the comparative data as a continuous check on routine semen analysis in order to validate and control CASA rather than as a retrospective assessment of the comparability of the methodologies.

The use of aliquots of a cryopreserved sample to provide routine quality control of sperm penetration assays, based on 95% confidence limits, has been outlined by Muller (1992), although the number of measurements was very small. Difficulties experienced were in the lack of consistency between vials and in the limited number of vials available. Cooper *et al.* (1992) utilized three cryopreserved controls to assess inter- and intra-technician variation in reporting manual concentration and motility with time, and again, encountered

inconsistency between aliquots. A similar attempt to employ a cryopreserved control in CASA was made in this study. Although a larger number of aliquots were measured over a 6 month period (6/8/93–27/1/94), similar problems were encountered (Table II, Figure 3a,b).

In contrast to most of these methods of assessing analytical error, the system which has been introduced into this laboratory is modelled largely on traditional methods which have long been employed by pathology disciplines such as clinical chemistry. We have reduced the emphasis on the quality control of technicians. Inter-technician comparison exercises, held on an approximately 2-weekly basis in this laboratory, have now been largely replaced by routine methods of quality control and are used only if a need is seen to arise, such as when a technician commences semen assessments for the first time or after a period of absence from the bench. Training exercises are also used to provide data which can be used in the estimation of achievable goals in day-to-day quality control. For example, our data from the videotaped control suggest that most of the differences obtained between technicians analysing the same sample on the HTM analyser can be accounted for by instrument imprecision and random error inherent in the analysis of a relatively small number of cells.

The laboratory has focused on methods which monitor the overall analytical process, in order to control assay performance. This has been achieved by using a variety of control materials, some of which, however, have been found to be more effective than others. Wall-mounted control charts have enabled the laboratory to display its commitment to quality control and hopefully assisted in the interpretation of the laboratory's results by its users.

The use of a single positive control for the immunobead-binding test (Table I, Figure 1) for an extended period (currently more than 12 months) has provided the assurance that, despite the obvious variability of the method, a highly positive sample is unlikely to be found negative, that is, to show <20% binding (WHO, 1992). Conversely, no results for the negative control were found to be positive (data not shown as binding was rarely observed). However, the quality control data described here suggest that this test is less reliable in discriminating clinically significant levels of antibody-binding in a given sample, i.e. in excess of 50% for either class of antibody (WHO, 1992). Some of the variability could perhaps be ascribed to matrix effects resulting from the use of a pooled sample of seminal plasma. A decrease in the value of the positive control was shown by a cusum plot (Figure 1d), accompanied by a simultaneous increase in the CV (Table I), indicating a decrease in precision with time. Possible explanations for these changes include a change of operator in October 1993, a change to the methodology by increasing the final BSA concentration of the immunobead suspension from 0.4 to 5% to prevent clumping of the beads in March 1994 (Mortimer, 1994), and possible deterioration of the control samples. It was not found necessary to repeat batches due to poor performance as indicated by the control chart during the period of this study. This was because a batch was not repeated if the positive controls were out of limits but no binding was observed in any of the patient samples, provided that the controls showed a minimum binding of >20%. (It has, however, been found necessary to repeat batches subsequent to this study where binding has been observed in patient samples and the controls have been below the lower limits.) A direct consequence of running the long-term quality control samples has been to alert technicians and medical staff to the imprecision of the method, which has led to the mandatory re-checking of all suspected antisperm antibody-positive samples by another technician. Although well-trained technicians will usually agree on classifying a sample as positive, we feel that a second opinion is invaluable, especially in weakly positive samples and those in which mucus interference and poor sperm motility make interpretation more difficult. In response to the poor performance of the immunobead test revealed by our quality control procedures, we have introduced a second positive control prepared from a single serum specimen, and also changed the technical staff performing the assays. Although we have shown that control samples may be used to monitor indirect immunobead tests, the routine quality control of direct tests is not currently practised in this laboratory. Our overall assessment of the immunobead test is that it is of limited reliability and of dubious value in infertility investigations (see Tomlinson *et al.*, 1993).

Long-term precision of the HTM analyser was monitored by the videotaped sample (Table II, Figure 2). Data from this study shows that, when 200 cells are analysed, there is a degree of unavoidable error due to the instrument, represented by a CV which is at least 5% for concentration measurements and significantly higher for percentage motility values. This compares with a CV of ~10% which has been regularly demonstrated in this laboratory by repeated concentration measurements of fresh samples (data not shown). This information is of value in interpreting between-operator error. A cusum plot (Figure 2d) showed small but consistent differences from the expected values for the percentage of rapidly moving and static cells. A decrease in precision was also noted (Table II). However, these changes were relatively small, and may have been due to some decline in the tape quality after at least 34 playings. Measurements of concentration were shown by the cusum plot to be consistent over a 9 month period. This routine quality control procedure provided assurance of the analytical reliability of the instrument.

The use of aliquots of a cryopreserved ejaculate for routine quality control purposes was shown to be compromised by the wide variation between straws, especially with respect to percentage motilities (Table III, Figure 3a,b). This exercise was designed principally to control the analysis of post-thaw samples, which present special difficulties for CASA due to their poor optical quality, clumping of cells, and a relatively low motile concentration. The quality control charts showed that the HTM analyser can be used to measure the motile concentration of post-thaw samples more precisely than the total concentration. One reason for this may be the variable clumping to which post-thaw immotile cells are prone. Immotile cells are also slightly less bright than motile cells. They are therefore more easily lost to analysis, more so because post-thaw spermatozoa are smaller than fresh spermatozoa and are also suspended in a buffer which is more opaque than

seminal plasma, as it contains 25% egg yolk. Any attempt to fine-tune the image to increase the pick-up tends to bring interference from the lipid globules in the buffer. There may also have been an operator bias toward picking up the motile cells, as it is the motile concentration which is used to accept or reject post-thaw samples. (If the motile concentration is below a threshold value, the samples are discarded.) As control samples should ideally be chosen to reflect as closely as possible the samples being assayed, it is questionable whether cryopreserved specimens may be used validly in routine semen analysis using CASA for fresh specimens. As a result of this demonstration of inter-straw variability, however, several straws from potential semen-donor samples are now thawed and pooled before analysis instead of relying on a single straw.

The introduction of routine manual checks of CASA (Figure 3c) has led to an improvement in accuracy relative to the manual method of estimating spermatozoa concentration (Figure 3d). Thus integrated quality control procedures are shown here to contribute to technician training. The laboratory is now able to report CASA concentration values with greater confidence. For example, from the 92 manual checks performed until the end of December 1993, 24 out of 92 CASA values (26%) showed differences >20% of the manual method, whereas from January to May 1994 inclusive, only five out of 81 CASA results (6%) showed this magnitude of discrepancy. There has been an increased awareness of the need to perform manual concentrations on samples unsuitable for analysis by CASA. However, the results obtained in this study suggest that CASA may be used with a greater degree of confidence than is suggested by some studies, as illustrated by the reluctance of the WHO to recommend CASA for patient diagnosis (WHO, 1992).

The use of multiple sets of five slides which were read at intervals of approximately 2 weeks was found to be a successful way of introducing routine quality control into the assessment of morphology (Table IV). Although some slides were found to fade more quickly than others, only one slide (no. 19) was discarded for this reason. The quality control data acquired here suggest that the use of CVs to characterize the precision of morphology assessments is of limited value. This is because the number of values obtainable is inevitably small due to the increasing risk of bias due to familiarity with a slide, and also because the CV is clearly related to the mean (Cooper *et al.*, 1992). The introduction of stricter criteria has involved a reduction in the mean values obtained, with a corresponding increase in the CV, and has not been shown to improve the consistency of morphology assessments in this laboratory. A disadvantage of computing the CV as a quality control technique is that it is a retrospective test. Analysis of the difference in score from the previous reading may be a more informative technique, as this reduces the effect of long-term changes, such as the adoption of different criteria, the fading of slides, and change of technicians, and also offers a more immediate check on operator consistency. The fact that these differences were not related to the number of readings shows that the influence of familiarity using this system was negligible. (In order to concentrate on this method, we have recently modified the system. A large pool of different control slides are read in sets of five on two occasions only. This is felt to reflect clinical practice, whereby more than two semen analyses are rarely performed per patient.) Despite the generally small average difference between readings, the potential for large discrepancy was shown (Figure 4a). This may depend on the nature of the samples to some degree, such as those with large numbers of borderline forms, or with high background staining due to chromophilic seminal plasma, which may be expected to be less amenable to repeatable analysis. As a consequence of this demonstration of variability, all routine slides with a count of <10% ideal forms are now re-read by a second technician and the mean value reported. Data accumulated by routine quality control allows inter-operator comparison under normal working conditions and can help in assessing consistency in applying criteria.

Calculation of the monthly mean is subject to the same kind of criticism as that directed at the use of the daily mean in clinical chemistry analyses, as being a relatively insensitive method which ignores changes in the patient population over time (Broughton, 1978; Statland and Westgard, 1991). It was, however, found to be a useful indicator of long-term changes in the assessment of morphology (Figure 4b). It allowed us to monitor the effect of adopting the new WHO (1992) criteria, and has been used to respond to queries from doctors about the significance of reduced morphology scores. Although the changes in the description of a normal spermatozoon are relatively small, we feel that the classification of all borderline forms as abnormal, as specified by the new criteria, represents a major shift in emphasis towards the strict selection of only ideal forms of normal cells. Our internal quality control was used to attempt to control and standardize the degree of strictness applied by technicians in the laboratory. A disadvantage of this method is that relatively large numbers of data are required, although smaller laboratories could use longer time-periods for batching results.

In conclusion, it is possible to integrate routine procedures into the andrology laboratory which actively control the quality of patient results. A variety of control materials are available for use on a long-term basis. Valuable information is obtained from an on-going quality control programme that can be utilized in the interpretation of patient results by users of the laboratory. Together with effective technician training, these methods allow the andrology laboratory to assure both control over its analyses and the quality of its results.

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Clinical value of sperm morphology for in-vivo fertility: comparison between World Health Organization criteria of 1987 and 1992

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The World Health Organization (WHO, 1992) has suggested new criteria for scoring sperm morphology. This study compares the clinical value of the new criteria, i.e. classification of a man as fertile or infertile, to those previously established by the WHO (1987). Papanicolaou-stained semen smears from 166 men attending our infertility clinic, whose fertility status was known, were scored using both methods. Using logistic discriminant analysis for compositional data, no difference between these two sets of criteria with respect to predicting pregnancy outcome was observed. The categorization of the abnormalities (head, midpiece, tail) provides no extra clarification. The WHO (1992) cut-off point of 30% for normal forms is not appropriate, as approximately half of the men in the fertile group had a normal sperm morphology below this limit. In conclusion, the present WHO (1992) classification of sperm morphology is of no additional clinical value. Studies on sperm morphology should concentrate on obtaining biological data on, and measurements of, spermatozoa which are functionally active. Only then can the definition of normal be achieved and clinically useful criteria be adopted.

Key words: in-vivo conception/semen analysis/sperm morphology/WHO

Introduction

Recently, the assessment of sperm morphology at the light microscope level has received considerable attention (see Kruger *et al.*, 1986; Menkveld *et al.*, 1990; Liu and Baker, 1992). Data from several independent laboratories clearly show that the assessment of sperm morphology using a modified method of strict scoring is one of the most significant predictors for sperm fertilizing ability *in vitro* (Kruger *et al.*, 1986; Liu and Baker, 1988, 1992; Menkveld *et al.*, 1990; Kobayashi *et al.*, 1991). In these studies, a biological end-point has been used to aid the definition of a normal spermatozoon, e.g. examination of morphological characteristics of spermatozoa recovered from cervical mucus and/or those binding to the zona pellucida. Inherent in these methods of strict scoring is an emphasis on quality control procedures to reduce technician error in reading the slides. Previously, inter- and intra-technician variability was a significant problem when scoring sperm morphology (see Dunphy *et al.*, 1989).

In keeping with the move to a more strict definition of sperm morphology, the World Health Organization (WHO) has redefined what they consider to be a normal spermatozoon and subsequently set an 'empirical reference value of 30% normal forms and above as normal' (WHO, 1992). However, the definition of a normal spermatozoon as described by the WHO (1992) is different from that used by other authors (see Kruger *et al.*, 1986; Liu and Baker, 1988, 1992; Menkveld *et al.*, 1990). In contrast to the more strict scoring methods of sperm morphology adopted by Kruger and Liu, there appears to be no biological or clinical basis for the derivation of a normal spermatozoon given in the WHO (1992) laboratory manual. In addition, there are no clinical data to suggest that the WHO (1992) definition of normal and cut-off point at 30% are of clinical value, or that they are an improvement upon the previous definition (WHO, 1987). It is important to emphasize that the *WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction* is used as a standard textbook throughout the world for laboratories performing basic semen assessments; therefore, the collection of biological data and the derivation of normal values are critical exercises.

The aim of this study was to determine if the adoption of the WHO (1992) criteria was of any value for predicting pregnancy *in vivo*, and to compare this to the previous standard set by the WHO (1987). Using the WHO (1992) criteria for sperm morphology, we re-analysed our Papanicolaou-stained semen smears from 166 men attending our infertility clinic whose fertility status was known. Values for semen morphology using the WHO (1987) method of scoring were already known. The details describing the original semen characteristics of these groups of men and their relation to fertility have been published previously (Barratt *et al.*, 1993; Tomlinson *et al.*, 1993).

Materials and methods

Experimental design

Sperm morphology smears from men whose partners had achieved a treatment-independent conception (n = 42) and those who had not (n = 124) were re-evaluated using the new criteria described by the WHO (1992) for the examination of

sperm morphology. These smears had previously been scored using criteria described by the WHO (1987). By determining which method of scoring sperm morphology (WHO 1987 versus 1992) provided the most prognostic information we aimed to ascertain which method was the most suitable for routine clinical laboratory practice.

Patients

Sperm morphology smears were examined from subjects referred for the investigation of subfertility between April 1990 and January 1992. Couples were investigated according to the WHO protocol for the management of the infertile couple (WHO, 1993). Couples with a clinical diagnosis of sexual dysfunction in either partner or ejaculatory dysfunction in the male partner were excluded from this study as the relative contribution of semen variables in these couples would have been impossible to estimate. Men with azoospermia were excluded from the analysis. In order to assess the predictive value of the semen variables, the influence of the female partner on fertility needs to be minimized (Barratt *et al.*, 1992a,b). Therefore, in this study we only used data from men where the female partner had the following characteristics: normal in terms of history and examination, a regular menstrual cycle, was ovulatory (mid-luteal serum progesterone >18 nmol/l) and the outcome of the hysterosalpingogram was normal. All patients had normal plasma prolactin and thyroid function tests. The median follow-up time was 13 months (5–22 months). In all, 166 couples were included in the study. If the subject indicated that a spontaneous conception had occurred and the pregnancy had not been managed at the Jessop Hospital for Women, Sheffield, UK, confirmation of the pregnancy was sought. If the subject indicated that a spontaneous abortion had occurred, then this was only accepted as a conception if evidence was available from histology, ultrasound examination visualization of a fetus or gestational sac by a reliable witness or an elevated serum β-human chorionic gonadotrophin (βHCG) (Barratt *et al.*, 1993). The median female age was 31 years (range 20–44) and the median male age was 30 years (range 21–54). Eighty-eight per cent of the women had primary infertility, 12% had secondary infertility.

Semen characteristics

Sperm morphology was determined using brightfield illumination at ×1000 magnification, after preparing air-dried Papanicolaou-stained smears (see Barratt *et al.*, 1988, 1992a,b, 1993). Care was taken to obtain an even distribution of spermatozoa for examination. All slides were read blind by an experienced highly trained technician who produced consistent and reliable results. At least 200 spermatozoa were examined. Assessment of morphology was carried out using the criteria of the WHO (1987) and then the same slides were re-examined using the WHO (1992) criteria.

Criteria of the WHO (1987)

A normal spermatozoon was defined as described by the WHO (1987), i.e. an oval head shape with regular outline and acrosomal cap covering more than one-third of the head surface. The head length was 3–5 μm and width ranged between 2 and 3 μm; the width was between one-half and two-thirds of the length. The midpiece was slender, less than one-third of the width of the head, straight and regular in outline and ~7–8 μm long. The tail was slender, uncoiled and regular in outline and at least 45 μm in length. Spermatozoa were classified into % normal, % head defects (amorphous, small, large pyriform, tapering), % midpiece defects (including cytoplasmic droplets) and % tail defects (Table I). Two counts were performed on each occasion and if the difference was >10% (e.g. 30 and 34% normal forms), then a repeat count was performed and the mean value calculated. At least 200 spermatozoa were examined in an attempt to reduce technical variation. In contrast to the WHO (1987), our morphological assessment was multiparametric, i.e. tallying each defect separately (see Jouannet *et al.*, 1988). We used this system because previous data in our laboratory had indicated that the priority system (when multiple defects were present priority was given to defects of the sperm head over the midpiece and to defects of the midpiece over those of the tail) had little predictive value (Barratt *et al.*, 1992a). The percentage of normal spermatozoa was not affected by the multiparametric method of scoring. The coefficient of variation (CV) of scoring four morphology smears 10 times is presented in Table II.

Table I. Normal sperm criteria—comparison between WHO (1987) and (1992) methods

	WHO (1987)	WHO (1992)
Head shape	Regular oval shaped	Oval; borderline forms abnormal; pinheads not counted
Normal frequency	50%	30%
Acrosome	>1/3 of head surface	well defined, 40–70% head area
Head size	3–5 μm long; 2–3 μm wide	4.0–5.5 μm long; 2.5–3.5 μm wide
Length/width ratio	1.5–2.0	1.5–1.75
Vacuoles	No details	<20% of head area
Midpiece	Slender, <1/3 width of head, straight and regular, aligned with longitudinal axis of head; 7–8 μm	No dimensions; no description of normal midpiece (defects only given, e.g. insertion of tail >90% to head long axis is abnormal)
Tail	Slender, uncoiled, regular at least 45 μm	No dimensions; no description of normal tail (defects only given)
Cytoplasmic droplets	No details	<1/3 normal head

Criteria of the WHO (1992)

Sperm morphology was examined as described by the third edition of the WHO manual (1992). Sperm morphology was split into normal, head, neck or midpiece defects and tail defects. Morphological assessment was parametric for each spermatozoon, tallying each defect separately. The numerical values of normal spermatozoa described by the WHO (1992) are different from the 1987 description (see Table I for summary). For the WHO (1992) method, the length of the head should be 4.0–5.5 μm and the width 2.5–3.5 μm, and the length-to-width ratio should be 1.5–1.75, with a well-defined acrosomal region covering 40–70% of head, no neck, midpiece or tail defects and no cytoplasmic droplets more than one-third the size of a normal sperm head. Using this classification scheme, all borderline forms were considered abnormal. Head defects included large, small, tapering, amorphous, pyriform, vacuolated or double heads or any combination of these. Neck or midpiece defects included absent tail, non-inserted or bent tail, distended irregular or bent midpiece or abnormally thin midpiece. Tail defects included short, multiple hairpin, broken, irregular width or coiled tails or tails with terminal droplets or any combination of these. An abnormal cytoplasmic droplet was taken as greater than one-third the area of the normal sperm head. A minimum of 200 spermatozoa was counted. A stage micrometer was used to aid interpretation. The coefficient of variation (CV) of scoring four morphology smears 10 times is presented in Table II.

Table II. Results of 10 repeated morphology evaluations performed by one observer on four separate semen samples of the percentage normal forms using WHO (1987) and (1992) criteria

	Semen sample			
	1	2	3	4
WHO (1987)				
Mean	22	22	50	63
SD	2.5	2.8	5.5	5
Range	19–26	20–26	39–58	59–72
CV	11.6	12.7	11.0	8.0
WHO (1992)				
Mean	14	28	25	32
SD	1.3	2.7	2.0	5.4
Range	13–17	25–33	22–29	24–44
CV	8.9	9.5	7.9	16.7

SD = standard deviation; CV = coefficient of variation.

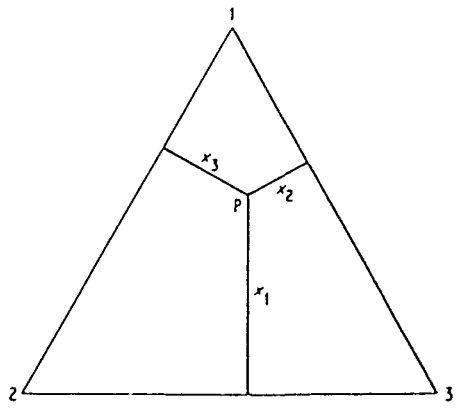

Figure 1. Representation of a three-part composition p: (x_1, x_2, x_3) in a triangle.

Measurements of sperm motion and concentration

Briefly, all semen samples were obtained by masturbation after a period of 48–72 h of sexual abstinence from men attending the University Research Clinic at the Jessop Hospital for Women, Sheffield, UK. Sperm concentration and motion characteristics were measured using a Hamilton–Thorn Analyser (2030 version 7; HTM Hamilton–Thorn Research Inc., Beverly, MA, USA). The details of the analysis for these patients has been described previously (Barratt *et al.*, 1993; Tomlinson *et al.*, 1993). Appropriate quality control procedures were implemented to obtain accurate results; for example the play-back and Alt 22 graph facilities were used to check correct cell identification. In addition, manual concentrations, using an improved Neubauer chamber, were performed periodically for validation purposes. In this analysis only samples with $>5\times10^6$ motile spermatozoa/ml semen were analysed. Men with antisperm antibodies (>20% binding) were excluded.

Statistical analysis

In order to compare the 1987 and the 1992 WHO sperm morphology classification with respect to predicting pregnancy outcome, we utilized logistic discriminant analysis for compositional data implemented by the GLIM (1986) and CODA (Aitchison, 1986a) statistical packages.

Some semen variables, e.g. sperm morphology and sperm motility, are in the form of proportions in the whole semen, and the sum of them is equal to a constant value, namely 100. Because of this unit-sum constraint, crude statistical analyses are often not valid for analysing such data (Pearson, 1897).

Aitchison (1986b) has introduced a very simple and straightforward strategy for analysing data in the form of proportions of a whole. He suggests converting the compositions by the additive log ratio transformation and reformulating the underlying problem in terms of these log ratio compositions and making use, wherever possible, of standard statistical procedures. Therefore, in this study, the composition (% ideal, % abnormal) is transformed into the variable

$$v = \log\left(\frac{\%\ \text{ideal}}{\%\ \text{abnormal}}\right)$$

and then logistic discriminant analysis is applied for this variable.

Ternary diagrams are used for visual display of the variation of three-part compositions. Each ternary diagram is a reference triangle corresponding to the vector (χ_1, χ_2, χ_3) where $\chi_1, \chi_2, \chi_3 \geq 0$ and $\chi_1 + \chi_2 + \chi_3 = 100$. There is a unique point in each triangle with perpendicular values χ_1, χ_2, χ_3 (e.g. χ_1 = % of ideal forms, χ_2 = % of head defects, χ_3 = % of other

abnormalities). Therefore, each point (such as p) in the triangle shows a three-part composition. Figure 1 is an illustration of a ternary diagram.

Results

The coefficient of variation using both methods of scoring sperm morphology was within acceptable limits when utilizing our strict quality control procedures (see Table II).

Table III shows the semen variables, excluding morphological characteristics, of the fertile (men whose partners achieved a conception) and infertile (men whose partners did not achieve a conception within the study period) groups. Many variables were noticeably higher in the fertile group compared to the infertile group. These results have been discussed previously (Barratt *et al.*, 1993).

Table III. Semen characteristics of men whose partners had normal menstrual history, normal hysterosalpingogram and were ovulatory. Values are median (range)

Characteristics	Fertile[a] (n = 42)	Infertile[b] (n = 124)
Volume (ml)	4 (1–8)	3 (0.5–7)
Sperm concentration ($\times 10^6$/ml)	51 (11–241)	24 (10–337)
Rapid progressive motility (%)	28 (0–76)	13 (0–71)
Moderate progressive motility (%)	9 (2–42)	7 (0–50)
Slow motility (%)	7 (0.25)	4 (0–42)
Static (%)	54 (10–87)	71 (10–98)
Progressive motility (%)	38 (9–86)	22 (0–86)
Concentration of rapid progressive spermatozoa ($\times 10^6$/ml)	11.7 (0–102)	3.1 (0–72)
Concentration of progressively motile spermatozoa ($\times 10^6$/ml)	17.5 (9–149)	4.4 (0–104)
Total number of spermatozoa ($\times 10^6$)	168 (30–70)	64 (0–134)
Total number of progressively motile spermatozoa ($\times 10^6$)	59.7 (3.6–382)	13 (0–417)
VAP (μm/s)	45 (23–72)	39 (0–60)
VCL (μm/s	57 (28–81)	48 (0–91)
VSL (μm/s)	37 (19–99)	34 (0–99)
ALH (μm)	3 (2–5)	3 (0–9)

[a]Fertile is defined as pregnancy achieved within the period of study.
[b]Infertile is defined as no pregnancy within the period of study.
VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity; ALH = amplitude of lateral head displacement.

Table IV. Morphological characteristics of 166 men whose partners had normal menstrual history, normal hysterosalpingogram and were ovulatory. A comparison between WHO (1987) and (1992) methods. Values are median (range)

	Morphology (%)	
	Fertile (n =42)	Infertile (n =124)
WHO (1987)		
Normal	41 (11–88)	30 (0–75)
Head	40 (2–82)	46 (1–93)
Midpiece	16 (1–39)	19 (0–60)
Tail	10 (0–82)	14 (0–69)
WHO (1992)		
Normal	30 (7–58)	20 (0–62)
Head	62 (33–93)	68 (0–99)
Midpiece	31 (14–48)	39 (0–77)
Tail	11 (1–28)	14 (0–54)

Table IV shows the morphological characteristics of the study group using the two WHO methods of classification. The percentage of normal forms was higher in the fertile group using both methods.

Figure 2 shows the variation of the morphology classification [% ideal, % head defects, % other defects (tail and midpiece)] obtained using the WHO (1987) criteria in the fertile and infertile groups. The corresponding display for measurements detailed by the WHO (1992) is Figure 3. These figures clearly show that the variation was considerably reduced using the WHO (1992) criteria. In addition, there was considerable overlap between the fertile and the infertile group for each composition. For other morphology classifications, i.e. (% ideal, % midpiece, % other defects) and (% ideal, % tail defects, % other defects), similar analyses were performed and similar results arose (data are not shown).

To examine whether morphology composition was a good discriminator for defining infertility we attempted to build a logistic regression model. The composition (% normal, % abnormal) for sperm morphology was the explanatory variable.

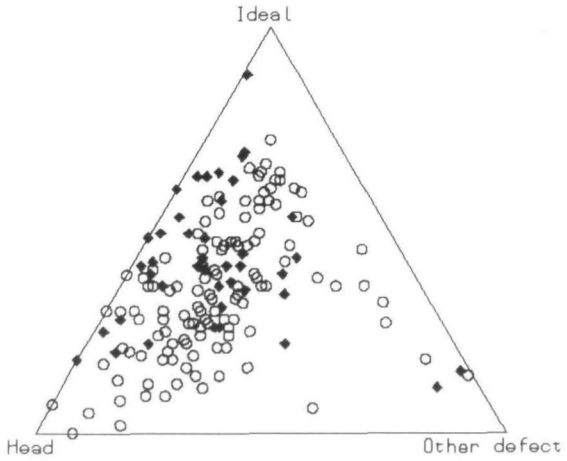

Figure 2. Representation of morphology classification (ideal, head defects, other defects) using WHO (1987) criteria. ◆ = fertile; ○ = infertile.

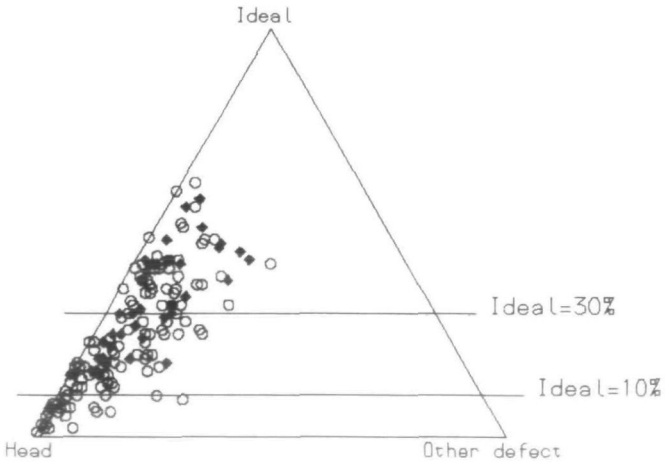

Figure 3. Representation of morphology classification (ideal, head defects, other defects) using WHO (1992) criteria. ◆ = fertile; ○ = infertile.

Two logistic models (WHO 1987 and 1992) were fitted using the iterative Newton–Raphson method. Estimated coefficients for the parameters of each model are shown in Table V. The Wald's statistic $[\hat{\beta}/\text{se}(\hat{\beta})]$ has been calculated in the last column of Table V. In relation to the hypothesis that the individual coefficient is zero, this statistic will follow the standard normal distribution (Hosmere and Lemeshow, 1989). Therefore, using an appropriate level of significance at 0.05, we conclude that the coefficients are significant for both models, demonstrating that there is a positive relationship between percentage normal forms and pregnancy. To quantify this correlation, goodness-of-fit tests were used to assess the model (Hosmere and Lemeshow, 1989). A fitted logistic model can be summarized in a classification table (Table VI). The estimated probabilities of pregnancy outcome were used to predict group membership, i.e. Table VI cross-classifies the observed fertility outcome, with a dichotomous variable. To obtain this dichotomous variable a cut-off point is defined. If the estimated logistic probability exceeds that cut-off point, then we let the dichotomous variable be equal to 1 (classified as fertile); it is equal to 0 (classified as infertile) otherwise. Here, 0.25 was chosen as a cut-off point because in our data set we had 42 fertile and 124 infertile men, and therefore the probability of being fertile can be estimated as $42/166 \approx 0.25$. Table VI shows the results of the fitted logistic model, through classification, for WHO (1987) and WHO (1992) respectively. There was a very poor classification. The overall rates of correct classification were 0.57 and 0.58 for the WHO (1987) and (1992) criteria respectively.

Logistic regression analysis shows that morphology is not a good predictor of pregnancy outcome. In addition, there was no improved predictive power to determine those patients who would achieve a conception or not using the WHO (1992) method compared with the WHO (1987) method.

For the above classification our covariate was the two-part composition (% ideal, % abnormal). To test the effectiveness of each of the three defects on classification (head, midpiece and tail), different logistic models were obtained for each of the three defect compositions. Log maximum likelihoods of the models are shown in Table VII. The log ratio test statistic has been calculated in the last column. Comparing this statistic against upper percentage points of the $\chi^2_{(1)}$ distribution, it demonstrates that using % abnormal and % ideal is justified and that further categorization of the abnormalities provides little extra clarification.

Figure 3 demonstrates that the limit of normality for the WHO (1992) of 30% normal forms is not useful in discriminating fertile and infertile men as, in the fertile group, approximately half the points are under and half are over this limit. Interestingly, only one man whose partner conceived had a normal morphology of <10% compared to 30% in the infertile group (Figure 3).

Table V. Estimated coefficient for logistic regression model using morphology composition (% ideal, % abnormal)

Parameter	Coefficient estimate	Standard error	Wald's statistic[a]
WHO (1987)			
1	−0.7409	0.21111	−3.5097
$v = \log\left(\frac{\%\text{ ideal}}{\%\text{ abnormal}}\right)$	0.6130	0.2373	2.5832
WHO (1992)			
1	−0.2973	0.2887	−0.8566
$v = \log\left(\frac{\%\text{ ideal}}{\%\text{ abnormal}}\right)$	0.6130	0.2373	2.5832

[a]Wald's statistic = $\frac{\text{coefficient estimate}}{\text{standard error}}$

Table VI. Classification based on fitted logistic model for WHO (1987) and (1992) criteria. The figures in brackets denote the percentages of correct classification

	Classified as fertile	Classified as infertile
WHO (1987)		
Observed as fertile	29 (69%)	13
Observed as infertile	58	66 (53%)
WHO (1992)		
Observed as fertile	28 (66%)	15
Observed as infertile	55	68 (55%)

Discussion

To our knowledge this is the first study to compare the two WHO methods of examining sperm morphology using data from in-vivo conceptions as a biological endpoint. This study

Table VII. Maximized log likelihoods and values of test statistics for investigating the discriminatory power of morphology compositions

Code	Composition	Log likelihood	Log ratio statistic $2\log(c, -d)$
WHO (1987)			
c_1	ideal, head, not head	−86.85527	6.0475
c_2	ideal, midpiece, not mid	−89.1756	1.4068
c_3	ideal, tail, not tail	−89.6929	0.3682
d	ideal, abnormal	−89.879	
WHO (1992)			
c_1	Ideal, head, not head	−87.45242	−2.196
c_2	ideal, midpiece, not mid	−86.05633	2.57254
c_3	ideal, tail, not tail	−87.91721	1.1492
d	ideal, abnormal	−87.3426	

clearly showed that, even though there was less variation when the criteria of the WHO (1992) method for morphology scoring were adopted, there was no change in the predictive power to determine which patients would achieve a conception. Thus, we conclude that sperm morphology determination using the new WHO method is of no greater clinical value than the previous method.

In this study we used appropriate quality control procedures when examining sperm morphology: (i) one trained observer was used to examine the morphology smears, (ii) each slide scored was examined twice and if the percentage difference was >10% a repeat examination was performed, (iii) we established our variation using both methods (Tables I and II). In addition, in order to assess the potential benefit of the new definition by the WHO (1992), we examined the same semen smears which were examined using the WHO (1987) method of scoring, thus the known variation which exists between semen samples was reduced. In our study we evaluated the new definition using the biological endpoint of in-vivo pregnancy, which has been successfully used in our laboratory and by others to determine the clinical value of semen parameters (Jouannet *et al.*, 1988; Barratt *et al.*, 1992a,b, 1993).

It is interesting that the main finding of this study suggests that the new definition of normal was of no additional clinical benefit. The primary reason for this lack of improvement is the arbitrary definition of normality adopted by the WHO (1992). It would appear prudent when defining normal (functional) spermatozoa that a biological assay is used as an endpoint. This has been described by several authors, e.g. penetration into cervical mucus (Kruger *et al.*, 1986) and binding to the human zona (see Liu and Baker, 1992). In such cases subsequent clinical data have justified the determination of normal. The new WHO (1992) method was not based on biological data so it is no surprise that there is no improvement in clinical value.

It is important to recognize that the WHO clearly recommends that each laboratory recruits fertile men to help establish their own limits. However, it would appear that only a few laboratories actually perform this analysis, as these men are very difficult to recruit (Barratt *et al.*, 1988). In view of this, the WHO should provide guidelines for morphology scoring that are based on biological data.

Data now suggest that the dimensions of normal spermatozoa can be determined by examining them on the zona, or those which have successfully penetrated cervical mucus. Quantitative methods to measure sperm morphology can now be developed using this information as a starting point. Some quantitative systems exist but, at present, are in their infancy and they may only be applicable for specialized laboratories (Schrader *et al.*, 1990; Wang *et al.*, 1991; Kruger *et al.*, 1993). These systems, or adaptations, must become available to non-specialized laboratories so that a potential improvement in clinical value can be addressed.

In this and other studies we have consistently demonstrated that morphology scoring at the light microscope level has only a weak association with in-vivo conception. In contrast, in several previous studies we have demonstrated that the measurement of sperm motion by eye and by computerized analysis is of clinical value (Barratt *et al.*, 1992a, 1993; Tomlinson *et al.*, 1993). In view of this, we would suggest that studies on sperm morphology should concentrate on obtaining biological data on, and measurements of, spermatozoa which are functionally active; only then can the definition of normal be achieved. If an inexpensive computer system can be developed which is shown to be of noticeable clinical value compared to traditional methods, this would be a major advance. However, in the meantime, measurements of sperm motion would appear the most clinically useful estimates in determining the fertilizing potential of a semen sample.

Acknowledgements

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Paper 12:

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Development of a novel home sperm test

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BACKGROUND: The majority of men find the production of a semen sample an embarrassing and stressful experience. Consequently, the availability of an over-the-counter home sperm test, which would reliably and accurately allow the patient to obtain an assessment of fertility potential at their convenience, would be a major benefit. Our objective was to develop and evaluate a home sperm test that provides a visual estimate of the concentration of progressively motile sperm in a semen sample. METHODS: Three particular challenges are described (i) developing a visualization system; (ii) optimization of the detection limit; and (iii) controlling variation due to changes in ambient temperature. The accuracy of the device was tested against two reference methods: computer-assisted sperm analysis (CASA) and a hyaluronate migration test (HMT). RESULTS: In 129 semen samples, where both reference methods agreed (positive or negative), the accuracy of the device was 95%. The observed likelihood ratio of 8.8 indicated that a sample showing a red line in the device was over eight times more likely to have a positive (normal) result in CASA and HMT than a sample without a red line. CONCLUSIONS: The final device provides a visual estimate of the concentration of progressively motile sperm in a semen sample using a test that is completed within approximately 1 h of production of the sample and can be used by the man in the comfort of his own home.

Key words: cervical mucus/home sperm device/hyaluronic acid/sperm function test/sperm penetration test

Introduction

Semen analysis is the cornerstone of infertility investigation in the male. On the basis of the result, the couple are provided with prognostic and diagnostic information to assist in their management. Many men find the production of a semen sample an embarrassing and stressful experience. In addition, there is often a significant waiting time for an initial appointment and an additional delay before the results are available. All these factors heighten the anxiety associated with infertility investigations. Consequently, the development of an over-the-counter home sperm test has been a primary objective in andrology for a number of years. An effective home sperm test would allow the patient to obtain an assessment of fertility potential at their convenience.

The concentration of progressively motile sperm is one of the most predictive parameters for estimating natural fertility in both subfertile (Ayala *et al*., 1996; Tomlinson *et al*., 1999) and normal couples (Larsen *et al*., 2000; Zinaman *et al*., 2000). For example, Larsen and colleagues examined 358 normal couples planning to conceive with no previous history of subfertility. The concentration of motile sperm (curvilinear velocity >25 μm/s) was the most predictive parameter for *in vivo* conception and, not surprisingly, differences in the concentration of motile sperm in the low range of the scale made the largest difference with regard to fertility (Larsen *et al*., 2000). Our objective was to develop a simple and reliable test for the concentration of progressively motile cells. In addition, we wanted an assay that would provide an assessment of the functional capacity of the sperm as a small proportion of men with normal semen parameters (e.g. $>10 \times 10^6$ progressively motile sperm/ml semen) have dysfunctional sperm (Schats *et al*., 1984; Mortimer *et al*., 1986; Barratt *et al*., 1989).

Sperm penetration into human cervical mucus *in vitro* is known to provide important predictive information about sperm function (Barratt *et al*., 1989; Eggert-Kruse *et al*., 1989; Abu-Heija *et al*., 1996; Eggert-Kruse *et al*., 1996). Hyaluronic acid has been used extensively as a human cervical mucus substitute and penetration of sperm into it is highly correlated with semen characteristics (Mortimer *et al*., 1990; Neuwinger *et al*., 1991; Tang *et al*., 1999) and sperm function testing (Aitken *et al*., 1992). For example, Aitken and colleagues compared sperm penetration into hyaluronic acid with quantitative motility and the zona-free hamster penetration assay (Aitken *et al*., 1992). Seventy five per cent of the variation in quantitative motility and 65% of the variation in the zona-free hamster penetration assay could be accounted for by penetration into hyaluronic acid and, importantly, patients with zero oocyte penetration could be identified. In addition, Tang and colleagues demonstrated that antisperm antibodies significantly impair sperm penetration into hyaluronic acid (Tang *et al*., 1999). In summary, penetration into hyaluronic acid is regarded as a simple and objective means of measuring the functional competence of sperm and was chosen as the basis of our assay.

Figure 1. Home test device for progressively motile sperm.

In this study we describe brief details of the scientific principles employed in the development of the assay based on the separation of progressively motile sperm and detection using a lateral flow nitrocellulose strip, and the performance of the final device on 150 semen samples.

Materials and methods

Development of the device was a multidisciplinary collaborative project managed by Genosis Ltd with contributions from The Birmingham Women's Hospital (UK), The University of Birmingham (UK), Pearson Matthews Design (UK), British BioCell International Ltd (Wales) and Plextek Ltd (UK). The final device that was evaluated—the home sperm test (trade name Fertell; Figure 1)—was provided by Genosis Ltd (UK).

Principles of the assay

The device can be regarded as performing two distinct operations (Figure 2). The first operation involves separation of progressively motile sperm from liquefied semen by a direct swim-up through hyaluronic acid. During this step, the temperature of the hyaluronic acid buffer is maintained at 37 ± 3°C by a thermostatically controlled heating collar. In the second operation and detection step, the sperm fraction of the swim-up is reacted with a monoclonal antibody conjugated with colloidal gold and the labelled sperm then collect at the interface of a nitrocellulose lateral flow strip, while the free, unreacted, conjugate is washed clear. The appearance of the clear red line (test result) due to the colloidal gold label on the antibody-bound sperm is indicative of a concentration of progressively motile sperm in the semen sample of 10×10^6/ml or greater. To ensure that the device is functioning properly, at various stages, feedback is provided to the consumer by means of a simple light emitting diode (LED). The LED is controlled by a microprocessor integral to the device.

Development of the assay

Three specific challenges were encountered in the development process: visualization of sperm, optimization of the detection limit, and robustness to variation in ambient temperature.

Visualization of sperm

A major challenge of any home test system is the visual detection of sperm without the use of any specialized equipment, thus allowing the man to obtain results in the comfort of his own home. The device uses a lateral-flow nitrocellulose strip using anti-CD59 antibody conjugated to colloidal gold to generate a visible red line when sperm are present.

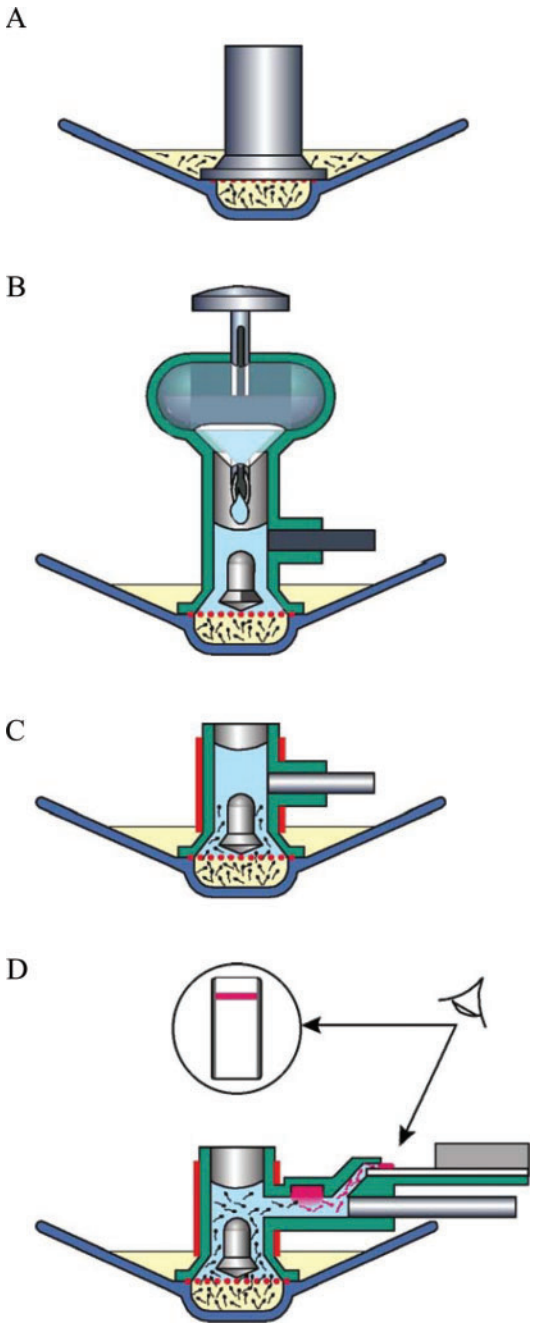

Figure 2. (A) Schematic drawing of the test device with 600 µl at the bottom sealed off by a swim-up chamber with mesh at the top of the semen volume. The figure shows the situation after the lid has been put in place following 30 min of liquefaction. (B) The swim-up process is initiated by the depression of a button which releases a hyaluronate (hyaluronic acid) solution (blue) on top of the mesh at the semen surface. (C) During the half-hour swim-up phase, the swim-up chamber is heated with a heating collar (red line) to 37°C. (D) After the swim-up phase, progressively motile sperm in the hyaluronic acid solution are released into capillary channel, labelled with anti-CD59 colloidal gold conjugate (red sperm) and trapped on the nitrocellulose strip, where a visible line is formed if sufficient numbers of progressively motile sperm have migrated into the hyaluronic acid solution.

Following the separation of progressively motile cells in hyaluronic acid, an aliquot is moved by capillary action under an absorbent polyester pad impregnated with anti-CD59 antibody labelled with colloidal gold (see below). Wetting of this pad releases the conjugate, which reacts with the sperm in the capillary channel. As the reaction proceeds, the fluid is carried onto the surface of a lateral-flow nitrocellulose membrane at the end of the capillary channel. The labelled sperm become trapped at the interface of this membrane while the free, labelled antibody is washed clear and drawn along the nitrocellulose by an absorbent wick in fluid contact with the nitrocellulose. The appearance of a clear red test line is formed on the surface of the nitrocellulose in the presence of a defined concentration of sperm.

Anti-CD59 antibody, which detects a 18–20 kDa GPI-linked glycoprotein thought to play a role in protecting cells (sperm) from attack by complement (Rooney *et al*., 1993), was used to label the sperm. Anti-CD59 binds to spermatids in the human testis (Simpson and Holmes, 1994), to both acrosome-intact and reacted sperm (D'Cruz and Hass, 1992; Fenichel *et al*., 1994; Simpson and Holmes, 1994) and, following mild detergent treatment, to internal structures. Preliminary experiments showed that anti-CD59 bound to practically all sperm (>95%) and no noticeable difference was observed between men from different ethnic groups (data not shown). CD59 is also present on white blood cells, immature and dead sperm and in seminal plasma (Rooney *et al*., 1993). Separation of progressively motile sperm, which was an integral part of the assay, therefore allowed the detection of only progressively motile cells on the nitrocellulose strips, avoiding potentially erroneous results, e.g. from non-progressively motile cells or seminal plasma (i.e. azoospermic men).

Optimization of detection limit

It was important to maximize the number of progressively motile sperm recovered (yield) in order to optimize the detection limit. To increase sperm yield, an inverted conical swim-up chamber was used with an internal space filler. This maximizes the interface between hyaluronic acid and semen (i.e. collisions of sperm in semen with hyaluronic acid) and, by reducing the volume of hyaluronic acid, increases the concentration of sperm. Preliminary experiments tested aspirating sperm from several different heights (0.65, 1 and 2 cm) above the semen–hyaluronic acid interface. A height of 0.65 cm was finally used as this provided the most effective differentiation between normal and abnormal (definition from WHO, 1999) semen samples (Ivic *et al*., 2002).

Robustness to variation in ambient temperature

Temperature control is a particularly significant challenge as progressive sperm motility is highly dependent on temperature (Milligan *et al*., 1978; Ford *et al*.,1992) and the home test environment by definition introduces significant variation in ambient temperature. To address this, a thermostatically controlled heating coil was devised that covered the outer surfaces of the swim-up chamber. The temperature at the height where the sperm are automatically aspirated is maintained at 37 ± 3°C. Conduction of heat warmed the liquefied semen sample to 30 ± 2°C when ambient temperature was ≥20°C. At any ambient room temperature between 18° and 30°C, the swim-up chamber would equilibrate to 37 ± 3°C within 5 min.

The assay was designed to provide a reflection of the concentration of progressively motile sperm in semen as this is the primary diagnostic and prognostic semen parameter (see Introduction). A reference value (cutoff point) of 10×10^6 progressively motile sperm/ml semen, which reflected two of the three primary WHO criteria of normality (20×10^6 sperm/ml and 50% progressive motility; WHO, 1999), was chosen.

Study population

The results of the present study incorporate testing of the final device in 150 men selected from research donors (n = 132), subfertile males (n = 7) and post-vasectomy donors (n = 11). The subfertile males had an abnormal semen analysis, with a concentration of $<10 \times 10^6$ progressively motile sperm per ml of semen. The clinical diagnosis of subfertility was based on unsuccessful attempts of the couple to achieve a pregnancy within 12 months of unprotected sexual intercourse, but no data on the medical status of the female was known. Semen samples were obtained from research donors and from patients attending the andrology laboratory at the Birmingham Women's Hospital, Birmingham, UK [Human Fertilisation and Embryology Authority (HFEA) centre 0119], as part of fertility investigations. Consent for the use of semen samples were taken in accordance with the HFEA Code of Practice. Local ethical approval from South Birmingham Research Ethics Committee (0472) was obtained. During the development period, prior to testing the final device reported here, we analysed over 3000 semen specimens. Semen samples were obtained from Caucasian, Asian and black African men and men of Middle Eastern origin.

In order to achieve the desired distribution of specimens with high and low concentrations of progressively motile sperm, respectively, the research donors were screened in a previous semen analysis and post-vasectomy subjects were included in order to increase the proportion of negative (concentration of progressively motile sperm below the test cutoff) subjects. It was intended that a target minimum of 25% of the subjects should have progressively motile sperm concentrations below the test cutoff. In the final analysis of the results, only data for samples with concordant reference method results (see below) were included.

Reference methods

The concentration of progressively motile sperm, obtained by computer-assisted sperm analysis (CASA) (Hamilton Thorne, Beverley, MA, USA), and a modified Kremer test [hyaluronate migration test (HMT)] was performed on each semen sample. Due to the expected variability in the reference methods, not least the HMT due to the relatively low numbers of cells assessed for samples close to the calculated cutoff, the results from the device were compared with those for samples where the reference methods were concordant [i.e. both showing low values (negative) or both showing high values (positive)].

Quantitative motility

CASA. Quantitative sperm motility was assessed using a Hamilton-Thorne IVOS, version 10.9. The CASA equipment was operated using the standard setup (37°C working temperature; cell depth 20 μm; 30 frames acquired at 60 Hz; starting analysis at least 3 mm from opening of chamber; magnification calibrated with micrometer scale on microscope slide; average path velocity low cutoff 5.0 μm/s and high cutoff 25.0 μm/s). High-concentration samples could not be quantified by the instrument when the system warned that the concentration of progressively motile cells was too high for accurate assessment. This typically occurred for samples with progressively motile concentrations exceeding $60–70 \times 10^6$/ml. Therefore, all the samples with a very high concentration of progressively motile sperm were verified by observation on the TV display and classified as positive. All analyses were performed at 37°C. Preheated (37°C) MicroCell 20 μm fixed-depth chambers were filled with 3 μl well-mixed semen. At least five fields with 200 motile sperm (defined by standard settings of the CASA equipment) were assessed. The IVOS software calculated the concentration of progressively motile sperm. Concentrations of progressive and motile sperm were recorded together with donor code and time of analysis. A concentration of progressively motile sperm of 10×10^6/ml or above was designated as a positive result.

Hyaluronate Migration Test. Microslide capillaries (0.4 × 4.0 mm internal diameter; VitroCom, Mountain Lakes, NJ, USA) were filled with the same hyaluronate solution (hyaluronic acid) as used in the final device, sealed at one end with Cristaseal (Hawksley, Scientific laboratory supplies, Nottingham) and heated to 37°C in a humid chamber with air containing 5% CO_2. Migration tests were performed in duplicate: two capsules (BEEM Capsules, Agar Scientific, Stanstead, Essex) were filled with 50 µL semen and one capillary was placed vertically into each capsule so that the hyaluronic acid was in direct contact with the semen. Capsules with capillaries were then placed in an incubator (37°C, 5% CO_2) for 1 h, after which the capillaries were removed from the capsules and placed on graded microscope slides and assessed immediately. Assessment was done at ×200 total magnification using phase-contrast microscopy and a calibrated reticulum in the ocular (Ivic *et al*., 2002). The number of sperm per mm^2 was assessed at 20 mm from the lower end of the capillary. The sperm in four microscopic fields were counted and the average number of sperm per mm^2 was calculated. To decrease the uncertainty of the results near the calculated cutoff point (61 sperm/mm^2), eight fields in total were assessed when the number of sperm in any of the initial four fields was less than 21. A concentration of 61 sperm/mm^2 or above was designated as a positive result.

Semen collection and processing

Semen samples were produced in a room adjacent to the laboratory and collected in a specimen pot of the type used in diagnostic andrology and the therapeutic assisted reproductive procedures (60 ml Biotite Container, A1 LW5465; Alpha Laboratories, Eastleigh, Hampshire UK,). In order to mimic the home environment, the samples were allowed to liquefy at ambient temperature (20°C) and then carefully mixed before two 3 µl droplets were sequentially withdrawn for CASA assessment, followed by two 50 µl aliquots for the HMT. The remaining semen was transferred to the test device. From this point the handling of the test device followed the instructions of the device.

Statistical analysis

Contingency tables were analysed with the Fisher exact test. All calculations were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Results

Of the 150 subjects enrolled, five were excluded due to insufficient volume and a further two due to technical problems. For the comparison with the combined reference method (CASA and HMT), 14 subjects were excluded because the results of the two reference tests were not concordant, i.e. not valid. The device gave a positive, red test line in 95 of the 129 valid experiments and no test line in the remaining 34. Of the 95 positive results, 91 (95.8%) also showed positive results with the reference tests and of the 34 negative device results, 32 (94.1%) were negative in the reference tests. Thus, the device gave results with high sensitivity and specificity (Table I). The observed likelihood ratio of 8.8 indicated that a sample rendering a red line in the device was over eight times more likely to have a positive (normal) result in CASA and HMT than a sample without a red line.

The accuracy of the device in relation to the reference methods was very high (accuracy 95.3%; Fisher's exact test, $P < 0.0001$).

Table I. Results of the device in comparison with concordant reference methods [computer-assisted sperm analysis (CASA) and hyaluronate migration test (HMT)] in a selected population of men ($n = 129$)

		95% confidence interval
Accuracy	95.3%	90.2–98.3
Sensitivity	97.8	92.5–99.7
Specificity	88.9	73.9–96.9
Likelihood ratio	8.8	

Samples with reference results close to the cutoff were excluded (see text) to decrease random influence due to uncertainty in reference methods.

Discussion

The objective of this work was to develop and evaluate the effectiveness of an over-the-counter home sperm test. In this study, which analysed 129 semen samples with valid reference method results, the overall accuracy was 95%. To our knowledge this is the first time that such a device, providing a visual signal representing the concentration of progressively motile cells in the semen, has been developed and evaluated.

Semen analysis is known to have many sources of variation. For the investigation of the analytical reliability of a new diagnostic device it is essential to use appropriate reference methods. To avoid errors due to uncertainty in results from the reference methods, measures must be taken to reduce their imprecision. Here we optimized the numbers of cells assessed in the reference methods (Kvist and Björndahl, 2002) and we excluded samples where the reference methods gave contradictory results. Using such measures, we estimate that we have eliminated, as far as possible, errors in the reference methods. Therefore, in the present study ($n = 129$) the reliability of the device could be rigorously tested.

A key aspect of this study was the establishment of a robust and effective visualization system. We used a novel rapid test system utilizing a nitrocellulose strip and a conjugate (anti-CD59 antibody conjugated to immunogold) dried down on a polyester pad. Numerous modifications to this system were evaluated in order to allow (i) the automatic transfer of fluid onto the nitrocellulose strip, (ii) the correct releasing system for the anti-CD59 conjugate, and (iii) effective fluid flow within the device. As CD59 is also present on other cells in semen as well as free in seminal plasma [possibly associated with proteosomes (Rooney *et al*., 1993)], it was essential to separate progressively motile sperm from other cells and seminal plasma in order to avoid erroneous results. Separation of the sperm based on their motility, i.e. penetration through hyaluronic acid, ensured the effective separation of progressively motile sperm. Hyaluronic acid was chosen as the sperm separation media as it is widely used in andrology and is regarded as an effective alternative to human cervical mucus and an objective means of measuring the functional competence of sperm (Aitken *et al*., 1992). Our initial experiments used several different media for potential sperm separation. Some of these gave very good separation of sperm, e.g. methyl cellulose 4000 c.p.i. (concentration 10 mg/ml) (Ivic *et al*., 2002) but were unsuitable for use in a rapid home sperm test. Hyaluronate was an effective method to separate

progressively motile sperm and allowed rapid visualization of the strip test results.

Infertility is a couple problem and as advancing age has a significant negative impact on future fecundity it is essential that couples attempting conception have rapid and reliable information about their potential fertility at the earliest opportunity. This device has a significant number of advantages. Firstly, as a home test it allows the man to examine his potential fertility rapidly and in the comfort and privacy of his own home. Secondly, it provides the man with a potential warning sign (concentration of progressively motile sperm in semen less than 10×10^6/ml) so that he can seek detailed investigations and potential treatment at the earliest opportunity. Thirdly, it allows the patients to take an active part in their management and potentially allows them to make a more informed choice about the use of putative self-administered drug therapy, e.g. antioxidant treatment (Lenzi *et al*., 2004). However, although our results are very encouraging and in home trials (unpublished data) patients have found the device easy to use, this device should only be used as a screening test and should not be seen as an alternative to a detailed, high-quality, comprehensive semen assessment.

In summary, the final device provides an accurate, rapid and easily visualized estimate of the concentration of progressively motile sperm in a semen sample that can be used by a man in the comfort of his own home.

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Conflict of interest

C.L.R.B. is a member of the Scientific Advisory Board of Genosis Ltd.

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REPRODUCTION
REVIEW

Counting sperm does not add up any more: time for a new equation?

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Abstract

Although sperm dysfunction is the single most common cause of infertility, we have poor methods of diagnosis and surprisingly no effective treatment (excluding assisted reproductive technology). In this review, we challenge the usefulness of a basic semen analysis and argue that a new paradigm is required immediately. We discuss the use of at-home screening to potentially improve the diagnosis of the male and to streamline the management of the sub-fertile couple. Additionally, we outline the recent progress in the field, for example, in proteomics, which will allow the development of new biomarkers of sperm function. This new knowledge will transform our understanding of the spermatozoon as a machine and is likely to lead to non-ART treatments for men with sperm dysfunction.
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Introduction

This review starts with the premise that there is a clear need to dramatically improve our understanding of the cellular and molecular basis of sperm function. This knowledge is fundamental for two key developments in male fertility: firstly, to provide the basis for effective diagnostic tools and, secondly, to facilitate the study of the physiology of abnormal/dysfunctional cells, which is central for developing rational, non-ART therapy. We initially discuss the developments in male fertility testing with particular reference to the potential role of at-home testing in the sub-fertile patient's pathway. Secondly, we discuss advances in potential markers involved in key physiological processes, such as capacitation and sperm proteomics, which are likely to transform our understanding of the normal functional cell and lead to improvements in diagnosis and developments of rational therapy.

Epidemiological data show that 1:7 couples are classed as sub-fertile (Hull *et al.* 1985, Templeton *et al.* 1990). Sperm dysfunction is the single most common cause of infertility and affects approximately 1:15 men (HFEA 2005, www.hfea.gov.uk). Studies using semen assessment as the criteria for sub-fertility (sperm concentration $<20\times10^6$/ml) show that 1:5 18-year-olds are classed as sub-fertile (Andersen *et al.* 2000). This is a high proportion of the population compared with other prevalent diseases such as diabetes (2.8% of the population; Wild *et al.* 2004). Thus, male sub-fertility is a very significant global problem and, what is most worrying is that the recent reports suggest that its prevalence is increasing (Sharpe & Irvine 2004).

Improving and evaluating the diagnosis of male fertility, in particular, sperm dysfunction

There is an urgent requirement to develop new and robust tests of sperm function to accurately diagnose male infertility. The value of traditional semen parameters (concentration, motility and morphology) in the diagnosis and prognosis of male infertility has been debated for 60 years and, perhaps not surprisingly, the debate intensifies (see Bjorndahl & Barratt 2005 for detailed discussion). There are clear issues over concordance with standardized procedures (Holt 2005, Pacey 2006) with differences undoubtedly leading to uncritical reporting of results and thus precipitous

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decline in usefulness of the tests. A stark warning presents itself: if the professionals cannot successfully turn around the current low performance in the majority of Andrology laboratories, semen analysis will become undervalued and a redundant procedure. Unquestionably, even with appropriate quality assurance, traditional semen parameters can only provide a limited degree of prognostic and diagnostic information for the infertile couple (Tomlinson *et al.* 1999), primarily at the lower ranges of the spectrum (Comhaire 2000). The insanity that surrounds perceived absolute values, e.g. 5% 'normal' forms is just that – insanity. Continual discussion and generation of putative cut-off values will lead to further irrelevant and misleading information.

It is, therefore, necessary to develop simple, robust and effective tests of sperm function. Yet, despite the plethora of potential assays available, results have been very disappointing (Muller 2000, Aitken 2006). Recent data suggest that only three potential tests of sperm function have sufficient data to support their routine use: penetration into cervical mucus (or e.g. hyaluronate; Ivic *et al.* 2002), measurement of reactive oxygen species production/lipid peroxidation (Ford 2004, Williams & Ford 2005) and estimate of sperm chromatin/DNA damage (Seli & Sakkas 2005); however, promising initial data for the latter is now being questioned (see Bungum *et al.* 2004, Gandini *et al.* 2004, Erenpreiss *et al.* 2006, Makhlouf & Niederberger 2006).

After decades of research, the primary reason for the paucity of effective and robust estimate of sperm function is our limited basic understanding of the functioning of the spermatozoon (Ford 2001, Conner *et al.* 2007). Additionally, there has been inappropriate and uncritical use of the current tools (Barratt 1995, Tomlinson *et al.* 1999, Conner *et al.* 2007). However, there are a number of new developments which promise to transform our diagnostic and treatment pathways (for examples see Aitken 2006, Jimenez-Gonzalez *et al.* 2006, Publicover *et al.* 2007). Two of these are 1) development of at-home testing and 2) biomarkers for sperm function.

A new paradigm in male fertility diagnosis: the development of at-home testing

Many men find the production of a semen sample for infertility investigations an embarrassing, difficult and stressful experience. To add to this, there can be a significant waiting time for an initial appointment at the hospital and an additional time delay before the results are available. All these factors heighten the anxiety associated with infertility investigations, often delaying investigations and subsequent treatment and consequently reducing the chances of success (HFEA 2005, www.hfea.gov.uk). The development of an over-the-counter home sperm test, which would allow the patient to obtain an assessment of fertility potential at their own convenience in the comfort of their home, has a number of benefits including potentially increasing the number of men who are tested and speeding up the diagnosis.

Several putative tests are available but the primary questions regarding home sperm testing are accuracy and reliability. In our laboratories, we have concentrated on developing a test (Fertell; Bjorndahl *et al.* 2006) based on assessing the concentration of progressively motile sperm, which is one of the most predictive parameters for estimating natural fertility in both sub-fertile (review Tomlinson *et al.* 1999) and normal couples (Larsen *et al.* 2000, Zinaman *et al.* 2000; Fig. 1). In addition, we wanted an assay that would provide an assessment of the functional capacity of the sperm as a small proportion of men with normal semen parameters (e.g. >10 million progressively motile sperm/ml semen) have dysfunctional sperm (see Barratt *et al.* 1989). Briefly, we mimicked penetration into human cervical mucus *in vitro* using hyaluronic acid, a known cervical mucus substitute for sperm function studies (Aitken *et al.* 1992). Our analysis show that the test is accurate ($\sim$95%) and patients find it easy to use (see Bjorndahl *et al.* 2006 for details).

Currently, we do not know what proportion of men with normal semen parameters but defective cells (so called hidden male factor patients – see Barratt & Publicover 2001) would be detected by our assay. However, as the number of these men is small, it is not a pivotal issue. What is a critical factor is where (and how) such home testing fits in the diagnostic pathway. Providing home testing is accurate then it should have a well-defined place in the screening of sub-fertile couples. The evaluation of diagnostic tests is now subject to renewed critical examination (Gluud & Gluud 2005). Diagnostic tests can be used as replacement, triage or add-on with their usefulness being dependent on a large number of factors (Bossuyt *et al.* 2006). Home sperm testing has not been evaluated in this critical manner and thus we are uncertain where in the pathways it fits best. Our analysis suggests it as a first-line initial investigation. Based on our preliminary data of $\sim$95% accuracy, the likelihood is that men who test positive (red line – thus >10 million progressively motile sperm/ml semen) will not require a semen assessment unless specific, rare circumstances suggest otherwise. The couples (if the female is normal) may be encouraged to try longer (Steures *et al.* 2006). Men with a red line would certainly be suitable for intrauterine insemination and possible *in vitro* fertilization (IVF), but not intracytoplasmic sperm injection (ICSI). This paradigm would have a significant cost saving ($\sim$28% men in our tertiary referral centre would be positive) as no initial screening semen analysis would be required. Men who were negative (no red line) would urgently require a high-quality comprehensive semen assessment (WHO 1999). This diagnostic pathway requires validation to determine if home testing can be used to improve the pathway of

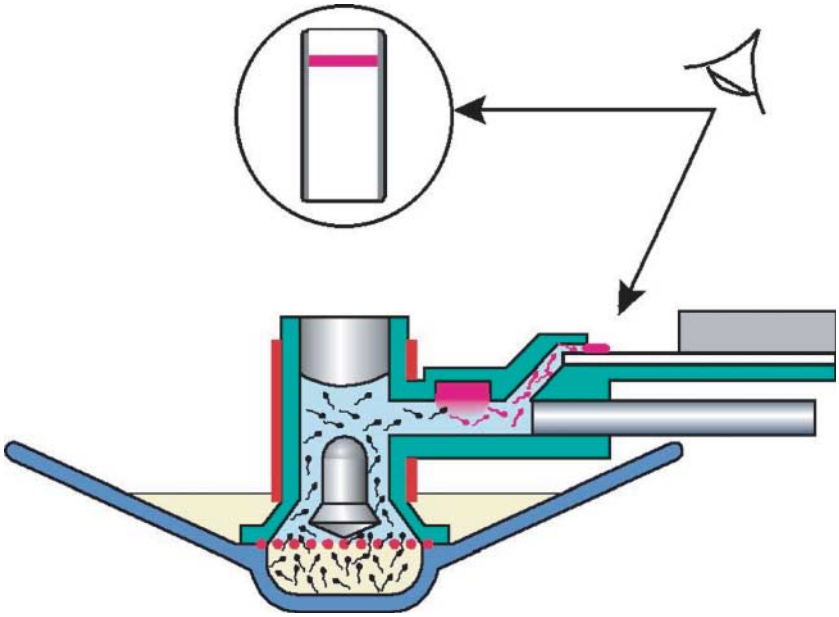

Figure 1 Diagrammatic representation of the home sperm test. Progressively motile sperm swim from semen (yellow) into hyaluronate (HA) solution (blue) via a mesh (red dotted line). Sperm are then released into capillary channel, labelled with anti-CD 59 colloidal gold conjugate (red sperm) and trapped on the nitrocellulose strip where a visible line is formed if sufficient numbers of progressively motile sperm have migrated into the HA solution. Red solid line around the green capsule is the heater to keep the cells at 37°C. A red line is visible if >10 million motile cells/ml of semen are present in the ejaculate (Adapted from Bjorndahl *et al.* 2006).

infertility investigations and provide cost-effective analysis (Fig. 2).

Home fertility testing for the man is now a reality and combined with the plethora of tests available for women: urinary luteinizing hormone (Barratt *et al.* 1989, Robinson *et al.* 1992), follicle-stimulating hormone dipsticks (www.fertell.co.uk) and complex urinary monitors; there is the potential, as yet untested, to revolutionize the diagnostic and treatment pathways for the sub-fertile couple.

Biomarkers for sperm function

Events associated with sperm capacitation

After deposition in the female tract, mammalian sperm undergo a number of functional and structural changes termed 'capacitation', which render the cells competent to fertilize. Capacitation can also be induced *in vitro* by incubation, usually for some hours, in suitable medium. The most widely accepted functional definition of *in vitro* capacitation is acquisition of ability to undergo acrosome reaction in response to a biological agonist, such as zona pellucida or progesterone. Studies on sperm incubated under capacitating conditions have revealed numerous biochemical and physiological changes that accompany the process including an efflux of plasma membrane cholesterol, an increase in the activity of adenylate cyclase (both soluble and membrane localized), elevated levels of cAMP and protein kinase A (PKA) activity, a rise in intracellular pH, hyperpolarization of membrane potential and increased serine/threonine and tyrosine phosphorylation of some proteins (Tash & Means 1983, Leclerc *et al.* 1996, Cross 1998, Osheroff *et al.* 1999, Lefièvre *et al.* 2002, Visconti *et al.* 2002, O'Flaherty *et al.* 2004, Fraser *et al.* 2005, Moseley *et al.* 2005). These observations provide useful indicators of the occurrence of capacitation in spermatozoa *in vitro,* but it is still far from clear how the various events relate to each other or whether all of them must occur for the acquisition of fertilization competence to occur. It is likely that sperm possess the ability to regulate the signalling pathways involved in capacitation, thus minimizing over-capacitation and premature acrosome reaction (Visconti *et al.* 2002, Ecroyd *et al.* 2004, De Jonge 2005, Fraser *et al.* 2005), but it is not clear to what extent the changes that have been observed in capacitating cells are reversible and whether they are capacitation endpoints or part of the capacitation process (such that reversal does not prevent subsequent induction of acrosome reaction).

We have recently demonstrated that upon transfer of cells from non-capacitating to capacitating media (CM), PKA activity, serine/threonine phosphorylation and $[Ca^{2+}]_i$-signalling 'switch' between minimum and maximum rapidly (as fast as detectable; Fig. 3; Bedu-Addo *et al.* 2005, Moseley *et al.* 2005). In contrast, the occurrence of tyrosine phosphorylation of tail proteins requires up to 3 h to reach its maximum. Progesterone-induced acrosome reaction also rises much more slowly, possibly because of the latency of tyrosine phosphorylation of head proteins (Fig. 3; Bedu-Addo *et al.* 2005). We have also shown that different CM vary strikingly in their efficacy as IVF fertilization media was shown to accelerate sperm capacitation where both tyrosine phosphorylation and acrosome reaction reached a maximum after only 90 min (Moseley *et al.* 2005). This effect did not seem to be associated with enhanced activation of PKA or increased levels of serine/threonine phosphorylation suggesting factor(s) acting through signalling pathways other than the well-characterized

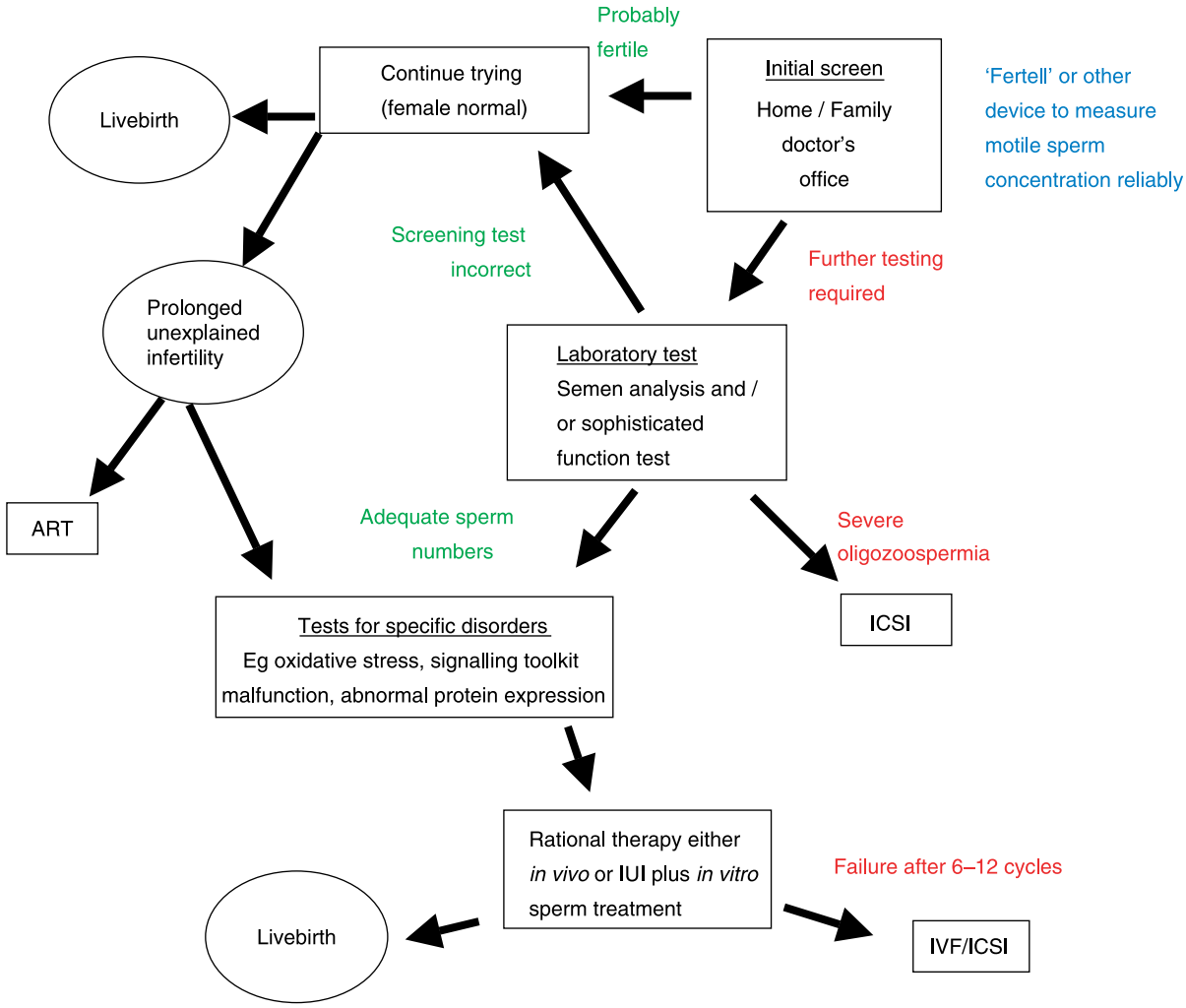

Figure 2 A new paradigm for sperm testing. The first step is a simple screening test (Fertell or similar), designed to distinguish men who are probably fertile from those where further investigation is indicated. If the man's semen passes the test and his partner is normal they can continue trying to conceive. The minority that experience prolonged infertility should be offered ART but in future improved tests that lead to other effective treatment. If the semen fails the screening test then the result will need to be confirmed in the laboratory, perhaps by a standard semen analysis supplemented by a sperm function test. If the screening test proves to be incorrect (i.e. the man has a large number of motile cells), the couple can continue trying. In some cases, men may have severe defects e.g. severe oligozoospermia, globozoospermia, in which case immediate referral for ICSI will usually be the best option. However, most men who reach this stage are likely to have low but adequate sperm numbers with impaired sperm function. Proteomics and other novel research tools will allow development of a battery of tests to diagnose the underlying cause of poor sperm function and to direct them to appropriate therapy (see section – Biomarkers of sperm function in text). It may be possible to treat the man, but it may be easier to develop ways to treat sperm *in vitro* to increase success rates in IUI. Only if appropriate therapy remains unsuccessful after a reasonable period (probably 6–12 cycles depending on age and other factors), would the couple be referred for IVF/ICSI.

activation of soluble adenylyl cyclase/cAMP/PKA (Moseley *et al.* 2005). Moreover, we have also demonstrated that, in human spermatozoa, progesterone-induced $[Ca^{2+}]_i$ signalling, protein serine/threonine phosphorylation, protein tyrosine phosphorylation and progesterone-induced acrosome reaction are all reversibly regulated by the external environment (Fig. 3; Bedu-Addo *et al.* 2005). Human spermatozoa are therefore capable of repeated and reversible cycles of many of the events that occur in response to capacitating conditions and have a high degree of plasticity and adaptability in their responses to events which signal ovulation.

Tyrosine phosphorylation of sperm proteins is a well-established and widely-used marker for cells undergoing capacitation (Visconti *et al.* 1995*a*, 1995*b*, Leclerc *et al.* 1996, Osheroff *et al.* 1999). In our recent experiments, we showed that when spermatozoa were incubated in CM for 6 h and then resuspended in CM without bicarbonate or CM without albumin for an additional hour, the cells lost their ability to undergo acrosome reaction (Bedu-Addo *et al.* 2005). However, the absence of either albumin or

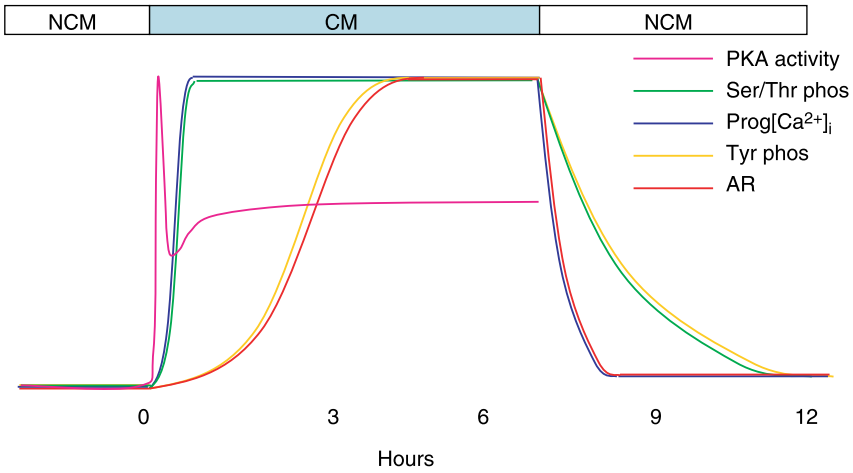

Figure 3 Proposed sequence of events during induction and reversal of capacitation *in vitro*. Under capacitating conditions, PKA activity (pink line), serine/threonine phosphorylation (green line) and $[Ca^{2+}]_i$-signalling (blue line) 'switch' between minimum and maximum in minutes. In contrast, acquisition of competence to undergo progesterone induced AR (red line) and the occurrence of tyrosine phosphorylation (yellow line) of tail proteins requires up to 3 h to reach maximum. PKA levels drop over the subsequent 5 min before reaching a plateau at 30 min. Tyrosine phosphorylation (yellow line) and progesterone-induced acrosome reaction (AR, red line) rises more slowly, taking up to 3 h to reach a maximum. Upon return to NCM, serine/threonine tyrosine phosphorylation reverses slowly (approximately 3 h), but the progesterone-induced $[Ca^{2+}]_i$ signal falls to minimal levels within minutes. Progesterone-induced AR also falls to levels similar to those seen in uncapacitated cells in less than an hour of re-suspension. The kinetics of this change are shown to follow those of the $[Ca^{2+}]_i$ response such that AR is dependent on the development of tyrosine phosphorylation during capacitation and on the decay of the $[Ca^{2+}]_i$ signal during decapacitation. (Adapted from Bedu-Addo *et al.* 2005, see also Bedu-Addo *et al.* 2007).

bicarbonate did not prevent protein tyrosine phosphorylation in these cells. Levels of tyrosine phosphorylation were comparable to the ones observed in CM medium (Bedu-Addo *et al.* 2005). These results support the use of sperm protein tyrosine phosphorylation as a marker of cells undergoing capacitation (Visconti *et al.* 1995*a*, 1995*b*, Leclerc *et al.* 1996, Osheroff *et al.* 1999), but suggest that tyrosine phosphorylation is not a diagnostic marker of capacitated cells.

There is little doubt that failure of capacitation is a cause of infertility in a subset of patients. However, the complexity of the process involved is such that it is not yet possible to identify a reliable marker of capacitated (competent to fertilize) sperm. The complexity of 'capacitation' may even be such that no single reliable marker exists.

Calcium oscillations in sperm – a new signalling system?

We have recently reviewed and discussed at length the exciting developments in calcium (Ca^{2+}) signalling in the spermatozoon (Harper & Publicover 2005, Jimenez-Gonzalez *et al.* 2006, Publicover *et al.* 2007). We believe that sperm intracellular calcium ($[Ca^{2+}]_i$) oscillations are functionally significant, modulating flagellar activity in a way that promotes functional sperm motility changes (Harper & Publicover 2005). Intriguingly, the ability of cells to generate these complex $[Ca^{2+}]_i$ signals does not seem to be noticeably related to capacitation. Perhaps, this should not be surprising. The prime marker of capacitation is acrosome reaction, and the Ca^{2+} store mobilization that occurs during oscillations raises $[Ca^{2+}]_i$ in the neck and midpiece regions of the sperm and affects the flagellum but does not induce acrosome reaction (Harper *et al.* 2004, Bedu-Addo *et al.* 2007). Similarly, hyperactivation, though often associated with capacitation, can be induced separately and occurs by a separate, Ca^{2+}-dependent, signalling pathway (Marquez & Suarez 2004). Our recent findings suggest that two factors, prior filling of the Ca^{2+} store in the sperm neck region and nitrosylation of sperm proteins, predispose the sperm to generate $[Ca^{2+}]_i$ oscillations and consequent effects on flagellar activity. Thus, the ability of the sperm to generate complex Ca^{2+} signals is apparently regulated, but by mechanisms separate to those that underlie classical 'capacitation'. Identification of features that are diagnostic of this regulation may provide a new way to identify sperm that are capable of generating complex patterns of flagellar activity and provide the first step to being able to manipulate these patterns using specific drugs.

The sperm proteome: the potential for new biomarkers of male fertility and a transformation in our understanding of the spermatozoon as a machine

The comprehensive and systematic identification and quantification of proteins expressed in cells and tissues are providing important and fascinating insights into the dynamics of cell function (Chu *et al.* 2006, Rifai *et al.* 2006). Yet, although spermatozoa are ideal to study from a proteomic perspective, until very recently, there have

been relatively few studies examining the proteome of the spermatozoa (Ainsworth 2005, Conner *et al.* 2007). By virtue of the rudimentary technology available, initial human sperm proteomic studies were relatively crude. Many studies used antisperm antibody sera in an attempt to detect potential novel (and functional) sperm targets for male contraception. Unfortunately this rational approach met with limited success (Naaby-Hansen *et al.* 1997, Shetty *et al.* 1999, Shibahara *et al.* 2002; review in Conner *et al.* 2007). Perhaps surprisingly, recent studies employing more sophisticated proteomic profiling have similarly failed to identify interesting targets (Bhande & Naz 2007). It is likely that we need to redefine the nature of immunological infertility before this strategy will yield meaningful results. The more recent experiments have followed four main themes: (1) identification of proteins associated with specific events in sperm function, (2) examination of specific structures and associated signalling complexes, (3) whole proteome investigation and (4) comparison with normal versus abnormal (fertile versus sub-fertile).

Identification of proteins associated with specific events in sperm function. It has been 12 years since the discovery of tyrosine phosphorylation as a putative marker of capacitation (Visconti *et al.* 1995*a*, 1995*b*; see above), but the role of these proteins and their sequence of activation is still very sketchy with the exception of only a small number of candidate proteins (see Naz & Rajesh 2004). Until recently, we were a long way from obtaining even a minimal 'picture' of events. However, several recent proteomic studies have provided a wealth of information which has the capacity to transform our potential understanding of protein changes associated with capacitation. For example, examining the phosphoproteome of human sperm during capacitation has provided some interesting novel targets. In addition to A-kinase-anchoring proteins (AKAPs; AKAP3 and AKAP4) and calcium-binding tyrosine-phosphorylation regulated protein, several other interesting proteins: heat shock protein 70 and 90α suggested as being involved in sperm egg interaction and valosin-containing protein (VCP/p97) a member of the ATPases associated with various cellular activities were detected (Ficarro *et al.* 2003). Interestingly, tyrosine phosphorylation of VCP/p97 regulates its subcellular localization and its localization changes from the neck to the head after capacitation (Ficarro *et al.* 2003). Several other groups of proteins such as enzymes, structural proteins and channels were also identified. Moreover, with the pivotal role of tyrosine phosphorylation in sperm capacitation it is important to identify the kinases involved. Lalancette *et al.* (2006) used an affinity chromatography approach combined with proteomic analysis on the cytosolic fraction of bovine sperm to enrich tyrosine kinase activity. Tyrosine kinases from three families were identified: Src (Lyn), Csk and Tec (bmx, Btx). In addition, other important proteins involved in different cellular events associated with sperm, such as capacitation and acrosome reaction were also identified (a total of 126 proteins). These proteins include: actin polymerization, chaperones, proteases, proteases inhibitors, metabolism, vesicle transport/membrane fusion, signal transduction, sperm–egg interaction and structural proteins. Surprisingly, a number of sperm RNA binding/transport proteins were also identified. In view of the recent suggestions of the potential role of RNA in early embryo developed, these could be an important surreptitious finding (Miller *et al.* 2005).

Stein *et al.* (2006) performed a selected proteomic analysis of mouse sperm of the regions that are likely to be involved in mediating sperm–egg interactions. Subcellular fractionation included enrichment of acrosomal contents and plasma membrane. In the acrosomal contents, 166 proteins were identified and, as expected, two of the largest groups were enzymes and their inhibitors and secretory proteins. Twenty-five percent of the proteins were novel. Two hundred twenty-nine proteins were identified from the vesicle fraction and 171 from biotinylation fraction. Interestingly, a significant number of the biotinylated proteins were ion channels, most of which had unknown function.

Examination of specific structures and associated signalling complexes. Nomura & Vacquier (2006) performed immunoprecipitation experiments for soluble adenylate cyclase (sAC) to determine the proteins associated with sAC in the sea urchin flagellum. Ten proteins were tightly associated with sAC including guanylyl cyclase and cGMP specific phosphodiesterase (PDE5A). This approach provides a critical starting point demonstrating the potential role in protein phosphorylation and ion channel activities in sperm motility.

There are a number of studies examining the proteomic composition of the 9+2 arrangement of the cilium. For example, Pazour *et al.* (2005) performed a proteomic analysis of *Chlamydomonas reinhardtii* flagella documenting the basic building blocks (for updates go to http://labs.umassmed.edu/chlamyfp/index.php). As the 9+2 structure is relatively conserved throughout evolution, you would expect a similar proteome across species. Yet, although there is remarkable conservation across the phyla, detailed proteomic studies on, for example, the flagella of trypanosomes show surprising diversity among the 300 or so proteins forming the axoneme (Broadhead *et al.* 2006). Interestingly, comparing the proteomes of *Trypanosoma brucei* with *C. reinhardtii* classified only 49 proteins to be common. 249 proteins were present but not in C. *reinhardtii* and 203 proteins present in *C. reinhardtii* but not in *T. brucei*. Further studies are required to determine the role of 'organism specific elaborations'. Sperm proteomic techniques are still refining the composition of the axonemal structures, e.g. outer dynein arms (Hozumi *et al.* 2006). Such studies, used in comparisons across species, are likely to provide a detailed understanding of the basic molecular motor apparatus. For example, comparisons between mutants of *Chlamydomonas* can

provide a powerful analysis of the defects present in the sperm of sub-fertile men and identify potential interactions of novel signalling complexes (see Zhang *et al.* 2004). Detailed imaging of the flagella motion (Ohmuro & Ishijima 2006) will allow a clear understanding of how the dynamics of the signalling complexes affect waveform. In addition to the studies on the axoneme, there is also data on the sperm accessory structures (fibrous sheath, outer dense fibres). For example, Cao *et al.* (2006) identified 50 proteins associated with these structures dramatically enhancing our knowledge of localization of both expected and unexpected complexes. This study confirmed that the fibrous sheath is a dynamic structure having a fundamental role in signalling, metabolism and oxidative stress. A proteomic study of the human fibrous sheath identified a unique ADP/ATP carrier protein and seven glycolytic enzymes previously unreported in the human fibrous sheath confirming that the fibrous sheath is a complex structure intimately involved in energy transduction (Kim *et al.* 2007). Such studies will lead to a new understanding of sperm metabolism and open exciting avenues for research potentially addressing 'old' questions such as the role of glucose in sperm energy metabolism (Ford 2006).

Whole proteome investigation. So far, there are only two studies addressing the complete human sperm proteome (Johnston *et al.* 2005, Martinez-Heredia *et al.* 2006). Using a two-dimensional gel analysis followed by mass spectrometry, Martinez-Heredia *et al.* (2006) identified 98 different proteins. A significant number was associated with proteosome turnover providing further evidence for the existence of the proteosome in human sperm function (Sutovsky *et al.* 2004). Interestingly, the second most abundant group of proteins were those involved with transcription, protein synthesis and turnover and protein transport/folding. As the spermatozoon is supposed to be transcriptionally and translationally silent, this may reflect a role for sperm RNA in the egg (see above and Lalancette *et al.* 2006, Martinez-Heredia *et al.* 2006). Alternatively, the dogma that the sperm are translationally dormant may need to be reassessed in the light of recent preliminary evidence that they can both synthesize and turnover proteins (Gur & Breitbart 2006).

Johnston *et al.* (2005) adopted a direct one-dimensional SDS–PAGE approach with liquid chromatography tandem mass spectrometry performed on the various protein bands from detergent soluble and insoluble fractions of human spermatozoa. They identified 1760 proteins with high confidence – representing 76% of the proteins predicted to be in sperm. This represents the most comprehensive sperm proteome to date. Interestingly, the greatest number of proteins characterized was either novel proteins or proteins for which no ontology was available providing a wealth of new information. Of particular interest was the significant number of tissue-specific proteins as these could potentially be used as targets for male contraception. It is likely that a number of studies reporting/refining the whole-sperm proteome will become available in the next 12 months allowing a comprehensive first draft of the mature cell to be available. In addition to studies on the sperm proteome, a number of authors have examined the protein profile of seminal plasma. In the most comprehensive study to date, Pilch & Mann (2006) identified 923 proteins with high confidence. Not surprisingly, the most abundant proteins were those associated with involvement in clot formation, metabolism and protection of the sperm cell. Potentially, this dataset could be used for biomarker discovery for testicular cancer, prostate cancer and for male infertility. Once a subset of protein markers is available, it may be feasible to perform protein chip diagnoses as used for cancer (Ciordia *et al.* 2006).

Comparison with normal versus abnormal (fertile versus sub-fertile). In our laboratory, we have been using proteomic strategies to identify defects in sperm function responsible for fertilization (Lefièvre *et al.* 2003, Pixton *et al.* 2004, reviewed Conner *et al.* 2007). Specifically, we are interested in identifying differences in sperm protein expression between control (fertile) men and patients with spermatozoa that failed to fertilize oocytes *in vitro*. Our initial studies showed, surprisingly, relatively little intra- and inter-donor variation and we have categorized one man (Pixton *et al.* 2004) where we identified 20 differences from the control that we are confident would represent true differences. Examination of five more men has found several consistent differences in protein expression levels (Conner *et al.* 2007) but further studies are required to determine if these differences relate to sperm function and where in the pathways these defects are manifested. Interestingly, with the clear need to identify (and improve) the fertility potential of animals in the agricultural industry, there is a concentration of effort to identify factors responsible for high fertility. For example, a study in bulls showed a significant effect of seminal plasma proteins on the function of the sperm, i.e. seminal plasma from high fertility bulls can act to improve the fertilizing potential of low-fertility bulls (Henault *et al.* 1995). Detailed proteomic studies of accessory gland fluids have shown consistent differences in high-/low-fertility groups, where four interesting proteins were identified: bovine seminal plasma 30 kDa (sperm capacitation), osteopontin (sperm-oolema interaction), phospholipase A2 (acrosome reaction) and spermadhesin Z13 (sperm motility) as contributing to the higher fertility (Moura *et al.* 2006, 2007*a*, 2007*b*). Examination of fertility in bulls is a very powerful approach as it allows *in vitro* studies to be complemented with data *in vivo*. Such complementary experiments cannot be performed in humans. The identification of proteins in the accessory glands of bulls contributing to fertility will allow a detailed understanding of how the sperm cell is modified by extrinsic factors.

How can all this detailed information be used to improve diagnosis and treatment of male infertility?

There has been a dramatic increase in our knowledge of the protein composition of the spermatozoon, its structures and the surrounding fluids (epididymal, vesicular, prostate) contributing to its function. Additionally, we have preliminary insight into the signalling complexes involved in key physiological processes, e.g. capacitation and the regulation of sperm motility. The difficulty is that this knowledge represents only but a starting point, somewhat analogous to knowing all the genes in the human genome but uncertain of their role and interactions. Detailed physiological experiments are required to fully understand the biological function of these proteins (and complexes) in the mature cell and its interaction with its functional environment – the female tract and human egg. The data generated by proteomic analysis is leading to a dramatic increase in our understanding (and by its nature re-evaluation) of sperm cell function. As the current methods to assess sperm function are inadequate, a key objective will be to develop more effective biomarkers as has been done in other diseases (reviewed in Rifai *et al.* 2006). For example, if we can successfully adopt redox proteomics to sperm then we may be better able to identify men who will benefit from antioxidant therapy. If we apply critical validation methods to evaluate these new biomarkers, we can expect a dramatic change in the way we diagnose male fertility.

In addition, knowledge of the cell proteome (combined with metabolomics) will allow us, for the first time, to be able to develop rational therapies for sperm dysfunction. Remarkably, although, male infertility is an important health issue, there are no drug treatments to enhance sperm function that have been shown to be effective in randomized controlled trials (Kamischke & Nieschlag 2002, cf. Greco *et al.* 2005). The only treatment option for the sub-fertile man is IVF/ICSI. ART is very expensive, invasive, has limited success, a number of side effects, is not widely available, and poses significant concerns about the long-term health of children (Maher 2005; review Hansen *et al.* 2005). This means that, put simply, as a result of our ignorance of the causes of sperm dysfunction, we are currently subjecting an increasing proportion of women to inappropriate invasive therapy in order to treat their partners. Hopefully, in the near future, perhaps using high throughput screening tools, we will be able to exploit our new knowledge base to develop and test new drug regimes for the enhancement of sperm function and avoid unnecessary ART treatment.

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The polypeptide backbone of recombinant human zona pellucida glycoprotein-3 initiates acrosomal exocytosis in human spermatozoa *in vitro*

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Human gamete interaction is of fundamental biological importance, yet the molecular interactions between spermatozoa and the zona pellucida are poorly understood. Surprisingly, the role of the polypeptide backbone of zona pellucida glycoprotein 3 (ZP3), the putative ligand for spermatozoa activation, has been largely overlooked. Purified recombinant human ZP3 was expressed in *Escherichia coli* as a C-terminal fusion to the dimeric glutathione *S*-transferase (GST) from *Schistosoma japonicum* and was shown to induce acrosomal exocytosis in live, capacitated human spermatozoa. The level of exocytosis is comparable with that obtained using purified, glycosylated, recombinant human ZP3 [van Duin, M., Polman, J. E. M., DeBreet, I. T. M., Van Ginneken, K., Bunschoten, H., Grootenhuis, A., Brindle, J. and Aitken, R. J. (1994). Biol Reprod. **51**, 607–617]. These data imply that the polypeptide chain of human ZP3 contributes to recognition of spermatozoa during acrosomal exocytosis *in vitro*.

INTRODUCTION

Fertilization is the most important cell adhesion event in the life time of any living organism that reproduces by sexual means. Furthermore, in a diverse range of organisms (from echinoderms to mammals), the recognition of carbohydrate epitopes by complementary protein receptors has been proposed to be a critical factor in gamete interaction [1–3].

At the molecular level, the survival of an organism can depend upon the success of interactions between proteins and carbohydrates. For example, lectin-containing adhesion molecules termed selectins, resident on both vascular endothelium and leucocytes, are vital for the phenomenon of leucocyte rolling and extravasation into damaged tissue during the inflammatory response [4]. Conversely, protein–carbohydrate interactions may also bring an untimely end to the organism's existence. Examples of this include the binding of some viral particles (influenza) and bacterial toxins (cholera toxin from *Vibrio cholerae* and pertussis toxin from *Bordetella pertussis*) to the plasma membrane of eukaryotic cells that is mediated by protein–carbohydrate interactions (microbial recognition of target-cell glycoconjugates has been reviewed in [5]). However, protein–protein interactions also provide fundamental triggers for a great number of inter- and intra-cellular signalling processes, perhaps the best characterized example being that of the growth hormone–growth hormone receptor complex [6].

A comprehensive understanding of the molecular mechanism of human gamete interaction is a pre-requisite for the development of novel methods of contraception aimed at blocking fertilization. In mammals such as mice and pigs, it is generally accepted that carbohydrate epitopes displayed by zona pellucida glycoprotein 3 (ZP3) play a pivotal role in spermatozoa–ZP3 interaction [2,7]. However, although both native human zona pellucida and purified, glycosylated recombinant human ZP3 (rhuZP3) secreted by Chinese Hamster Ovary (CHO) cells have been shown to stimulate acrosomal exocytosis in human spermatozoa [8–11], it has yet to be firmly established whether the ability to initiate this exocytotic event resides in the carbohydrate moieties of the ZP3 glycoprotein alone, the polypeptide backbone of ZP3 or elements of both. One method to examine the role of the protein backbone in gamete interaction has been to deglycosylate zona proteins either enzymatically or chemically [2,7]. Although both approaches successfully remove carbohydrate from the polypeptide, there are a number of limitations in such experiments. For example, enzymatic removal of carbohydrate can often be incomplete [12] and moreover chemical deglycosylation employs harsh conditions which may modify the polypeptide backbone itself [2]. Recombinant proteins expressed in *Escherichia coli* are free from carbohydrate modifications and therefore provide a homogenous molecular population for the experimental analysis of ZP3 interacting with spermatozoa.

MATERIALS AND METHODS

General reagents

The reagents used for the following procedures were of the highest standard commercially available and were obtained from Sigma (Poole, Dorset, U.K.), Fisher (Loughborough, Leics., U.K.) and BDH (Lutterworth, Leics., U.K.). Restriction enzymes were from New England Biolabs (Beverly, MA, U.S.A.) or MBI Fermentas (Vilnius, Lithuania). Glutathione–agarose affinity chromatography resins, polyclonal rabbit-anti-*S. japonicum* GST antiserum and FITC-labelled goat anti-mouse IgG antiserum were purchased from Sigma. Hybond-ECL™ nitrocellulose membranes, horseradish peroxidase conjugated to donkey-anti-rabbit immunoglobulins and ECL Western blotting detection reagents were supplied by Amersham International (Slough, Bucks., U.K.). *E. coli* culture media were supplied by Oxoid and Difco Laboratories. Mouse monoclonal antibody ab 18.6 was a kind gift from Professor Harry Moore (University of Sheffield, U.K.). Rabbit anti-porcine ZP3 antiserum was a kind gift of Professor A. G. Sacco (University of Michigan, U.S.A.); this antiserum had been raised against purified porcine ZP3 and had

Abbreviations used: IPTG, isopropyl–β-D-thiogalactopyranoside; OD_{595}, optical density at 595 nm; rhuZP3, recombinant human ZP3; GST-HuZP3, glutathione *S*-transferase-recombinant human ZP3 fusion protein; TTB, Towbin Transfer Buffer.

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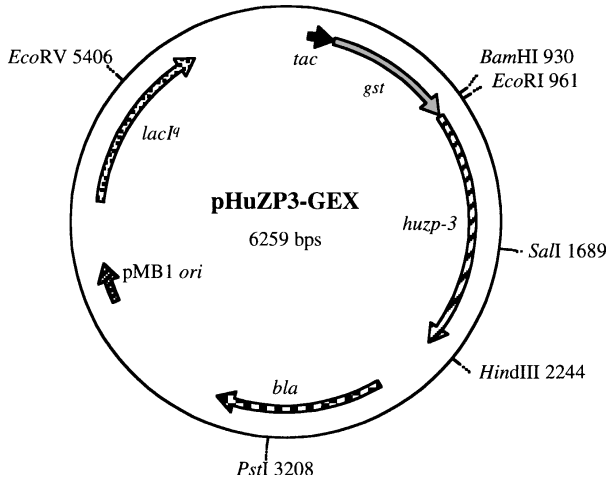

Figure 1. pHuZP3-GEX, the plasmid used for expression of recombinant human ZP3

Expression of GST-HuZP3 is under the control of the IPTG-inducible *tac* promoter. Positions in base pairs (position 1 is 118 bp upstream from the start of the *tac* sequence) of unique restriction sites are given.

been shown to cross-react strongly with both intact human zonae pellucidae [13] and purified recombinant human ZP3 [14]. The Hamilton–Thorne motility analyser (HTM model 2030; Hamilton–Thorn Research Inc, Danvers, MA, U.S.A.) was used to assess spermatozoa motility before and after the following experimental procedures (see Clements et al. [15] for HTM set up and quality control measures).

Expression and purification of GST-HuZP3 in *E. coli*

The cDNA encoding the full length human ZP3 [16] was a generous gift from Professor Jurrien Dean (NIDDK). The Polymerase Chain Reaction (PCR) was employed to engineer convenient restriction sites at the 5′ and 3′ ends of ZP3 open reading frame to facilitate insertion of the PCR product into the expression vector pGEX-KG [17] forming the expression plasmid pHuZP3-GEX. Oligonucleotide primers were synthesized on an Applied Biosystems 392 DNA synthesizer (Perkin–Elmer, Warrington, Cheshire, U.K.). Primer sequences were as follows:

HuZP3.1:
5′-CGC***GAATTC***GGATGGAGCTGAGCTATTA-3′
(*Eco*RI site bold-italicized).

HuZP3.2:
5′-GCGCC***AAGCTT***TTATTCGGAAGCAGAC-3′
(*Hin*dIII site bold-italicized).

PCR reactions were carried out for 30 cycles of: 94 °C for 45 s; 52 °C for 2 min and 72 °C for 2.5 min on a Techne PHC-2 thermal cycler. The PCR products were digested with 10 U of both *Eco*RI and *Hin*dIII, purified from an agarose gel using GeneClean (Anachem, Luton, U.K.) and were ligated into pGEX-KG (cut with the same enzymes) using 40 U of T4 DNA ligase at 16 °C overnight. pHuZP3-GEX is shown schematically in Figure 1.

pHuZP3-GEX was used to transform *E. coli* DH5αMCR (Genotype: F^- *endA1 supE44 thi-1*λ^- *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)*U169* ϕ*80dlacZM15 mcrA* Δ[*mrr hsdRMS mcrBC*]) [18,19].

Single colonies of *E. coli* DH5αMCR harbouring pHuZP3-GEX were then used to inoculate 5 ml M9 medium which was then incubated overnight at 37 °C. M9 minimal medium was prepared as follows: M9 salt solution: 49 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl and 7.57 mM $(NH_4)_2SO_4$. The pH of the salt solution was adjusted to 7.35 with 10 M NaOH and then autoclaved. Stock solutions of $MgSO_4$ (1.0 M) and $CaCl_2$ (0.5 M) were autoclaved separately and were used at working concentrations of 1 mM and 0.5 mM, respectively. Thiamine (vitamin B_1) was prepared as a stock solution of 10 mg/ml, filter sterilized through a 0.22 m sterile acrodisc filter (Gelman Sciences) and then used at a working concentration of 0.05 mg/ml. D-Glucose was used as the carbon source at a working concentration of 1.5% (w/v). Ampicillin at a final concentration of 200 μg/ml was added to all cultures. All solutions were made up using MQ water. The following day an overnight 5 ml culture was used to inoculate 50 ml of M9 medium. This culture was again grown overnight at 37 °C, then subsequently used to inoculate 1 l of M9 medium. This culture was allowed to attain an OD_{595} of 0.35 before induction of glutathione *S*-transferase-recombinant human ZP3 fusion protein (GST-HuZP3) expression was initiated by addition of isopropyl–β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The cells were then grown for a further 3.5 h at 37 °C. Control cultures of non-transformed *E. coli* DH5αMCR were grown in parallel. IPTG was also added to these cultures at the same stage of growth.

Cells were harvested by centrifugation at 17000 ***g*** for 10 min at 4 °C in a Beckman J2-HS centrifuge (JA-10 rotor), spent medium was aspirated and the cell pellet re-suspended in 6 ml phosphate buffered saline (PBS pH 7.4; 136 mM NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4 and 10.14 mM Na_2HPO_4). A cocktail of protease inhibitors was then added (final concentrations given in parentheses) to this cell suspension; these included leupeptin (100 μM), PMSF (200 μM) and EDTA (1 mM). The cell suspension was then lysed by sonication and Triton X-100 was added at a final concentration of 1% (v/v). The mixture was then gently vortexed and incubated for a further 25 min on ice. Soluble and insoluble fractions were then separated by centrifugation at 12100 ***g*** for 10 min at 4 °C in a Beckman J2-HS centrifuge (JA-20 rotor). The soluble fraction was then applied to a glutathione–agarose affinity matrix to purify GST-HuZP3 as detailed in [20].

SDS/PAGE and Western analysis

The purity of GST-HuZP3 eluted from the affinity matrix was determined by 10% SDS/PAGE [18] and the identity of the protein determined by Western analysis. The gels used a discontinuous buffer system with a 5% stacking gel and 10% resolving gel. Protein samples (usually 5 μl) from crude cell lysates (both transformed and non-transformed) were diluted 1:6 with SDS loading buffer (prepared to a final volume of 24.0 ml: 3.0 ml 500 mM Tris pH 6.8, 2.4 ml glycerol. 4.8 ml 10% (w/v) SDS, 1.2 ml β-mercaptoethanol, 600 μl 0.05% (w/v) bromophenol blue and 12.0 ml MQ water). Column samples were diluted with SDS loading buffer to obtain equivalent protein concentrations and were then boiled for 10 min, briefly centrifuged (in a benchtop micocentrifuge) to collect condensed water and then between 10 and 40 μl were electrophoresed at 100 V (40 mA) until the dye front had migrated from the resolving gel. Gels were then either stained immediately in Coomassie blue (450 ml methanol/distilled water (1:1 (v/v)), 50 ml glacial acetic acid and 1.25 g Coomassie brilliant blue R250) or electroblotted on to Hybond-ECL™ nitrocellulose as described below. Proteins were subsequently visualized after destaining the gel overnight in destain (30:10:60 methanol:glacial acetic acid:water). Gels were calibrated with high molecular mass standards obtained from Sigma.

The amount of purified recombinant protein recovered after each induction was determined using a Bio-Rad Protein Assay kit. The protocol was based on the method of Bradford [21] and was supplied by the manufacturer. A standard curve was prepared using BSA as the standard. The yield of GST-HuZP3 was $\sim 120\ \mu g/l$ of culture medium.

For Western analysis both the gels and nitro-cellulose membranes were first equilibrated in Towbin Transfer Buffer (TTB; 25 mM Tris pH 7.4, 192 mM glycine and 20% (v/v) methanol) for 15 min. Proteins were then transferred to nitro-cellulose membrane (Hybond-ECL™; Amersham International, U.K.) for 30 min at 9 V (constant current) using a Trans Blot SD™ Semi-Dry Transfer Cell (Bio-rad). Once the gel had been blotted, the membrane was washed (all subsequent washes and incubations were carried out at room temperature) for 15 min in Tris Buffered Saline (TBS; 20 mM Tris pH 7.4 and 500 mM NaCl) and then blocked in 10% blocking buffer (TTBSM; TBS containing 0.05% (v/v) Tween-20 and 10% (w/v) low fat dried milk powder (Boots pharmaceuticals)) for two 30 min sessions. Excess TTBSM was removed from the membrane with thorough washing in TTBS (TTBSM without the milk powder) for a total time of 35 min (15 min plus 4×5 min).

The membrane was then probed for the presence of GST-HuZP3 with either a rabbit antiserum raised against porcine ZP3 (1:1000 dilution in TTBS for 1 h) or rabbit anti-*Schistosoma japonicum* GST antiserum (1:1000 dilution in TTBS for 1 h) (hereafter these antisera are referred to as the 'primary ab'). Excess primary ab was then removed by washing in TTBS as described above. The membrane was probed for the presence of rabbit immunoglobulins with a 1:2000 dilution of secondary ab (a horseradish peroxidase-linked donkey antiserum raised against rabbit IgG) in TTBS for 45 min. Excess secondary ab was then removed by washing in TTBS as described above. The membrane was then quickly rinsed in TBS and then developed according to the ECL detection protocol described by the manufacturer (Amersham).

Preparation of spermatozoa

Human semen samples were obtained from healthy, fertile donors attending the University Clinic at the Jessop Hospital for Women, Sheffield, U.K. Motile spermatozoa were isolated using a direct swim-up technique into Earle's balanced salt solution (EBSS; Gibco–BRL Paisley, Scotland) supplemented with 3.5% (w/v) human serum albumin (HSA) as described in [14]. The motility and concentration of the spermatozoa in the swim-up sample and throughout the experiments described in this paper were examined on a Hamilton–Thorne motility analyser. The percentage of motile spermatozoa in the swim-up sample was 94 ± 2 (mean $\pm$ S.E.M.). The spermatozoa were then capacitated *in vitro* for 3 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After this time, spermatozoa were centrifuged at 500 ***g*** for 6 min at room temperature in a MSE Mistral 2000 centrifuge. Spent medium was discarded and the pellet then re-suspended in fresh EBSS supplemented with 0.3% (w/v) HSA and the spermatozoa concentration adjusted to 10×10^6/ml. These spermatozoa were then used to assess the biological activity of GST-HuZP3.

Induction of acrosomal exocytosis in human spermatozoa by GST-HuZP3

Separate 200 μl aliquots of spermatozoa (prepared as detailed above) were challenged with either EBSS (supplemented with 0.3% (w/v) HSA), or 10 μM A23187 (a bivalent Ca^{2+} ionophore which was prepared fresh each day as follows: A frozen stock solution of 10 mM A23187 in dimethyl sulphoxide (DMSO) was diluted to 1 mM with EBSS. The latter would then be diluted to a final concentration of 10 μM upon addition to the spermatozoa-containing medium), or GST-HuZP3 (5 ng/μl; final concentration; the amount of ZP3 resident in a human zona pellucida has been estimated at ~ 5 ng/zona [11]) or GST-M.*Msp*I (5 ng/μl; final concentration) for 3 h and 18 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Affinity-purified GST-M.*Msp*I (prepared as detailed for GST-HuZP3 but using the M.*Msp*I expression vector pGEX-M.$Msp\mathrm{I}_{cass}$ supplied by Dr. Paul Hurd [22]; data not shown) was an ideal control fusion protein for use in the following experiments to determine the level of non-specific acrosomal exocytosis induced by a non-physiological agonist (GST-M.*Msp*I is of similar molecular mass to GST-HuZP3; ~ 72 kDa). Spermatozoa were also challenged with heat-denatured (samples incubated at 100 °C for 15 min) GST-HuZP3 and GST-M.*Msp*I to investigate whether the tertiary structure of GST-HuZP3 was necessary for induction of acrosomal exocytosis in human spermatozoa.

Viability and acrosomal status of spermatozoa

Viability and acrosomal status of spermatozoa was evaluated simultaneously using the hypo-osmotic swelling (HOS) test and indirect immunofluorescence using a mouse anti-hamster primary monoclonal antiserum (mab 18.6). This was essentially as detailed in reference [23], with the sole modification being that mab 18.6 was used neat in the present study and not as a 1:4 dilution. The HOS test facilitates discrimination between live and moribund spermatozoa on the basis that live cells have swollen, coiled tails, dead spermatozoa have straight tails [23]. Mab 18.6 is an antiserum that specifically binds both to an antigen within the inner face of the outer acrosomal membrane and acrosomal content. In fixed spermatozoa one observes a decrease in fluorescence intensity over the spermatozoon acrosome due to the loss of the antigen-mab 18.6 complex as acrosomal exocytosis proceeds [24]. Using a fluorescence microscope (Laborlux S, Leitz) at $\times 1000$ magnification spermatozoa were scored as follows: (1) viable, acrosome-intact (uniform fluorescence over the acrosome region with coiled tails); (2) viable acrosome-reacted (fluorescence over the equatorial segment or no fluorescence with coiled tails); (3) non-viable, acrosome-intact (uniform fluorescence over the acrosome region with straight tails); (4) non-viable acrosome-reacted (fluorescence over the equatorial segment or no fluorescence with straight tails).

Viable spermatozoa exhibiting patchy fluorescence were scored as partially acrosome-reacted but were discounted from the data analysis in this study. At least 100 spermatozoa were scored for each agonist and all experiments were done blind with the slides being coded to avoid any bias in data interpretation. The percentages of motile spermatozoa at the end of the 3 and 18 h incubation periods were 92 ± 2 and 76 ± 9 (mean $\pm$ S.E.M.), respectively.

Statistical analysis

Results from at least seven experiments using different fertile donors are presented as mean percentage of live acrosome-reacted spermatozoa $\pm$ S.E.M. To calculate the significance of any differences observed between the means of each spermatozoa/agonist sample at each time point, paired *t* tests were performed after the data had firstly been transformed using the following equation: $\log(x+0.5)/(500-x)$ to normalize percentage data (such as the acrosome reaction data) [23]. In this equation x represents the mean percentage of live, acrosome-reacted sperma-

tozoa observed with a given agonist after a set time course. A result was considered significant when $P < 0.05$.

RESULTS

Expression and purification of GST-HuZP3

Figure 2A shows an SDS gel analysis of expression and purification of GST-HuZP3 in *E. coli* DH5αMCR grown in M9 medium. A protein corresponding to the M_r of GST-HuZP3 (72000) can clearly be seen in lane 8 following purification on glutathione-agarose. In addition, a second band of $\sim$ 26 kDa co-purifies with this species. This band was identified as *S. japonicum* GST by protein sequence analysis. The results of this sequence analysis are given in Table 1. The sequence obtained is identical to the N-terminal sequence of *S. japonicum* GST (M-S-P-I-L-G-Y-W-K).

To verify that the molecular species observed in lane 8 (Figure 2A) was GST-HuZP3, Western analysis was performed as described in materials and methods. Figure 2B illustrates the Western blot probed with polyclonal anti-GST antiserum as the primary ab. Two bands show strong cross-reactivity with this antibody in lane 8. The high molecular weight band is GST-HuZP3, while the stronger reacting, lower molecular weight species corresponds to GST. Figure 2C shows an identical Western blot probed with anti-porcine ZP3 antiserum. A cross-reacting species corresponding to GST-HuZP3 is observed in lane 8 which is not present in the control fractions, see lanes 2 and 3. No cross-reactivity was observed against GST with this antiserum. It can be clearly seen that the vast majority of GST-HuZP3 expressed in *E. coli* partitions into the insoluble phase as judged by the presence of a prominent band of $\sim$ 72 kDa in lane 5 (the induced, insoluble fraction from *E. coli* DH5αMCR harbouring pHuZP3-GEX) and not the control insoluble fraction (lane 3). The inherent insolubility of human ZP3 has proved to be a major obstacle in purifying milligram quantities of this protein. A single band of $\sim$ 60 kDa is present in lanes 2–6 of Figure 2C. This is an *E. coli* protein that cross-reacts with this anti-porcine antibody. The identity of this protein was not determined in the present study.

It is clear that there is strong cross reactivity between both the rabbit anti-porcine ZP3 and rabbit anti-*S. japonicum* GST antisera and a number of lower molecular weight species which may derive from GST-HuZP3 (Figure 2B and 2C, lanes 4 and 5). The evidence for this is that these cross reacting species are exclusive to lanes containing the insoluble fractions from strains

Table 1 N-terminal amino acid composition of the 26 kDa band as determined by automated Edman degradation

Two bands have been sequenced (labelled major and minor). The sequence of the former is in strong agreement with the database N-terminal sequence for the first nine amino acid residues of *S. japonicum* GST (M-S-P-I-L-G-Y-W-K). The latter is thought to represent an N-terminally truncated GST polypeptide since it lacks both the Met and Ser residues present at positions 1 and 2 in the major band sequence. N.A., not available; ? = identity of residue not determined at all or (when with identity letter) not determined unambiguously.

Major band			Minor band		
Residue	Amino acid	Amount (pmol)	Residue	Amino acid	Amount (pmol)
1	M	3.07	1	?	N.A.
2	S	1.82	2	P?	1.04
3	P	2.19	3	I	0.75
4	I	1.87	4	L	1.18
5	L	2.30	5	G	2.75
6	G	3.87	6	Y	1.06
7	Y	1.95	7	?	N.A.
8	W?	0.57	8	K	0.66

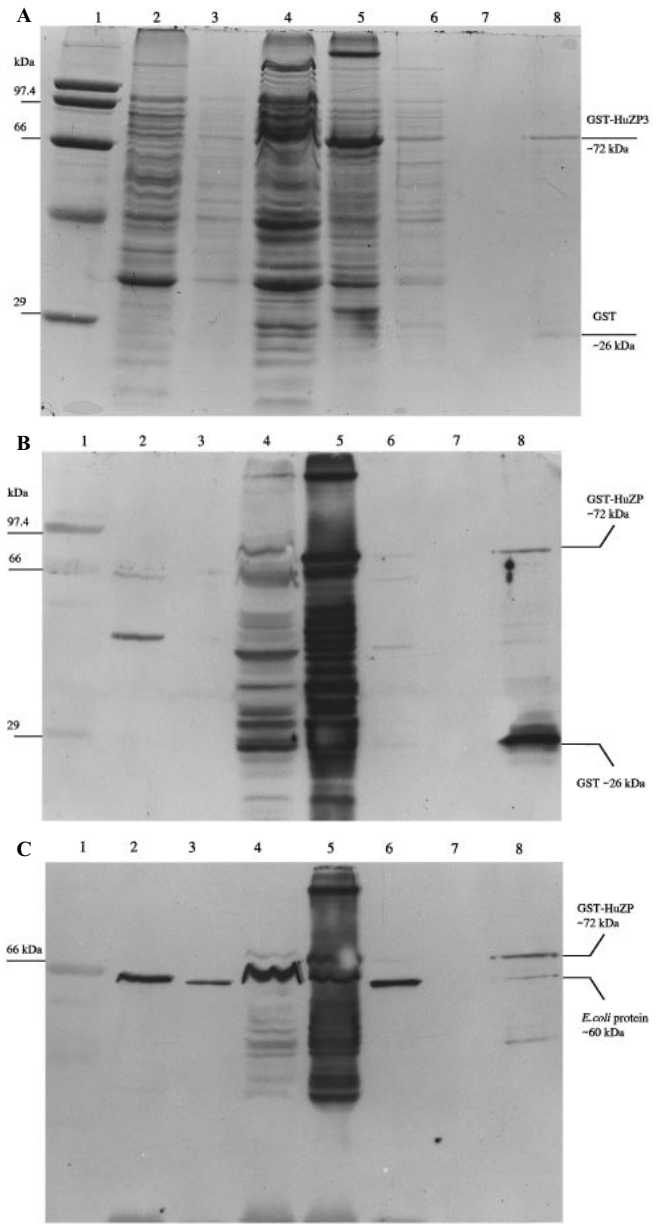

Figure 2. Analysis of expression of and purification of ZP3 by SDS/PAGE and Western blotting

A. Lane 1, Molecular mass markers; lane 2, induced, soluble extract from non-transformed *E. coli* DH5MCR; lane 3, as (2) but the insoluble fraction; lane 4, induced, soluble extract from *E. coli* DH5MCR harbouring pHuZP3-GEX; lane 5, as (4) but the insoluble fraction; lane 6, flow-through from glutathione–agarose column; lane 7, column wash in 50 mM Tris/HCl pH 8.0; lane 8, specific elution from glutathione–agarose affinity column using 50 mM Tris/HCl per 10 mM reduced glutathione pH 8.0. Identical gels to that presented in **A** were electroblotted and the blots probed for the presence of GST-HuZP3 with either a rabbit anti-*S. japonicum* GST antiserum (1:1000 dilution in TTBS; **B**) or a rabbit anti-porcine ZP3 antiserum (1:1000 dilution in TTBS; **C**) as detailed in materials and methods. The upper arrow on the right of each figure indicates the position of GST-HuZP3 ($\sim$ 72 kDa); the lower arrow on the right of A and B indicates the position of recombinant *S. japonicum* GST ($\sim$ 26 kDa). The molecular mass standards are indicated where visible.

Table 2 Ability of GST-HuZP3 to induce acrosomal exocytosis in alive, capacitated human spermatozoa

Spermatozoa were capacitated for 3 h and then incubated for a further 3 h with EBSS, 10 μM A23187, 5 ng/μl GST-HuZP3 or 5 ng/μl GST-M.*Msp*I as described in materials and methods. Values given are means $\pm$ S.E.M. n = number of semen samples from fertile donors; one ejaculate from each donor. No significant difference was observed in the level of acrosomal exocytosis in spermatozoa challenged with GST-HuZP3 compared to EBSS and GST-M.*Msp*I ($P > 0.05$).

Agonist	n	% live acrosome-reacted
EBSS	10	8.9 $\pm$ 2.29
10 μM A23187	10	58.3 $\pm$ 4.72
5 ng/μl GST-HuZP3	10	11.8 $\pm$ 2.4
5 ng/μl GST-M.*Msp*I	10	7.0 $\pm$ 1.15

Table 3 Ability of GST-HuZP3 to induce acrosomal exocytosis in live, capacitated human spermatozoa

Spermatozoa were capacitated for 3 h and then incubated for a further 18 h with EBSS, 10 μM A23187, 5 ng/μl GST-HuZP3 or 5 ng/μl GST-M.*Msp*I as described in materials and methods. Values given are means $\pm$ S.E.M. n = number of semen samples from fertile donors; one ejaculate from each donor. Significantly greater levels of acrosomal exocytosis were observed in spermatozoa challenged with GST-HuZP3 compared to EBSS (* $P < 0.001$) and GST-M.*Msp*I (** $P < 0.01$).

Agonist	n	% live acrosome-reacted
EBSS	10	16.5 $\pm$ 4*
10 μM A23187	10	65.1 $\pm$ 4.4
5 ng/μl GST-HuZP3	10	58.3 $\pm$ 4*, **
5 ng/μl GST-M.*Msp*I	10	30.6 $\pm$ 7.74**

harbouring the pHuZP3-GEX plasmid (but not strains that had not been transformed).

Induction of acrosomal exocytosis in human spermatozoa by GST-HuZP3

No significant difference in the level of acrosomal exocytosis was observed between spermatozoa challenged with EBSS, GST-HuZP3 or GST-M.*Msp*I for three hours (Table 2; 8.9 $\pm$ 2.29, 11.8 $\pm$ 2.40 and 7.0 $\pm$ 1.15 %, respectively, $P > 0.05$; $n = 10$). This suggests that the HuZP3 moiety of the GST fusion protein was unable to initiate a physiological acrosome reaction above control levels within this time course. Furthermore, acrosome reactions induced with GST-HuZP3 were significantly less than those induced using the ionophore A23187 (Table 2; 11.8 $\pm$ 2.40 vs. 58.3 $\pm$ 4.72 %; $P < 0.01$; $n = 10$). Studies using either purified recombinant glycosylated human ZP3 or purified non-glycosylated human ZP3 indicate that prolonged periods of incubation with ZP3 are necessary to induce significant levels of acrosomal exocytosis [11,14]. In order to determine if longer incubation times with the GST-HuZP3 were necessary to induce maximal stimulation (similar to levels obtained using the ionophore) spermatozoa were incubated for 18 h with GST-HuZP3 (5 ng/μl; final concentration); the amounts and nature of control agonists were as for the 3 h incubation. Spermatozoa incubated with GST-HuZP3 for 18 h were seen to have undergone significantly higher levels of acrosomal exocytosis to those observed for both the EBSS control (Table 3; 58.3 $\pm$ 4.0 vs. 16.5 $\pm$ 3.22 %; $P < 0.01$; $n = 10$) and the GST-M.*Msp*I control (Table 3; 58.3 $\pm$ 4.0 vs. 30.6 $\pm$ 7.74 %; $P < 0.01$; $n = 10$). Together these data strongly suggest that the polypeptide backbone of the HuZP3 moiety in the GST-HuZP3 fusion protein is biologically active and able to elicit acrosomal exocytosis in live, capacitated human spermatozoa.

Table 4 Ability of boiled GST-HuZP3 to induce acrosomal exocytosis in live, capacitated human spermatozoa

Spermatozoa were capacitated for 3 h and then incubated for a further 18 h with 5 ng/μl GST-HuZP3, 5 ng/μl boiled GST-HuZP3, 5 ng/μl GST-M.*Msp*I or 5 ng/μl boiled GST-M.*Msp*I as described in materials and methods. Values given are means $\pm$ S.E.M. n = number of semen samples from fertile donors; one ejaculate from each donor. A significantly reduced level of acrosomal exocytosis was observed in spermatozoa challenged with boiled GST-HuZP3 compared to non-boiled GST-HuZP3 (* $P < 0.05$). No significant difference in the number of acrosome reactions was recorded in live, capacitated human spermatozoa challenged with boiled GST-HuZP3, boiled GST-M.*Msp*I or non-boiled GST-M.*Msp*I ($P > 0.05$).

Agonist	n	% live acrosome-reacted
5 ng/μl GST-HuZP3	7	60.14 $\pm$ 5.68
5 ng/μl boiled GST-HuZP3	7	34.57 $\pm$ 7.69*
5 ng/μl GST-M.*Msp*I	7	39.7 $\pm$ 7.74
5 ng/μl boiled GST-M.*Msp*I	7	32.43 $\pm$ 8.06

The response of live, capacitated spermatozoa challenged with GST-HuZP3 for 18 h could be reduced to control levels (i.e. that observed for either heat inactivated GST-HuZP3, heat inactivated GST-M.*Msp*I or GST-M.*Msp*I) if this fusion protein was heat-denatured (Table 4; 34.57 $\pm$ 7.69 % for boiled GST-HuZP3, 32.43 $\pm$ 8.06 % for boiled GST-M.*Msp*I, 39.7 $\pm$ 7.74 % for GST-M.*Msp*I and 60.14 $\pm$ 5.68 % for GST-HuZP3, $P < 0.05$; $n = 7$).

DISCUSSION

Expression of GST-HuZP3 in M9 minimal medium

This study demonstrates that for the first time that human ZP3 can be expressed and purified from *E. coli*. A number of low molecular weight fragments which may derive from GST-HuZP3 are seen only in the lysate of *E. coli* DH5αMCR harbouring pHuZP3-GEX. The presence of multiple gene products from pHuZP3-GEX could arise for three reasons: (*a*) multiple initiations of translation on the mRNA, (*b*) partial proteolysis of the GST-HuZP3 and (*c*) contributions from both (*a*) and (*b*) [25].

The generation of multiple gene products associated with expression of heterologous genes in *E. coli* has been previously documented [25,26]. The reason(s) for this phenomenon are unclear but it is thought that the small sections of the nucleotide sequence within the mRNA of a given eukaryotic protein resemble those sequences utilized by prokaryotes when initiating synthesis of nascent polypeptides [25]. Such a sequence is the purine-rich Shine–Delgarno (SD) sequence (which is found within the ribosome binding site (RBS)) located approx. 5–13 nucleotides upstream of the initiating methionine codon [27]. This stretch of nucleotides is complementary to the 3′ end of the 16S rRNA that makes up the ribosomes within a prokaryotic cell. Examination of the HuZP3 cDNA reveals that a SD sequence (5′-AGGAGG-3′) occurs at two locations throughout the HuZP3 mRNA (positions 1108–1113; 5′-***AGGAGG***CCACUCUG***AUG***-3′ and 1968–1973: 5′-***AGGAGG***CAGCCUCAUGUC***AUG***-3′). Both stretches of nucleotides are complementary to the 3′ end of the 16S rRNA (3′-AU***UCCUCC***ACCA-5′) and are located 8 and

12 nucleotides upstream of a methionine codon respectively. Attachment to one or both of these sequences by the ribosome could result in the initiation of translation at incorrect sites. This would give rise to truncated proteins of molecular masses of ~ 41 and ~ 9.3 kDa respectively. Products around the size of the former are observed in Figure 2B and 2C. However, molecular species of other sizes are also present suggesting that partial proteolytic degradation of GST-HuZP3 cannot be completely discounted as reasons for the presence of multiple gene products from pHuZP3-GEX.

Non-specific proteolysis of the GST-HuZP3 fusion protein probably also accounts for the observed co-purification of free GST. Non-specific cleavage at the fusion joint between GST and the γ chain of the high affinity IgE receptor (FcϵRIγ) has been documented even in the presence of a broad range of protease inhibitors [28], suggesting that a non-specific bacterial protease is cleaving GST-HuZP3 at the fusion joint. Attempts to express GST-HuZP3 in protease deficient strains of *E. coli* are presently underway.

Biological activity of GST-HuZP3 *in vitro*

In a number of non-human mammals such as mice and pigs, it is generally held that glycosylation of ZP3 is required for successful gamete interaction to occur [2,7]. However, contrasting data concerning the role of carbohydrate in spermatozoa-ZP3 (or ZP3α) binding in both mice and pigs have been presented. For example, it has been documented that carbohydrates associated with mouse ZP3 or pig ZP3α mediate spermatozoa-zona binding in these mammals *in vitro* [2,7] yet fertilization in both mice and pigs can be blocked by specific monoclonal antisera directed against synthetic peptides based on the native ZP3 primary amino acid sequences. However, although such observations indicate a central role for the polypeptide backbone of ZP3 in the binding of spermatozoa to the zona in these species [29,30], one should exercise caution with such interpretations. The inhibitory activity of such antibodies could arise through non-specific steric hindrance of the target epitopes due to the large molecular size of the immunoglobulins. However, such complications, which have been reported previously during investigations into spermatozoa-zona binding in the mouse, can be circumvented by using Fab fragments as opposed to intact antibodies [31].

Similar data regarding the role of ZP3-associated carbohydrate in human gamete interaction are scarce. However, it has recently been documented that anti-peptide antibodies generated against ZP3 fail to cross-react with ZP3 from certain oocytes that failed to fertilize at IVF [32], suggesting a more significant role for the ZP3 polypeptide backbone in human fertilization.

The data presented above demonstrate that GST-HuZP3 stimulates significantly higher levels of acrosomal exocytosis in live human spermatozoa compared to a series of non-specific controls. However, this level of stimulation could only be attained after the spermatozoa had been challenged with GST-HuZP3 for a period of 18 h. A number of authors have commented that native, intact human zona pellucida can stimulate acrosomal exocytosis within 1–2 h in $> 45\,\%$ of a given population of live human spermatozoa [8,9]. Furthermore, recombinant mouse ZP3 (used at a final concentration of 5 ng/μl) can initiate acrosomal exocytosis in 51 % of mouse spermatozoa after only 20 min [33]. These data are in contrast to those observations made in the present study in that maximal acrosomal exocytosis was attained after spermatozoa had been incubated with GST-HuZP3 for a period of 18 h. However, our data are entirely consistent with those observed for spermatozoa incubated for 18 h with non-glycosylated rhuZP3 produced using an *in vitro* transcription and translation system [14]. Furthermore, the level of acrosomal exocytosis observed with our non-glycosylated GST-HuZP3 is considerably higher than studies using purified glycosylated rhuZP3 even though such studies used final concentrations of ZP3 of 15–20 ng/μl [11].

It is possible that the HuZP3 moiety of the GST-HuZP3 fusion protein has not adopted its native tertiary structure after expression in *E. coli*, thereby reducing its specific activity. The cytoplasm of *E. coli* does not favour the formation of di-sulphide bonds [34]; human ZP3 contains 15 Cys residues, two of which are located in the signal sequence [15]. Fourteen of the 15 Cys residues found in the human ZP3 polypeptide are conserved in ZP3 primary sequences from a number of mammals, suggesting they may be involved in maintaining the structure of ZP3 [35]. At the present time, it is not clear if any of these Cys residues participate in either inter- and/or intra-molecular di-sulphide bridge formation, both of which could potentially act to stabilize and maintain the tertiary structure of ZP3 in the intact zona. These interactions may be missing in the recombinant protein.

The spermatozoa used in this study may not have been capacitated sufficiently and as such were refractory to the effects of the GST-HuZP3 fusion protein. However, the method of capacitation employed in this study has been used on previous occasions and shown not to preclude the reactivity of spermatozoa toward rhuZP3 agonists [14,36].

S. japonicum GST is a homodimer [37], and the fusion proteins produced by the pGEX series of vectors are heterodimeric [38]. Thus, GST-HuZP3 is also probably dimeric. The presentation of a dimeric ZP3 molecule to the spermatozoa may partly mimic that occurring *in vivo* and may offer an explanation for the observation why this form of non-glycosylated HuZP3 can induce acrosomal exocytosis in human spermatozoa but only after 18 h. If the distance between the two juxtaposed HuZP3 moieties within the fusion protein are not in the correct spatial context, then one assumes it would be difficult for the second HuZP3 moiety to bind the spermatozoon in such a way so as to initiate possible receptor aggregation [6,39–42] and subsequently induce both rapid and maximal acrosomal exocytosis. This explanation for the time-lag seems unlikely in view of the fact that purified, non-glycosylated monomeric rhuZP3 is capable of stimulating acrosomal exocytosis in $\sim 53\,\%$ of capacitated human spermatozoa after a period of 18 h [14], implying that the state of the non-glycosylated HuZP3 polypeptide (monomeric or dimeric) is not a major determinant of whether the recombinant protein will stimulate the acrosome reaction.

The question of whether or not other components of the zona matrix are required either (*a*) directly or (*b*) indirectly to stimulate acrosomal exocytosis is not clear. Regarding (*a*), it is presently thought that in a number of mammals such as humans, mice and hamsters, only ZP3 possesses acrosomal exocytosis-inducing activity, not ZP1 or ZP2 [3,8]. However, it is possible that these other major zona components (namely ZP1 and ZP2) indirectly contribute to the bioactivity of ZP3 by maintaining the latter in a specific, three-dimensional conformation that facilitates induction of maximal exocytosis. This hypothesis has yet to be addressed experimentally. To our knowledge, minor zona-associated glycoproteins such as hyaluronic acid, have yet to be ascribed a role in human spermatozoa-zona interactions. This is in contrast to recent data, presented in reference [43], that demonstrates hyaluronic acid enhances the zona pellucida-induced acrosome reaction of cynomolgus macaque spermatozoa [43].

However, it is clear that authentic glycosylation of the HuZP3 polypeptide is required for this protein to possess full biological activity. As stated above, previously it has been shown that

glycosylated rhuZP3 purified from CHO cells could only initiate maximal acrosomal exocytosis in human spermatozoa after a period of ~20 h [11]. Glycosylation therefore appears to influence the kinetics but not the outcome of the ZP3-induced acrosome reaction.

In other glycoproteins, the presence of carbohydrate has a number of roles in protein function ranging from ensuring the correct folding of the α-subunit of the nicotinic acetylcholine receptor from *Torpedo californica* [44] to the secretion and dimerization of human interferon-γ [45]. More recently, Wang et al. [46] have suggested that a general function of protein glycosylation is firstly to stabilize the glycoprotein structure and secondly to ensure the glycoprotein adopts its native tertiary structure during its transit through the endoplasmic reticulum (ER) and Golgi body of the cell.

Non-specific induction of acrosomal exocytosis

Why a non-specific fusion protein such as GST-M.*Msp*I was able to elicit acrosomal exocytosis was not determined. It is unlikely that the size of GST-M.*Msp*I was sufficient to stimulate these cells to exocytose since one would have then expected to observe a comparable number of acrosome reactions in spermatozoa challenged with EBSS containing 0.3% (w/v) HSA (molecular mass = 66 kDa); this was not seen. One can also rule out the possibility that the GST moiety of both fusion proteins was responsible for initiating acrosomal exocytosis. This is because one would expect to observe similar levels of exocytosis in spermatozoa challenged with either GST-HuZP3 or GST-M.*Msp*I; again this was not seen. Presently it is believed that the ability of GST-M.*Msp*I to initiate acrosomal exocytosis reflects the inherent poor agonist specificity of the acrosome reaction assay. A number of other, non-physiological agonists can elicit the acrosomal exocytosis in spermatozoa, e.g. corticosteroid-binding protein [47], Epidermal Growth Factor [48] and immunoglobulins [49].

As detailed above, non-glycosylated HuZP3 has been shown to be capable of stimulating acrosomal exocytosis in live human spermatozoa after 18 h. This time course is similar to that reported for purified glycosylated rhuZP3 [11]. A detailed kinetic analysis of ZP3-induced acrosomal exocytosis is now required to clarify whether this is the case. Moreover, it would be interesting to compare the rates of acrosomal exocytosis induced by both CHO- and *E. coli*-derived ZP3 side by side. Until the nature of the carbohydrate in human ZP3 is established, bacterial-expressed ZP3 remains the best defined inducer of the acrosome reaction for the study of this process in humans.

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Biological activity of recombinant human ZP3 produced *in vitro*: potential for a sperm function test

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The human zona binding test is the most predictive test of sperm function yet the availability of human zona severely restricts its clinical use. The primary aim of this study was to use a commercially available in-vitro transcription and translation system to produce immobilized recombinant human ZP3 (rhuZP3) on agarose beads. The biological activity of this preparation was examined using sperm binding and the acrosome reaction. Significantly higher levels of sperm binding to rhuZP3 beads (n = 12, P <0.05) compared with controls were observed and there was a significant induction (n = 12, P <0.01) in the acrosome reaction after overnight incubation at 37°C in 5% CO_2 in air. In conclusion, the in-vitro transcription and translation system can produce sufficient quantities of purified immobilized biologically active rhuZP3. These preliminary experiments will enable further refinements to be made so that a solid-phase sperm function test based on rhuZP3 coated beads is likely to be developed in the near future.

Key words: acrosome reaction/spermatozoa/zona/zona binding/ZP3

Introduction

The human zona binding test, which is widely regarded as the most predictive test of sperm function (Liu and Baker, 1992, 1996), has been successfully used as a clinical sperm function test in in-vitro fertilization (IVF) (Liu and Baker, 1992; Oehninger *et al.*, 1992), to diagnose sperm defects in men with unexplained infertility (MacKenna *et al.*, 1993) and recently, to identify improvements in sperm function in men given antioxidant therapy (Kessopoulou *et al.*, 1995). In addition to predicting the success or failure of sperm function using the zona binding assay, Liu and Baker (1994, 1996) have shown that failure of spermatozoa to bind to the zona pellucida and/or failure of these spermatozoa to acrosome react, are significant causes of sperm dysfunction. However, despite its numerous advantages, the zona binding test is not widely used because of the difficulty in obtaining sufficient numbers of human zona. In theory the paucity of material can be overcome by the production of recombinant human zona proteins (providing the recombinant proteins are biologically active). Bearing in mind the advantages of the zona binding test, a welcome advance in the study of male infertility would be the development of a simple, inexpensive and widely available sperm function test using recombinant zona proteins.

Data from the mouse and pig demonstrate that the first stage of sperm–zona interaction is the binding of spermatozoa to ZP3 (see Wassarman, 1992). As a consequence, the production of recombinant ZP3 has been a major objective for several research groups. Large quantities of mouse recombinant ZP3 have been produced *in vitro* (Kinloch *et al.*, 1991; Beebe *et al.*, 1992). This recombinant product is biologically active, i.e. possesses sperm receptor activity, and induces the acrosome reaction. Biologically active human recombinant ZP3 produced by Chinese hamster ovary (CHO) cells was first reported by Barratt *et al.* (1993). However, purification of biologically active material that provides consistent results has presented significant problems in both our own and in other laboratories (Van Duin *et al.*, 1994; Barratt and Hornby, 1995; Chapman and Barratt, 1996). As a consequence, alternative expression systems have been sought. One widely used expression system, particularly for rapidly producing and purifying small quantities of protein, is in-vitro transcription and translation. This system is commonly used in mutagenesis experiments examining the interactions between proteins (Boyd *et al.*, 1994; Paroush *et al.*, 1994; Snyder *et al.*, 1994; King *et al.*, 1995). Preliminary results from our laboratory show that this system allows the rapid (within 8 h) production of purified ZP3. In addition, limited proteolytic digestion experiments suggest that the recombinant ZP3 protein has a similar folding pattern to that of mouse native ZP3 (Whitmarsh *et al.*, 1994), and cross-linking experiments show that the ZP3 protein can bind to at least four sperm glycoproteins (Kiani *et al.*, 1994).

The primary aim of the study was to use the in-vitro transcription and translation system to produce immobilized rhuZP3 (on beads) and to examine the biological activity of

Preliminary data presented at the American Andrology Society (Raleigh Durham, NC, USA, 1995) and The International Symposium of the Human Acrosome Reaction in Collioure, 1995

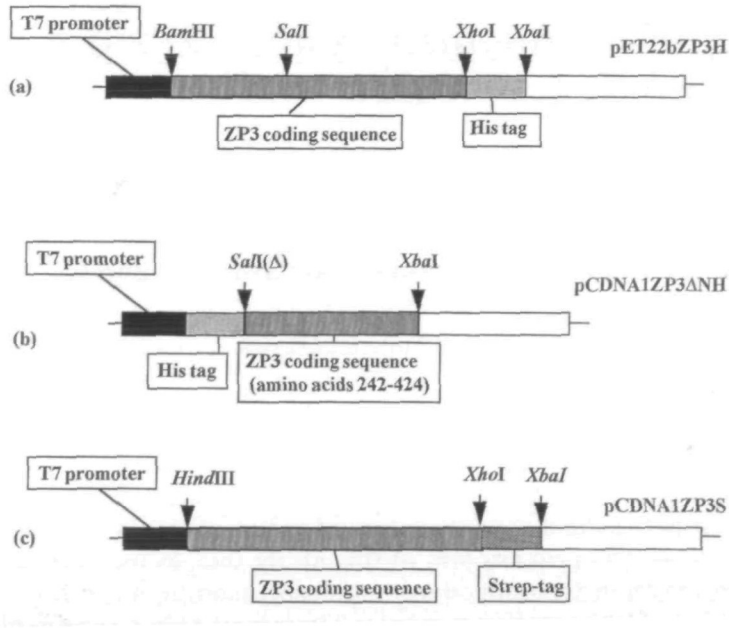

Figure 1. Plasmids used for the synthesis of human ZP3 *in vitro*. (**a**) The cDNA sequence encoding human ZP3 was amplified from a recombinant plasmid encoding the cDNA provided by Prof.J.Dean (NIH) using the following primers: 5′-CGCGGGATCCGGAGC-TGAGCTATAGGCTCTTC-3′ and 5′-CGCGGCTCGAGTTCGGAAGCAGACACAGGGTGG-3′. The product of the polymerase chain reaction (PCR) was digested with a combination of *Bam*HI and *Xho*I and the product ligated into the corresponding sites in pET22b in frame with the *C*-terminal His-tag. The recombinant plasmid is designated pET22bZP3H. (**b**) The cDNA sequence encoding human ZP3 was amplified from a recombinant plasmid encoding the cDNA provided by Prof. J.Dean (NIH) using the following primers: 5′-CGCG-GGATCCGGAGCTGAGCTATAGGCTCTTC-3′ and 5′-GCGCCAAGCTTTTATTCGGAAGCAGAC-3′. The product of the PCR reaction was digested with a combination of *Bam*HI and *Hin*dIII and the product ligated into the corresponding sites in pET14b in frame with the *N*-terminal His-tag. The recombinant plasmid pET14bZP3H was used as a template for a second PCR reaction using the primers: 5′-CGCGGGAAGCTTGCCACCATGGGCAGCAGCCATCATC-3′ and 5′-GCGCTCTAGAATTTATTCGGAAGCAGACACAGG-3′. The product of the PCR reaction was digested with a combination of *Hin*dIII and *Xba*I and the product ligated into the corresponding sites in pCDNA1. The recombinant plasmid pCDNA1ZP3 was then digested with a combination of *Bam*HI and *Sal*I, the ends filled in using the reaction catalysed by the Klenow fragment of DNA polymerase I and the product ligated to produce the plasmid pCDNA1ZP3ΔNH. (**c**) The cDNA sequence encoding human ZP3 was amplified from pET22bZP3H using the following primers: 5′-CGCCAAGCTTGCCACCAT-GGAGCTGAGCTATAGG-3′ and 5′-GCGCCTCTAGAATTTAGTGGTGGTGGTGGTGGTGC-3′. The product of the PCR reaction was digested with a combination of *Hin*dIII and *Xba*I and the product ligated into the corresponding sites in pCDNA to produce the plasmid pCDNA1ZP3H. The nucleotide sequence encoding the *C*-terminal His-tag was exchanged for that encoding a Strep-tag by first digesting pCDNA1ZP3H with *Xho*I and *Xba*I followed by the insertion of the following oligonucleotide duplex: 5′-CATGGGCCTCGAGAGCGCTT-GGCGTCACCCGCAGTTCGGTGGGGATCCGTAAT-3′ and 5′TAGATTACGGATCCCCACCGAACTGCGGGTGACGCCAAGCGCTCT-CGAGGCC-3′. The final plasmid encoding ZP3 with a *C*-terminal Strep-tag is pCDNA1ZP3S.

this preparation using sperm binding and the acrosome reaction as markers of sperm function.

Materials and methods

General reagents

Unless otherwise stated, reagents were of the highest quality available and were obtained from BDH (Lutterworth, Leicestershire, UK) or Sigma (Poole, Dorset, UK). Restriction and modification enzymes were from New England Biolabs (Beverly, MA, USA), MBI Fermentas (Vilnius, Lithuania) or NBL Gene Sciences (Cramlington, UK). Vent DNA polymerase was purchased from New England Biolabs. Oligonucleotide primers were synthesized on an Applied Biosystems 392 DNA synthesizer (Perkin Elmer, Warrington, Cheshire, UK) and the sequences (together with their specific application) are given in Figure 1. The method used for in-vitro transcription and translation of human ZP3 employed the TNT reticulocyte lysate, coupled in-vitro transcription–translation kit from Promega (TNT Rabbit Reticulocyte Lysate Coupled System; Promega, Southampton, UK). All radiochemicals were supplied by Amersham (Amersham Ltd, Slough, UK) and chromatography materials were from Sigma. All materials were used according to the manufacturers' instructions.

Production of recombinant human ZP3

The starting point for this work was a recombinant plasmid kindly provided by Professor Jurrien Dean (NIDDK, Maryland, USA). The cDNA encoding the human homologue of the murine zona pellucida protein (Chamberlin and Dean, 1990), rHuZP3, was provided in the multi-purpose cloning vector pBS. The published sequence of the cDNA clone (Chamberlin and Dean, 1990) was used to design oligonucleotide primers for the polymerase chain reaction (PCR) which was employed to engineer convenient restriction sites at the 5′ and 3′ ends of the huZP3 open reading frame (ORF) to facilitate the amplification of the coding sequence for insertion into appropriate cloning vector(s) as described below (see Figure 1). All PCRs were carried out for 30 cycles of: 94°C for 45 s; 52°C for 2 min; 72°C for 2.5 min on a Techne PHC-2 thermal cycler (Techne, Cambridge, UK). The PCR products were digested with suitable restriction enzymes, purified from an agarose gel using GeneClean (Anachem, Luton, UK) and ligated into the appropriate vector. Three cloning vectors were used in the experiments described here, all contain a T7 promoter sequence upstream of the ATG start codon of the hZP3 cDNA sequence, one was from InVitrogen, pCDNA1 (Leek, The Netherlands) and the others, pET22b and pET14b from Novagen (NBL Gene Science, Cramlington, UK). The sequence of the cDNA

inserted into these vectors following PCR amplification was determined by automated analysis in the Krebs Institute macromolecular sequencing and synthesis laboratory. In addition to amplifying the coding sequence of human ZP3, the addition of a 24 bp sequence at the 3 prime end of the ZP3 cDNA was appended, in order to generate a *C*-terminal octapeptide sequence in the translated protein which would act as a high affinity epitope for recognition by streptavidin (see Schmidt and Skerra, 1993). The polypeptide expressed from pET22bZP3 possesses a *C*-terminal hexahistidine tail, which was used in conjunction with a nickel chelating resin to immobilize and/or purify translated protein (see Hurd and Hornby, 1996). It was our intention to evaluate each affinity 'tag' for purification purposes and for assessment of biological activity.

In-vitro transcription and translation of human ZP3

The recombinant plasmid pET22bZP3H (formed by subcloning the ZP3 coding sequence from pBS into pET22b) and pCDNA1ZP3S (formed by subcloning the ZP3 coding sequence from pBS into pCDNA1) were purified by CsCl centrifugation and were used to programme a coupled in-vitro transcription/translation (TNT Rabbit Reticulocyte Lysate Coupled System; Promega) according to the manufacturer's instructions. The details of the plasmids and products are given in Figure 1. Briefly, the reaction components (as supplied by the manufacturer) were removed from storage at −70°C, and thawed on ice. They were assembled in an Eppendorf tube as follows; 25 µl TNT Rabbit Reticulocyte Lysate, 2 µl reaction buffer, 1 IU T7 RNA polymerase (stored at −20°C), 1 µl amino acid mixture (minus methionine), 1 µl RNasin ribonuclease inhibitor, 1 µg plasmid, 4 µl L-[methyl-^{3}H]-methionine or [^{35}S]-L-methionine (86 Ci/mole), and 15 µl nuclease-free water. This was incubated at 30°C for 2 h, allowing transcription and translation to occur. Products from the reaction were detected either by autoradiography following incorporation of [^{35}S]-methionine (Amersham International plc, Little Chalfont, UK) into newly synthesized protein or following sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) by Western blotting with anti-porcine ZP3 antiserum kindly supplied by Professor A.Sacco (Wayne State University, Detroit, MA, USA), which cross-reacts with human zona (Sacco *et al.*, 1981).

Affinity chromatography and immobilization of ZP3

Following translation of ZP3 *in vitro* from pET22bZP3H the reaction mixture was subjected to affinity chromatography on amino diacetic acid insolubilized on epoxy activated Sepharose 6B (Sigma) as described elsewhere (Whitmarsh and Hornby, 1994). When the template encoding the strep tag (pCDNA1ZP3) was used the product was purified with streptavidin–agarose as the affinity matrix. Immobilized proteins were used as described below, or were selectively eluted with either two column volumes (2×400 µl) of 1 M imidazole (in the case of the polypeptide with the hexahistidine tail) or with biotin at a concentration of 1 mM (in the case of the polypeptide with the strep tag).

Quantification of translated and immobilized rhuZP3

In order to quantify the amount of rhuZP3 produced in the in-vitro transcription and translation system and to determine the level of immobilization on the solid supports, scintillation counting was employed (Philips Liquid Scintillation Counter, Series PW4700, Beckmann International, High Wycombe, UK). Following in-vitro translation, the labelled protein was separated from the lysate proteins via SDS–PAGE. The band corresponding to the translated ZP3 was excised from the gel and the level of methionine incorporated (assuming all methionine residues were uniformly labelled) determined. From the level of incorporation of the [^{35}S]-amino acid, the yield of polypeptide could be calculated. In addition, the efficiency of ZP3 immobilization was determined by counting the incorporation of [^{35}S]-labelled protein into the streptavidin or nickel chelate beads.

Preparation of immobilized ZP3

Streptavidin coated agarose beads (diameter 60–140 µm) were obtained from Sigma. Aliquots of 5 µl were removed and washed in phosphate-buffered saline (PBS; Gibco BRL Life Technologies, Paisley, UK). This was done by adding 1 ml of PBS to the beads in an Eppendorf tube and mixing thoroughly. After centrifugation at 600 *g* for 5 s, the supernatant (~1 ml) was carefully removed and this washing procedure repeated twice more. The beads were then ready for coating with protein. Various aliquots of the washed beads were incubated with 100 µl of the TNT rabbit reticulocyte lysate containing strep-tagged rhuZP3, or with 100 µl of biotin at a final concentration of 100 µg/ml. (The latter concentration of biotin was chosen to act as a control at a level that had no influence upon spermatozoa motility.) After 1 h incubation, the beads were washed exhaustively a further eight times to ensure removal of any unbound protein. The beads were finally resuspended in 50 µl of PBS and were stable for up to 2 weeks if stored at 4°C.

Western blotting

In order to confirm the identity of protein expressed *in vitro*, antibodies raised against porcine ZP3 were used in Western blots. The porcine ZP3 antiserum was kindly provided by Professor Sacco and is known to cross-react well with human zona preparations. For Western blot analysis, proteins were separated by SDS–PAGE. Gels were equilibrated in transfer buffer (0.25 M Tris–Cl pH 8.3, 0.15 M glycine, 20% methanol, 0.1% SDS) and proteins were transferred to nitro-cellulose membrane (NC-Extra; Amersham) for 2 h at 400 mA with cooling. Membranes were washed in transfer buffer and blocked in blotto (2.5% dried milk powder, 0.3% Tween-20 in 1×PBS pH 7.4) overnight at 4°C with shaking. Primary antiserum was diluted 1:500 in blotto and incubated with the filters for 4–16 h at 4°C. After several washes with blotto, the second antibody [alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Ig)G; Sigma] or horse radish peroxidase-conjugated (Amersham) diluted 1:1000 in blotto, was incubated with the filters for 1 h at room temperature with shaking. After two 5 min washes in blotto and a 5 min wash in PBS/0.3% Tween-20, the filters were equilibrated in alkaline phosphatase buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 2 min. Colour or ECL film was developed by incubation with Nitro Blue Tetrazolium (NBT) and 0.3 mg/ml bichloro-phenyl indolylphosphate (BCIP) as described by the manufacturers (Boehringer Mannheim, Mannheim, Germany) at room temperature, in the dark or by exposure to Amersham's ECL film.

Preparation of the spermatozoa

Semen was obtained from fertile semen donors. The semen characteristics of the donors were: sperm concentration ≥80×106/ml, ≥60% ideal forms (World Health Organization, 1992), ≥65% progressive sperm motility (>10 µm/s). Semen samples were obtained by masturbation after a period of 48–72 h sexual abstinence. Specimens were allowed to liquefy (~30 min) at 37°C and semen analysis was performed, including analysis of sperm motility, using the Hamilton Thorn Research Motility Analyser (HTM model 2030; Hamilton Thorn Research, Danvers, MA, USA) (see Clements *et al.* (1995) for HTM set up and quality control measures). Spermatozoa were separated using a direct swim-up technique: 0.5 ml of semen was gently overlaid with 0.5 ml Earle's balanced salt solution (EBSS; Gibco) supplemented with human serum albumin (HSA; Sigma) and incubated at 37°C in 5% CO_2 in air for 45 min. The upper half of

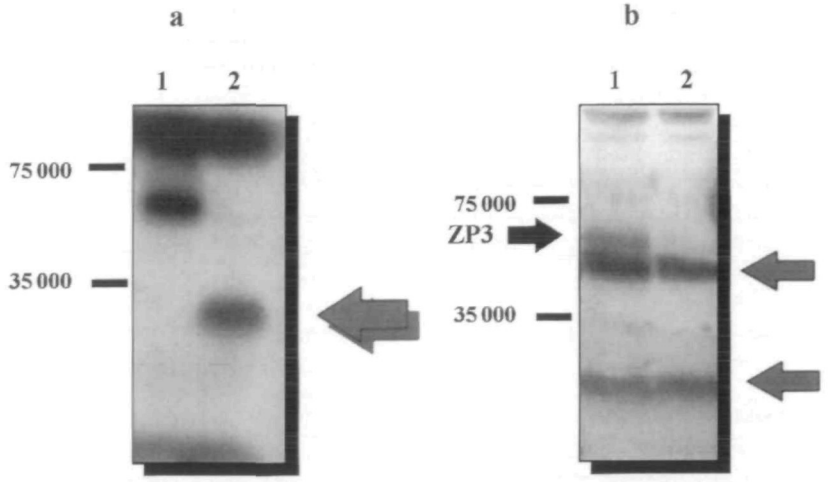

Figure 2. In-vitro translation of recombinant ZP3. (**a**) Autoradiograph of [^{35}S]-labelled polypeptides produced following in-vitro translation of plasmids pCDNA1ZP3S (lane 1) and pCDNA1ZP3ΔNH (lane 2; arrow). (**b**) A Western blot of two in-vitro translation reactions. In lane 1 the additional species detected following translation from the template pCDNA1ZP3S by anti-porcine ZP3 corresponds to a species of the same molecular weight as the translated species in lane 1 of Figure 2a (ZP3 arrow). When in lane 2 the template is pCDNA1 without the ZP3 cDNA, no such species is detected. The arrows on the right hand side of the figure (Figure 2b) indicate rabbit reticulocyte proteins which are detected using the rabbit anti-porcine ZP3 antiserum.

the supernatant was carefully aspirated and sperm motility analysed. The motile sperm concentration was adjusted to ~15–20×10^6/ml with EBSS and 0.3% HSA. The sperm suspension was incubated (in 5% CO_2 in air at 37°C) for a further 3 h. These sperm preparations were added to the beads as described below.

Spermatozoa binding assay

Sperm binding was assessed on one ejaculate from each of 12 fertile semen donors. Aliquots of 50 µl of capacitated spermatozoa were added to each of the three following groups: (i) 50 µl uncoated beads (streptavidin); (ii) 50 µl rhuZP3 coated beads; or (iii) 50 µl biotin coated beads. These were incubated at 37°C, in humidified 5% CO_2 in air. After 15 min samples were removed and sperm binding analyses were performed. An examination of sperm motility was also made. Preliminary experiments examining sperm concentration, bead:spermatozoa ratio in the incubations and times of incubations were performed to determine the best working system. A time point of 15 min for assessment was chosen as convenient which showed optimal levels of binding. Binding was not significantly increased with longer incubation times.

Sperm binding analysis was examined by placing 5 µl of the original mixed sample onto a microscope slide, covering with a coverslip and observing using phase contrast microscopy at a final magnification ×400. A total of 100 beads were counted and the number with spermatozoa attached recorded. For each sample, five slides were prepared and read, therefore, a total of 500 beads were observed and the percentage of beads with spermatozoa bound was calculated. Spermatozoa were considered to be bound only if there was significant tail movement while attached to the beads. Those spermatozoa that appeared to be attached, but were motionless, or weakly motile, were not recorded as bound. This ensured that only active binding was recorded. In all cases the number of spermatozoa exceeded the number of beads in the final mixture.

Acrosomal status

Aliquots of 50 µl of capacitated spermatozoa were added to each of the four following groups; (i) 50 µl uncoated beads; (ii) 50 µl rhuZP3 coated beads; (iii) 50 µl biotin coated beads; or (iv) 50 µl of EBSS plus 0.3% HSA with no beads (control for the effect of beads on the acrosome reaction). Samples were left for 3 h incubation and overnight (18 h) at 37°C, in an atmosphere of humidified 5% CO_2 in air. One ejaculate from five fertile donors (3 h capacitation) and one ejaculate from 12 fertile donors [18 h capacitation] was used in these experiments. Spermatozoa were not isolated from the beads to determine acrosomal status. Acrosomal status was evaluated using the technique described by Zhu *et al.* (1994) using primary monoclonal antibody 18.6 (Moore *et al.*, 1987) and indirect immunofluorescence. Acrosomal status was evaluated at ×1000 magnification. The HOS test was used in conjunction with acrosomal status so that only live acrosome-reacted spermatozoa were scored (Aitken *et al.*, 1993). Two slides were stained for each sample being analysed and for each slide two sets of 100 cells were scored as either: (i) viable acrosome intact (fluorescent green acrosome, curled tail); (ii) viable acrosome reacted (no fluorescence, thin fluorescent band in the post-acrosomal region or patchy fluorescence, all with a curled tail); (iii) non-viable acrosome intact (fluorescent green acrosome, straight tail); or (iv) non-viable acrosome reacted (no fluorescence, thin fluorescent band in the post-acrosomal region or patchy fluorescence, all with a straight tail). Results are expressed as percentage viable spermatozoa which are acrosome-reacted.

Statistical analysis

Differences in sperm binding and percentage of acrosome-reacted cells between rhuZP3 and any of the controls was calculated using the Wilcoxon rank sum test. This non-parametric test was used as not all the data were normally distributed.

Results

Production of recombinant human ZP3 (rhuZP3)

The rabbit reticulocyte lysate transcription/translation coupled system produced rhuZP3 as assessed by the appropriate synthesis of a polypeptide of the predicted molecular weight, as determined by fluorography and SDS–PAGE (Figure 2a) and by Western blotting using an antibody specific for porcine ZP3

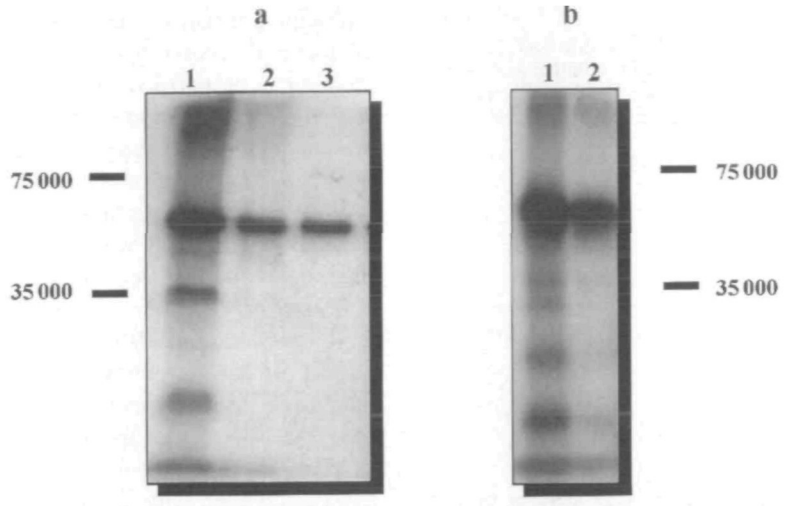

Figure 3. Purification of the products of *in vitro* translation from the templates pET22bZP3H and pCDNA1ZP3S. (**a**) The translation product from pET22bZP3H (lane1) was applied to 500 µl of metal chelate resin and ZP3H was eluted in two fractions following equilibration with 1 M imidazole (lanes 2 and 3). (**b**) The translation product from pCDNA1ZP3S (lane1) was applied to 400 µl of streptavidin agarose and ZP3S was eluted in a single fraction following equilibration with 1 mM biotin (lane 2).

(Figure 2b). The polypeptide gave very clear bands following translation, suggesting that little if any glycosylation had taken place during synthesis. Moreover, the products of the synthesis could be readily purified by a rapid affinity step using either metal chelate chromatography (see Figure 3a) in the case of ZP3H or by streptavidin chromatography in the case of ZP3S (see Figure 3b). In order to establish that the proteins produced by the TNT system were derived from the input template a control plasmid encoding amino acids 242–424 of human ZP3 was employed. All products were of the expected molecular weight as judged by SDS–PAGE (see Figure 2a). The percentage incorporation of L-[methyl-^{3}H]-methionine present in the lysate mixture into rhuZP3 was used as a measure of translational efficiency. Using a plasmid encoding bacterial luciferase as a control, the maximum incorporation of the labelled amino acid into luciferase was found to be 5%. The levels observed for rhuZP3 were slightly lower at 2–4%. The yield of ZP3 was estimated as ~100 ng per translation, practically all of which was immobilized on the beads under the conditions employed in these experiments.

In view of the deleterious effects of imidazole on sperm motility, at the high concentrations necessary for purification of the His-tagged protein ZP3H (data not shown), all of the biological experiments incorporated the use of the strep-tagged molecule, ZP3S.

Spermatozoa binding assay

The percentage of rhuZP3 coated beads bound by motile spermatozoa was significantly greater ($P < 0.05$) than either uncoated (streptavidin) or biotin-coated beads (Table I). In practically all cases (>95%) only one motile spermatozoon bound to the rhuZP3 coated beads. Binding to the rhuZP3 beads was always confined to the head region of the spermatozoa. Binding to both of the control groups of beads (uncoated and biotin beads) was not confined to the head region and sometimes more than one spermatozoa bound per bead.

Table I. Percentage of ZP3 coated, uncoated and biotin coated beads bound by capacitated spermatozoa. Values given are medians with ranges in parentheses

	n	Percentage of bound beads
ZP3 beads	12	21* (13–33)
Uncoated beads	12	5 (4–9)
Biotin beads	12	9 (6–12)

n = number of semen samples from fertile donors. One ejaculate from each donor.
*$P < 0.05$ difference between ZP3 coated beads and the other groups.

Table II. Percentage live acrosome reactions (AR) in fertile donor spermatozoa capacitated for 3 h and then incubated for a further 3 h with recombinant human ZP3 attached to the beads, biotin controls, uncoated beads or no beads. Values given are medians with ranges in parentheses

Treatment	*n*	Percentage AR
ZP3 beads	5	7 (3–12)
Uncoated beads	5	7 (3–16)
Biotin beads	5	8 (2–18)
No beads	5	4 (1–12)

n = number of semen samples from fertile donors. One ejaculate from each donor.

Assessment of the acrosome reaction

There was no significant induction of the acrosome reaction in capacitated spermatozoa after a period of incubation of 3 h with rhuZP3 coated beads (median levels <9%, see Table II). However, spermatozoa that were incubated with rhuZP3 coated beads overnight (18 h) showed significant induction of the acrosome reaction ($P < 0.01$) compared with the other controls (median 53% versus <20% for all the controls; see Table III).

Table III. Percentage live acrosome reactions (AR) in fertile donor spermatozoa capacitated for 3 h and then incubated for a further 18 h with recombinant human ZP3 attached to the beads, biotin controls, uncoated beads or no beads. Values given are medians with ranges in parentheses

Treatment	*n*	Percentage AR
ZP3 beads	12	53 (35–63)*
Uncoated beads	12	18 (8–44)
Biotin beads	12	19 (19–34)
No beads	12	14 (8–24)

n = number of semen samples from fertile donors. One ejaculate from each donor.
*P <0.01 significantly different to other treatments.

Discussion

This study shows that purified rhuZP3 was produced and successfully immobilized to beads, using a commercially available in-vitro transcription and translation kit. The addition of either a poly histidine or strep-tag provides a means of efficiently purifying the recombinant protein for structural and functional analysis. RhuZP3 was determined to be biologically active by the fact that: (i) the percentage of rhuZP3 coated beads bound by capacitated spermatozoa was significantly higher than controls (Table I); and (ii) significant levels of acrosome reactions were induced when rhuZP3 coated beads were incubated with capacitated spermatozoa for 18 h (Table III). This provides a firm basis for the further development of a sperm function test using rhuZP3 coated beads.

The production of recombinant human ZP3 by the in-vitro transcription and translation system was assessed by SDS–PAGE and subsequent Western blotting using an anti-porcine ZP3 antiserum. The use of several different ZP3 templates confirmed the origin of the translated protein. The glycoprotein was ~50 kDa (see Figure 2a). This is higher than the predicted molecular weight of the polypeptide chain (~46 kDa). In theory, the rhuZP3 produced by in-vitro transcription and translation should not be glycosylated as we did not incorporate canine pancreatic microsomal membranes which promote glycosylation. Preliminary analysis using a commercially available glycosylation detection kit (Amersham Ltd) failed to detect any glycosylation of the rhuZP3 (data not shown). These differences in molecular weight are probably due to post translational modifications, the nature of which are unknown. Native ZP3 migrates on SDS–PAGE with a molecular weight of 55–73 kDa (Bercegeay *et al.*, 1995). Interestingly, several other studies investigating the biological activity of recombinant zona proteins in the mouse, and the human, have demonstrated noticeably lower molecular weights of the recombinant product versus the native protein; however, the recombinant product retains biological activity (Kinloch *et al.*, 1991; Beebe *et al.*, 1992; Van Duin *et al.*, 1994; Barratt and Hornby, 1995).

In this study, rhuZP3 was tagged with both a strep affinity tag and poly histidine at the *C*-terminus allowing efficient purification with the appropriate affinity matrix. This system of tagging to purify recombinant proteins has been successfully used in many other cell systems (see Schmidt and Skerra, 1993). Whilst His-tagged ZP3 could be purified efficiently following translation, the use of the strep tag has the advantage of facile detection and biological compatibility. Although production of purified protein was relatively low, ~100 ng could be synthesized in each run. The concentration of rhuZP3 present on the beads was estimated, using scintillation counting, to be ~$2–8\times10^{-3}$ ng per bead. In the present study we did not determine the concentration of rhuZP3 on each bead or evaluate whether all the beads were coated in a homogeneous manner. In comparison with the amount of ZP3 in the zona, estimated to be 1–5 ng ZP3/zona in humans (see Van Duin *et al.*, 1994), the levels on the beads in this assay are lower. It is unclear at present what concentration of ZP3 is required to initiate binding to the zona pellucida. Interestingly, Wassarman *et al.* have recently shown, using transgenic mice, that a reduction of >50% in the number of functional sperm receptors in ZP3 can be achieved without affecting fertility (Liu *et al.*, 1995). Equivalent data for the human are not available but future studies using the ZP3 bead system are likely to be able to address this issue.

Biological activity in these experiments was evaluated using the percentage of rhuZP3 coated beads bound by capacitated spermatozoa and induction of the acrosome reaction. These two markers of sperm function are commonly used as assays of biological activity. Significantly higher levels of binding of spermatozoa to the rhuZP3 coated beads was observed compared with controls [uncoated (streptavidin) and biotin-coated beads; see Table I]. Interestingly, in practically all cases, motile spermatozoa bound to the rhuZP3 coated beads via the head region. Spermatozoa bind to the zona pellucida via the head region where complementary receptors for ZP3 are specifically located (e.g. zona receptor kinase, Burks *et al.*, 1995; Moore, 1995). The similarity in binding between the zona and the rhuZP3 beads strongly suggests that the latter are a valid tool for studying gamete interaction. Although binding to the rhuZP3 beads was significantly greater than the controls (P <0.01, Table I) the median level of binding was low (20%). These relatively low levels of binding could be due to several factors: (i) the concentration of rhuZP3 may not have been equal on all beads, hence some beads which did not bind spermatozoa may not have a high enough concentration of ZP3 (see above); (ii) when the rhuZP3 attaches to the beads some of the ZP3 binding sites for spermatozoa may have been masked. In the human, the region of the ZP3 molecule important for sperm binding is not known, although in-vitro experiments in mice have identified the importance of exon 7 for biological activity (Kinloch *et al.*, 1995); (iii) ZP2 and ZP1 may play a role in sperm attachment in the human; however, at present their role is unknown. Further studies using beads coated with rhuZP2 and rhuZP1 would clarify the biological role of these proteins.

Vasquez *et al.* (1989) using mice, covalently linked native mouse ZP3 to silica beads and examined the dynamics of sperm binding. Using this system only very low levels of binding (a maximum of 10% beads bound with spermatozoa) was observed. In addition, in practically all cases only one spermatozoon per bead was observed and binding to the beads was via the head. These observations are consistent with the result in our study, except that we achieved a higher degree

of binding. Whether these features are particular to this type of solid phase assay is, at this stage, unclear.

Induction of the acrosome reaction was not observed when the rhuZP3 coated beads were incubated with capacitated human spermatozoa for a short period of time (up to 3 h; Table II). However, significant induction of the AR was observed with longer incubations (see Table III). At present we can only speculate about these differences. The rhuZP3 product, although able to initiate binding and an acrosome reaction after long incubation times, may not have been sufficiently close to the native molecule to induce a rapid acrosome reaction. Experience using our rhuZP3 produced from CHO cells (see Barratt and Hornby, 1995; Brewis *et al.*, 1996) suggests that high levels of acrosome reactions (50%) can occur with relatively short incubation times (30 min); however, other laboratories report that overnight capacitation and/or long incubation periods with human rhuZP3 (produced from CHO cells) are necessary to achieve a significant response (for example, see Van Duin *et al.*, 1994). Interestingly, the levels of acrosome reactions are significantly lower when using solubilized zona pellucida as an agonist compared with intact zona pellucida (11 versus 32%, $P < 0.001$; Liu and Baker, 1996). Thus, the preparation of the zona proteins, and/or the nature of their presentation to spermatozoa (solubilized or solid phase) may be important factors to consider in the interpretation of acrosome reaction results. Further experiments examining induction of the acrosome reaction with the in-vitro transcription and translation rhuZP3 are necessary, e.g. varying the concentration of rhuZP3 and the capacitation conditions, before we can conclude that the rhuZP3 is unable to induce acrosome reactions after short incubation times (see Barratt and Hornby, 1995). In addition, comparisons with the kinetics of the acrosome reaction induced with solubilized human zona would provide interesting data.

In the present study the rhuZP3 was probably not glycosylated, yet retained some biological activity. This is perhaps surprising if we confine our thoughts to the mouse where conventional wisdom emphasizes the importance of carbohydrates in the binding of spermatozoa to the zona. The situation in the human is however more complex where the protein backbone also plays a significant role. For example, preliminary results show that rhuZP3 produced in *Escherichia coli* (non-glycosylated) stimulated a significant increase in phosphorylation of a 95 kDa membrane protein as assessed by the in-vitro kinase assay and Western blotting using anti-phosphotyrosine antibodies (Brewis *et al.*, 1995). In addition, several studies have demonstrated that antibodies against the polypeptide backbone of zona proteins significantly reduce sperm binding to the zona pellucida in humans (see Koyama *et al.*, 1994). Interestingly, Oehninger *et al.* (1996) using a specific peptide antibody to human ZP3, showed that abnormalities in the polypeptide backbone of the human zona pellucida are a significant cause of failure to fertilize at IVF. Furthermore, in pigs, peptide antiserum against the amino terminal of porcine ZPB (ZP3α) significantly blocks attachment of boar sperm to the zona (Yurewicz *et al.*, 1992), and monoclonal antibodies to peptide sequences in porcine ZPC (formally known as ZP3β) significantly block the attachment of boar sperm to the zona (Gupta *et al.*, 1995). In contrast, antibodies against mouse ZP3 do not block sperm binding to the mouse zona (see Vasquez *et al.*, 1989). We therefore think that the protein backbone of ZP3 may have a more significant role to play in sperm binding and subsequent acrosome reaction in the human than the mouse. We are examining further the role of the protein backbone in human gamete recognition (see Barratt and Hornby, 1995)

Binding of rhuZP3 to beads was chosen as a basis for a sperm function test because: (i) it allowed for easy purification and visualization of sperm binding; (ii) no specialized equipment was necessary; (iii) the test is somewhat analogous to the zona binding assay; and (iv) other successful laboratory andrology tests, e.g. immunobeads for antisperm antibodies, use beads and thus technical staff are familiar with the technology. These points will enhance the laboratory implementation of a recombinant human zona–sperm function test when it is developed.

Numerous questions about the development of a recombinant human zona assay remain. The region of binding of rhuZP3 to spermatozoa must be evaluated. Ideally, higher concentrations of rhuZP3 need to be attached per bead and the uniformity of attachment needs to be established. Perhaps a system more analogous to the zona, i.e. fewer beads (e.g. 10 per assay) which are saturated with recombinant zona proteins, should be developed. As well as examining binding characteristics to the rhuZP3 beads, the acrosomal status of the spermatozoa should be assessed. In addition, when an assay is closer to being refined, comparisons with the zona binding assay using clinical material (i.e. spermatozoa that have good and poor zona binding) need to be performed.

The results in this study clearly demonstrate the value of using recombinant proteins in comparison to native proteins. For example, in each run of the in-vitro transcription and translation system the equivalent of 200 zona were produced. In addition to producing sufficient quantities of rhuZP3 the in-vitro transcription and translation system allows rapid production (within 8 h) and is thus particularly suitable for performing mutagenesis experiments. Interestingly, in this context, Kinloch *et al.* (1995) have performed exon swapping and site directed mutagenesis experiments on recombinant mouse ZP3 produced in embryonal carcinoma cells. These studies suggested that exon 7 was important for sperm binding. Using the in-vitro transcription and translation system it should be possible to perform similar experiments in the human. The availability of several forms of recombinant zona proteins, e.g. produced in *E.coli*, bacculovirus and CHO cells, is likely to allow critical investigations of the molecules involved in human sperm–zona interaction (see recent examples in the rabbit; Prasad *et al.*, 1995, 1996; Yamasaki *et al.*, 1995).

In conclusion, the in-vitro transcription and translation system can produce sufficient quantities of purified immobilized biologically active rhuZP3. These preliminary experiments will enable further refinements to be made so that a solid phase sperm function test based on rhuZP3 coated beads is likely to be developed in the near future. In addition, this experimental system can be used to study the interaction

between spermatozoa and human zona proteins in greater detail, e.g. by generating ZP3 mutants (Chapman and Barratt, 1996).

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Short sequence-paper

Identification of the true human orthologue of the mouse *Zp1* gene: evidence for greater complexity in the mammalian zona pellucida?

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Abstract

The mammalian zona pellucida is a mixture of glycoproteins, believed to be encoded by three distinct genes, *ZP1*/*ZPB*, *ZP2*/*ZPA*, and *ZP3*/*ZPC*. We have now determined that the true human orthologue of the mouse *Zp1* gene is not *ZPB*, but that there is a distinct human *ZP1* gene. Comparison of the human *ZP1* and murine *Zp1* genes indicates significant conservation of nucleotide and amino acid sequences, of intron–exon size and organisation, and of regulatory sequences. In addition, the mouse and human *ZP1* genes are in a region of conserved synteny between human chromosome 11 and mouse chromosome 19.

Keywords: Oocyte; Zona pellucida; ZP1; ZP2; ZP3; ZPB

The mammalian oocyte is surrounded by a thick glycoprotein coat, the zona pellucida, that serves to mediate species-specific sperm binding, induction of the acrosome reaction, and to prevent post-fertilisation polyspermy [1,2]. In addition, the zp protects the developing embyro prior to implantation. The murine zp is composed of three distinct glycoproteins, ZP1, ZP2 and ZP3 (also known as ZPB, ZPA and ZPC, respectively [3]); the corresponding genes have been cloned [1,2], and shown to be conserved in other mammalian species [3]. The amino acid sequences of both ZP2 and ZP3 show significant conservation across mammals (e.g. mouse vs human, ZP2/A 57% identity, ZP3/C 67% identity); in contrast, mouse and human ZP1/B are only 33% identical [1]. A model has been proposed, based on the mouse zp, in which filaments of ZP2/ZP3 oligomers are cross-linked by dimers of ZP1 [2]. ZP3 is proposed to be the primary sperm receptor [1,2] and mice lacking a functional *Zp3* gene are infertile due to the absence of a zp [4,5]; surprisingly replacement with a human *ZP3* gene does not lead to human sperm binding activity, but instead restores mouse sperm binding [6]. In addition, sperm binding activity in the pig zp resides in a hetero-oligomer of ZP1/B and ZP3/C [7], suggesting that the mouse might not be a good model for mammalian sperm–egg interaction. Herein we suggest a resolution for these apparent discrepancies, with the observation that human *ZP1* and *ZPB* are distinct genes.

A BLAST search of the dbEST database (http://www.ncbi.nlm.nih.gov/BLAST/) was performed with

Abbreviations: aa, amino acids; bp, base pairs; kb, kilobase(s); PAC, P1 artificial chromosome; UTR, untranslated region; zp, zona pellucida

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the mouse *Zp1* cDNA sequence (accession no. U20448) and five human ESTs were identified that displayed significant sequence similarity (>80%), of which two (accession nos. AA426398 and AI208352) were from a human testis cDNA library, two from a pooled library derived from foetal lung, testis and B-cell cDNA libraries (accession nos. AA890119 and AI698605) and a fifth from a kidney cDNA library (accession no. AI766401). A BLAST search of the Genbank database with the human *ZP1* EST composite sequence identified a single genomic sequence entry with significant similarity (>90%, accession no. AC004126). The intron–exon organisation of the human *ZP1* gene was determined by comparison with the mouse *Zp1* sequence, the cDNA sequence generated from the ESTs, and manual identification of splice donor and acceptor sites (Table 1). As shown previously for the mouse *Zp1* gene [8], the human *ZP1* gene consists of 12 exons, with short 5′- and 3′-UTRs. Comparison of the sizes of the exons and introns of human *ZP1*, mouse *Zp1* [8] and human *ZPB* [3] reveals a greater similarity between human and mouse *ZP1* (Table 1).

The putative transcript encoded by the human *ZP1* gene is ~2 kb, predicting a protein of 638 amino acids, comparable in size to the mouse (623 aa) and rat (617 aa) ZP1 peptides [8,9]; in contrast, human ZPB encodes a peptide of 540 amino acids, similar in size to other ZPB isolates [3]. Similarly, the identity between the human ZP1 peptide and the mouse and rat ZP1 peptides is 67 and 68%, respectively; human ZPB and mouse ZP1 have previously been noted to be only 33% identical [1]. CLUSTALW alignment (http://biology.ncsa.uiuc.edu/BW30/BW.cgi) of the human, mouse and rat ZP1 peptide sequences demonstrates the degree of conservation (Fig. 1). Analysis of the promoter regions of mammalian *ZP* genes has identified a TATAA box at approximately −30 bp, and an E box at approximately −200 bp that is sufficient and necessary for oocyte specific expression, and to which a heterodimer of E12 and FIG-α binds [8,10]. Examination of the sequence upstream of the probable translation initiation codon of human *ZP1* identified both a TATAA box and a consensus E box at comparable positions (Fig. 2). The human *ZP1* gene is on a PAC from 11q12.2, a region of conserved synteny with mouse chromosome 19, to which mouse *Zp1* has previously been localised [8,11]. Comparison of the maps of human chromosome 11 and mouse chromosome 19 thus supports the assignment of this human *ZP1* as the orthologue of mouse *Zp1* (data not shown).

The presence of human genes for both *ZP1* and *ZPB* suggests that there should be a revision of both the gene nomenclature and the current model for zp structure and function. It is possible the mammalian zp contains four, not three proteins; the conservation of regulatory sequences makes it likely that ZP1 is expressed in the human oocyte. This may also serve to clarify the current confusion over the identity of human zona proteins [12]. The additional implication

Table 1
Exon and intron sizes of human *ZP1*, mouse *Zp1* [8] and human *ZPB* [3] genes

Exon	*ZP1*	*Zp1*	*ZPB*	Intron	*ZP1*	*Zp1*	*ZPB*
1	>195	229	175	1	1386	950*	283
2	122	122	122	2	270	312	84
3	364	364	103	3	430	89	1150*
4	144	144	153	4	481	384	750*
5	188	188	188	5	72	162	450*
6	98	98	98	6	1845	850*	1050*
7	128	128	131	7	86	94	175
8	190	190	190	8	69	98	83
9	144	145	151	9	1157	1300*	3500*
10	81	82	79	10	114	97	1850*
11	120	108	102	11	266	170	94
12	180	165	128				

Size given in bp, and determined by sequencing apart from fragments designated *.

```
hZP1     MAGGSATTWGYPVALLLLVATLGLGRWLQPDPGLPGLRHSYDCGIKGMQLLVFPRPGQTL
rZP1     ------MAWGCFVVLLLLVAAP--LRLGQHLHLKPGFQYSYDCGVQGMQLLVFPRPNQTI
mZP1     ------MAWGCFVVLLLLAAAP--LRLGQRLHLEPGFEYSYDCGVRGMQLLVFPRPNQTV

hZP1     RFKVVDEFGNRFDVNNCSICYHWVTSRPQEPAVFSADYRGCHVLEK-DGRFHLRVFMEAV
rZP1     QFKVLDEFGNRFEVNNCSICYHWVISEAQKPAVFSADYKGCHVLEKQDGRFHLRVFIQAV
mZP1     QFKVLDEFGNRFEVNNCSICYHWVTSEAQEHTVFSADYKGCHVLEK-DGRFHLRVFIQAV

hZP1     LPNGRVDVAQDATLICPKPDPSRTLDSQLAPPAMFSVSIPQTLSFLPTSGHTSQGSGHAF
rZP1     LPNGRVDTAQDVTLICPKPDHILTPESYLAPPTTPQPFIPHTFALHPISGHTLAGSGHTG
mZP1     LPNGRVDIAQDVTLICPKPDHTVTPDPYLAPPTTPEPFTPHAFALHPIPDHTLAGSGHTG

hZP1     PSPLDPGHSSVHPTPALPSPGPGPTLATLAQPHWGTLEHWDVNKRDYIGTHLSQEQCQVA
rZP1     LTTLYP---ETHPTPAPPSSEPGPVGPTVPQSQWGTLGSWELTELDSIGTHLLQERCQVA
mZP1     LTTLYPEQSFIHPTPAPPSLGPGPAGSTVPHSQWGTLEPWELTELDSVGTHLPQERCQVA

hZP1     SGHLPCIVRRTSKEACQQAGCCYDNTREVPCYYGNTATVQCFRDGYFVLVVSQEMALTHR
rZP1     SGHIPCMVKGSSEEACQQAGCCYDNTKEMPCYYGNTVTLQCFRSGYFTLVMSQETALTHG
mZP1     SGHIPCMVNGSSKEACQQAGCCYDSTKEEPCYYGNTVTLQCFKSGYFTLVMSQETALTHG
                Trefoil Domain
hZP1     ITLANIHLAYAPTSCSPTQHTEAFVVFYFPLTHCGTTMQVAGDQLIYENWLVSGIHIQKG
rZP1     VMLDNVHLAYAPNGCPPTQKTSAFVVFHVPLTLCGTAIQVVGKQLVYENQLVSNIEVQTG
mZP1     VLLDNVHLAYAPNGCPPTQKTSAFVVFHVPLTLCGTAIQVVGEQLIYENQLVSDIDVQKG
                ZP domain
hZP1     PQGSITRDSTFQLHVRCVFNASDFLPIQASIFPPPSPAPMTQPGPLRLELRIAKDETFSS
rZP1     PQGSITRDGVFRLHVRCIFNASDFLPIRASIFSPQPPAPVTRSGPLRLELRIATDKTFSS
mZP1     PQGSITRDSAFRLHVRCIFNASDFLPIQASIFSPQPPAPVTQSGPLRLELRIATDKTFSS

hZP1     YYGEDDYPIVRLLREPVHVEVRLLQRTDPNLVLLLHQCWGAPSANPFQQPQWPILSDGCP
rZP1     YYQGSDYPLVRLLQEPVYIEVRLLQRTDPGLALMLHQCWATPSASPFEQPQWPILSDGCP
mZP1     YYQGSDYPLVRLLREPVYVEVRLLQRTDPSLVLLLHQCWATPTTSPFEQPQWPILSDGCP

hZP1     FKGDSYRTQMVALD-GATPFQSHYQRFTVATFALLDSGSQRALRGLVYLFCSTSACHTSG
rZP1     FKGDNYRTQMVAADRATLPFWSHYQRFTIATFTLLDSSSQNALRGQVYFFCSASACHPVG
mZP1     FKGDNYRTQVVAADKEALPFWSHYQRFTITTFMLLDSSSQNALRGQVYFFCSASACHPLG

hZP1     LETCSTACSTGTTRQRRSSGHRNDTARPQDIVSSPGPVGFEDSYGQEPTLGPTDSNGNSS
rZP1     SETCSTTCDSEIARHRRSSGHHNSTIRALDIVSSPGAVGFEDAPKLE----PSGSTRNSG
mZP1     SDTCSTTCDSGIARRRRSSGHHNITLRALDIVSSPGAVGFEDAAKLE----PSGSSRNSS

hZP1     LRPLLWAVLLLPAVALVLGFGVFVGLSQTWAQKLWESNRQ
rZP1     SRPLLWVLQLL-ALTLVLGDGVLVGLSWAWAWA-------
mZP1     SR----MLLLLLAITLALAAGIFVGLIWAWAQKLWEGIRY
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Fig. 1. CLUSTALW alignment of the human (h), mouse (m) and rat (r) ZP1 peptide sequences. Human ZP1 has a transmembrane domain at the C-terminus, a putative signal cleavage site at amino acid 25, and highly conserved trefoil and zp domains (underlined). The predicted M_r of the protein is 70 061 Da, but it is known that the ZP proteins are highly glycosylated [1]; there are 97 possible *O*-glycosylation sites (S/T residues) and 4/6 of the *N*-glycoslation sites (N-X-S/T) in mouse ZP1 are conserved in human ZP1 (boldface). A potential furin cleavage site (R-X-R/K-R) is also conserved in all three ZP1 proteins (dotted underline).

is that it is likely that there are distinct genes for *ZPB* and *ZP1* in other mammals, if not indeed in other vertebrates. The identification of *ZPB* orthologues in *Xenopus* [13], chick (accession no. AB025428) and fish [14], would indicate the presence of a *Zpb* gene in mice, in particular. The initial characterisation of ZP3 as the primary sperm receptor was performed with SDS-PAGE purified protein; in other mammals, ZP3 and ZPB have been shown to comigrate [7,12], and indeed minor ($\sim$5%) ZP3 contamination of ZPB results in fully functional pig sperm receptors [7]. In addition, Rankin et al. [6] have demonstrated that human ZP3 is not sufficient to generate a zona pellucida capable of binding human sperm in transgenic mice, suggesting the involvement of other factors. We therefore suggest that the physiological receptor for sperm on the oocyte may be a hetero-oligomer of ZP3 and ZPB, and that this may function as such throughout vertebrates.

	E box		TATA box	
mZp1	agtcc**CAgcTG**ccaca	(-218) -	tATAA (-30) -	ATG (+53)
hZP1	agcca**CAgcTG**tgaga	(-188) -	cATAA (-30) -	ATG (+31)
mZp2	ttact**CAccTG**gagcc	(-216) -	tATAA (-31) -	ATG (+31)
hZP2	ttact**CAccTG**gagcc	(-220) -	tATAA (-30) -	ATG (+27)
mZp3	ggaat**CAcgTG**gagtg	(-181) -	tATAA (-29) -	ATG (+30)
hZP3	ggata**CAcgTG**ggggg	(-236) -	tATAA (-30) -	ATG (+13)

Fig. 2. Conservation of ZP gene regulatory sequences. Mouse (m) and human (h) ZP1, ZP2 and ZP3 upstream sequences were compared and critical motifs identified ([8,15,16], this study). The E box (boldface), TATA box and translation initiation codons are indicated; the transcription initiation site (nucleotide position 0) was either determined for the mouse genes [8] or inferred for the human genes ([15,16], this study). Nucleotide positions of the motifs are indicated in parentheses and conserved nucleotides are indicated in upper case.

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Paper 17:

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Four zona pellucida glycoproteins are expressed in the human*[1]

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BACKGROUND: The zona pellucida (ZP) is an extracellular glycoprotein matrix which surrounds all mammalian oocytes. Recent data have shown the presence of four human zona genes (ZP1, ZP2, ZP3 and ZPB). The aim of the study was to determine if all four ZP proteins are expressed and present in the human. METHODS: cDNA derived from human oocytes were used to amplify by PCR the four ZP genes. In addition, isolated native human ZP were heat-solubilized, trypsin-digested and subjected to tandem mass spectrometry (MS/MS). RESULTS: All four genes were expressed and the respective proteins present in the human ZP. Moreover, a bioinformatics approach showed that the mouse ZPB gene, although present, is likely to encode a non-functional protein. CONCLUSIONS: Four ZP genes are expressed in human oocytes (ZP1, ZP2, ZP3 and ZPB) and preliminary data show that the four corresponding ZP proteins are present in the human ZP. Therefore, this is a fundamental difference with the mouse model

Key words: human/mouse ZPB/oocyte/proteomics/zona pellucida

Introduction

Fertilization is a complex process which, to be successful, requires several steps. Critical early events include the binding of sperm to the zona pellucida (ZP) and subsequent induction of the acrosome reaction. The ZP is a matrix composed of glycoproteins which surrounds all mammalian oocytes and mediates several important roles such as binding sperm in a species-specific manner, inducing the acrosome reaction, preventing polyspermy and protecting the embryo prior to implantation. Experiments, primarily in the mouse, have led to the conclusion that the ZP is composed of three proteins (ZP1, ZP2 and ZP3). The murine ZP is the accepted model for ZP structure in higher vertebrates and is composed of repeating units of ZP2–ZP3 heterodimers arranged in filaments cross-linked by ZP1 dimers (Greve and Wassarman, 1985). Experiments in the mouse, using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)-purified zona proteins showed that ZP3 was the primary sperm ligand (Bleil and Wassarman, 1980a, 1983) and ZP2 was the secondary ligand binding to acrosome-reacted sperm and triggering events that are important for the prevention of polyspermy (Bleil and Wassarman, 1980b; Bleil *et al*., 1981). ZP1 is proposed to contribute to the structural integrity of the ZP matrix (Greve and Wassarman, 1985; Green, 1997; Wassarman, 1999).

Cloning of the ZP genes from a number of species has resulted in the introduction of an alternative nomenclature of ZPA (ZP2), ZPB (ZP1) and ZPC (ZP3) based on gene size (Harris *et al*., 1994). However, it has now become apparent that human ZP1 and ZPB genes are, in fact, paralogues (Hughes and Barratt, 1999), and thus the human genome contains four ZP genes and not three. This is consistent with the identification of both ZP1 and ZPB genes in chicken (Bausek *et al*., 2000) and rat (accession number AF456325), and with recent molecular phylogenetic analysis of vertebrate ZP genes (Conner and Hughes, 2003; Spargo and Hope, 2003). These recent findings show a greater complexity in the number of zona genes across vertebrates than previously anticipated (Conner and Hughes, 2003; Lefièvre *et al*., 2003). In this context it is surprising that the presence of the mouse ZPB gene has not been reported.

Current models for the structure of mammalian zona pellucida are based upon the existence of three ZP proteins

*Part of this work has been presented at ESHRE 2003 held in Madrid [L.Lefièvre *et al*. (2003), Hum. Reprod., 18 (Suppl. 1), 0–266: 91].

Table I. PCR primers used to amplify ZP1, ZP2, ZP3, ZPB and housekeeping genes β-*actin* and *HPRT*

Gene	Primer sequences 5′–3′[a]	References
ZP1	F1 GAAGCCTGTCAGCAGGCTGG	
	R1 GCTGTCCCGCGTGATGGAAC	
ZP2	F1 GCCTCCCAGGACCCATTCTC	
	R1 CAGGTAGCAGATGGAGCCTA	
ZP3	F1 GAGGCAGCCTCATGTCATG	
	R1 AGGCAAAGCCCACTGCTC	
ZP3	F1 CTGCTGCTCTGCAGGTACCATG	
	R1 TTTATTCGGAAGCAGACAGAGG	
β-*actin*	F1 CGGATGTCCACGTCACACTT	Ponte *et al.* (1984)
	R1 GTTGCTATCCAGGCTGTGCT	
HPRT	F1 AATTATGGACAGGACTGAACGTC	Gibbs *et al.* (1989)
	R1 GGCGATGTCAATAGGACTCCAGATG	
	R2 CGTGGGGTCCTTTTCACCAGCAAG	

[a]F1 = first round forward primers.
R1 and R2 = first and second round reverse primers; *HPRT* = hypoxanthine phosphoribosyl transferase.

(Wassarman, 1999). If all four ZP genes are transcribed and translated in the human oocyte, a re-evaluation would be required, both of the structure of the ZP and, potentially, of the mechanisms of sperm–zona interaction. Thus, the aim of the present study was to investigate the ZP gene expression in the human oocyte and the protein composition of the human ZP.

Materials and methods

mRNA purification and preparation of amplified oocyte cDNA in the human

Amplified cDNA preparations were prepared from human oocytes (four oocytes per sample). The oocytes were lysed in 3 µl of ice-cold lysis buffer (0.8% Igepal, Sigma, UK; 1 IU/µl RNase inhibitor, Gibco BRL, UK; and 5 mmol/l dithiothreitol, Gibco BRL, UK) and the released mRNA molecules bound to oligo-(dT)-linked magnetic beads (Dynabeads; Dynal UK). Synthesis of cDNA on the mRNA bound to the beads, and PCR amplification of the cDNA, were carried out using the SMART™ cDNA library construction kit (Clontech, UK). Further details of the methods are given in Holding *et al*. (2000).

PCR amplification of specific gene sequences in amplified cDNA preparations

Expression of four zona pellucida genes (ZP1, ZP2, ZP3 and ZPB), in the cDNA preparations derived from human oocytes was detected by PCR with the primers given in Table I. Primers for detection of the ubiquitously expressed genes β-*actin* and hypoxanthine phosphoribosyl transferase (*HPRT*) as control genes are also given in Table I. PCR amplification was carried out in total volume of 25 µl of reaction mixture, containing 1 µl of cDNA (concentrations were adjusted by ethidium bromide staining of diluted aliquots compared with standard DNA), 200 µmol/l each of dNTP (Pharmacia), 1 µg each of primers, 1×PCR buffer (Perkin Elmer), using 1.25 IU of AmpliTaq DNA polymerase (Perkin Elmer). With the exception of ZP3, the parameters for the PCR for the zona pellucida genes were one cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 1 min. For the detection of expression of the ZP3 gene, the PCR cycling parameters were one cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. PCR cycles for the detection of the housekeeping genes were as follows: β-*actin*, 35 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min and *HPRT*, 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. For the hemi-nested PCR for the expression of *HPRT*, 1 µl of the first round product was then transferred to 24 µl of reaction mixture for second round amplification, using the same conditions except for the substitution of the appropriate second round primers (Table I).

Gel electrophoresis

Ten microlitres of PCR product were mixed with 2 µl of loading buffer and electrophoresed for 90 min at 120 V on a 1 or 2% agarose gel and visualized under UV light.

Isolation of human ZP

Metaphase II human oocytes which failed to fertilize after ICSI were donated by patients attending the Assisted Conception Unit, Birmingham Women's Hospital (HFEA centre number 0119). All patients provided informed consent and the research was approved by the local research ethics committee (LREC 0554). Human ZP were mechanically isolated from oocytes. Briefly, several slits were made in the ZP using 10 µm subzonal insemination (SUZI) micropipettes (Conception Technologies, USA). The oocytes were then flushed out from one of the slits by rapidly introducing medium into the perivitelline space from behind the oocyte. The ZP were then washed five times with 50 mmol/l NH_4HCO_3, pH 7.2.

Solubilization of human ZP

Five isolated human ZP were solubilized in 300 µl of 50 mmol/l NH_4HCO_3, pH 7.2, for 1 h at 70°C. The sample was subsequently concentrated on a 10 kDa cut-off filter, transferred to a siliconized enzyme-linked immunosorbent assay plate and air-dried at 56°C. The dry spot was resuspended in trypsin solution (trypsin solution: 20 µg of trypsin was resuspended in 20 µl of 50 mmol/l acetic acid and then diluted to a final concentration of 12.5 ng/µl with 25 mmol/l NH_4HCO_3; sequencing grade modified trypsin, V5111, Promega UK Ltd) and incubated overnight at 37°C. The trypsin-digested human ZP were then resuspended in 6 µl of 1% formic acid and subjected to tandem mass spectrometry (MS/MS).

Mass spectrometry

Direct MS/MS analysis was used to identify the human ZP proteins. This allows direct identification of individual proteins from complex mixtures in very small samples. Lefièvre *et al*. (2003) previously identified the presence of tryptic peptides corresponding to the three known pig ZP proteins (ZP2, ZPB and ZP3) using only 10 isolated porcine ZP.

Tryptic peptides obtained from five isolated human ZP were subjected to MS/MS on a nanoESI Q-Tof mass spectrometer (Q-Tof Ultima GLOBAL; Micromass UK Ltd) following separation of peptides using capillary liquid chromatography (Waters Ltd, UK) with a 15 cm C18 PepMap column (75 µm i.d.×15 cm; Cat. No. 160396, Dionex Ltd, UK). Following MS/MS the raw data were processed using MassLynx 3.5 (Micromass). The resulting tryptic peptide *de novo* sequences data were then compared with non-identical protein sequence databases using MASCOT software (Matrix Science Ltd, UK). Finally, a BLAST search of the GenBank database was performed for each of the peptides obtained to confirm that the proteins identified by MASCOT was the unique match in the non-redundant protein database for a particular peptide sequence.

Mouse ZPB gene analysis

We adopted a bioinformatics approach to identify the mouse orthologue of the rat and human ZPB gene using available sequence data. A BLAST search of the non-redundant database with the rat ZPB

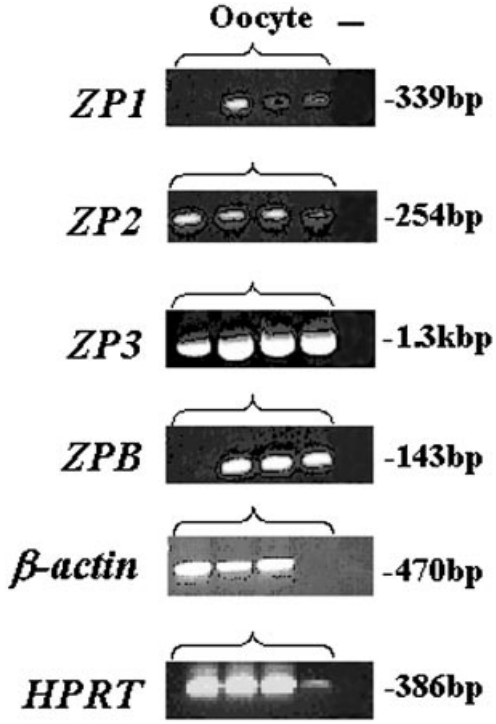

Figure 1. Analysis of expression by PCR, of human ZP genes ZP1, ZP2, ZP3, ZPB, and housekeeping genes β-*actin* and *HPRT* in amplified cDNA preparations from four samples of human oocytes. Sizes of expected PCR products are given to the right of the Figure in base pairs (bp). Blank (–): omitting DNA sample to the PCR reaction.

sequence (NM_172330) was used to identify murine genomic sequence with significant sequence similarity.

Results

ZP gene expression in human oocytes

To determine whether all four ZP genes were expressed, we amplified by PCR cDNA derived from four different samples of four human oocytes. PCR was performed with primers for human ZP1, ZP2, ZP3 and ZPB (Table I) on cDNA preparations from human oocytes. Figure 1 shows that, as expected, all four ZP genes are expressed in the human oocyte. This is the first demonstration that the ZP1 (Hughes and Barratt, 1999) and ZPB genes (Harris *et al*., 1994) are expressed in the human oocyte. Previously only expression of ZP2 and ZP3 had been demonstrated by PCR and serial analysis of gene expression (SAGE) of human oocytes (Neilson *et al*., 2000).

Human zona proteomics

In the present study we have used five human oocytes to investigate the number of distinct glycoproteins in the human ZP. The ZP were mechanically isolated from the oocytes, heat-solubilized, trypsin-digested and analysed using direct MS/MS. Results obtained from peptide sequencing and comparison with non-redundant protein sequence databases confirmed the presence of peptides from all four ZP proteins (ZP1, ZP2, ZP3 and ZPB) (Figure 2). Several tryptic peptide sequences were obtained for ZP2, ZP3 and ZPB (Figure 2), and one for

Human ZP1
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  1 MAGGSATTWGYPVALLLLVATLGLGRWLQPDPGLPGLRHSYDCGIKGMQL
 51 LVFPRPGQTLRFKVVDEFGNRFDVNNCSICYHWVTSRPQEPAVFSADYRG
101 CHVLEKDGRFHLRVFMEAVLPNGRVDVAQDATLICPKPDPSRTLDSQLAP
151 PAMFSVSIPQTLSFLPTSGHTSQGSGHAFPSPLDPGHSSVHPTPALPSPG
201 PGPTLATLAQPHWGTLEHWDVNKRDYIGTHLSQEQCQVASGHLPCIVRRT
251 SKEACQQAGCCYDNTREVPCYYGNTATVQCFRDGYFVLVVSQEMALTHRI
301 TLANIHLAYAPTSCSPTQHTEAFVVFYFPLTHCGTTMQVAGDQLIYENWL
351 VSGIHIQKGPQGSITRDSTFQLHVRCVFNASDFLPIQASIFPPPSPAPMT
401 QPGPLRLELRIAKDETFSSYYGEDDYPIVRLLREPVHVEVRLLQRTDPNL
451 VLLLHQCWGAPSANPFQQPQWPILSDGCPFKGDSYRTQMVALDGATPFQS
501 HYQRFTVATFALLDSGSQRALRGLVYLFCSTSACHTSGLETCSTACSTGT
551 TRQRRSSGHRNDTARPQDIVSSPGPVGFEDSYGQEPTLGPTDSNGNSSLR
601 PLLWAVLLLPAVALVLGFGVFVGLSQTWAQKLWESNRQ
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Human ZP2
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  1 MACRQRGGSWSPSGWFNAGWSTYRSISLFFALVTSGNSIDVSQLVNPAFP
 51 GTVTCDEREITVEFPSSPGTKKWHASVVDPLGLDMPNCTYILDPEKLTLR
101 ATYDNCTRRVHGGHQMTIRVMNNSAALRHGAVMYQFFCPAMQVEETQGLS
151 ASTICQKDFMSFSLPRVFSGLADDSKGTKVQMGWSIEVGDGARAKTLTLP
201 EAMKEGFSLLIDNHRMTFHVPFNATGVTHYVQGNSHLYMVSLKLTFISPG
251 QKVIFSSQAICAPDPVTCNATHMTLTIPEFPGKLKSVSFENQNIDVSQLH
301 DNGIDLEATNGMKLHFSKTLLKTKLSEKCLLHQFYLASLKLTFLLRPETV
351 SMVIYPECLCESPVSIVTGELCTQDGFMDVEVYSYQTQPALDLGTLRVGN
401 SSCQPVFEAQSQGLVRFHIPLNGCGTRYKFEDDKVVYENEIHALWTDFPP
451 SKISRDSEFRMTVKCSYSRNDMLLNINVESLTPPVASVKLGPFTLILQSY
501 PDNSYQQPYGENEYPLVRFLRQPIYMEVRVLNRDDPNIKLVLDDCWATST
551 MDPDSFPQWNVVVDGCAYDLDNYQTTFHPVGSSVTHPDHYQRFDMKAFAF
601 VSEAHVLSSLVYFHCSALICNRLSPDSPLCSVTCPVSSRHRRATGATEAE
651 KMTVSLPGPILLLSDDSSFRGVGSSDLKASGSSGEKSRSETGEEVGSRGA
701 MDTKGHKTAGDVGSKAVAAVAAFAGVVATLGFIYYLYEKRTVSNH
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Human ZP3
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  1 MELSYRLFICLLLWGSTELCYPQPLWLLQGGASHPETSVQPVLVECQEAT
 51 LMVMVSKDLFGTGKLIRAADLTLGPEACEPLVSMDTEDVVRFEVGLHECG
101 NSMQVTDDALVYSTFLLHDPRPVGNLSIVRTNRAEIPIECRYPRQGNVSS
151 QAILPTWLPFRTTVFSEEKLTFSLRLMEENWNAEKRSPTFHLGDAAHLQA
201 EIHTGSHVPLRLFVDHCVATPTPDQNASPYHTIVDFHGCLVDGLTDASSA
251 FKVPRPGPDTLQFTVDVFHFANDSRNMIYITCHLKVTLAEQDPDELNKAC
301 SFSKPSNSWFPVEGPADICQCCNKGDCGTPSHSRRQPHVMSQWSRSASRN
351 RRHVTEEADVTVGPLIFLDRRGDHEVEQWALPSDTSVVLLGVGLAVVVSL
401 TLTAVILVLTRRCRTASHPVSASE
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Human ZPB
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  1 MWLLRCVLLCVSLSLAVSGQHKPEAPDYSSVLHCGPWSFQFAVNLNQEAT
 51 SPPVLIAWDNQGLLHELQNDSDCGTWIRKGPGSSVVLEATYSSCYVTEWD
101 SHYIMPVGVEGAGAAEHKVVTERKLLKCPMDLLARDAPDTDWCDSIPARD
151 RLPCAPSPISRGDCEGLGCCYSSEEVNSCYYGNTVTLHCTREGHFSIAVS
201 RNVTSPPLLLDSVRLALRNDSACNPVMATQAFVLFQFPFTSCGTTRQITG
251 DRAVYENELVATRDVKNGSRGSVTRDSIFRLHVSCSYSVSSNSLPINVQV
301 FTLPPPFPETQPGPLTLELQIAKDKNYGSYYGVGDYPVVKLLRDPIYVEV
351 SILHRTDPYLGLLLQQCWATPSTDPLSQPQWPILVKGCPYIGDNYQTQLI
401 PVQKALDLPFPSHHQRFSIFTFSFVNPTVEKQALRGPVHLHCSVSVCQPA
451 ETPSCVVTCPDLSRRRNFDNSSQNTTASVSSKGPMILLQATKDPPEKLRV
501 PVDSKVLWVAGLSGTLILGALLVSYLAVKKQKSCPDQMCQ
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Figure 2. Human ZP1 (Hughes and Barratt, 1999), ZP2 (AAA61335), ZP3 (AAA61336) and ZPB (NP_067009) amino acid sequences. The bold underlined sequences represent the tryptic peptides obtained from the MS/MS analysis.

ZP1 (^{505}FTVATFALLDSGSQR^{519}) (Figures 2 and 3). This latter tryptic peptide was compared with the human ZP1 peptide sequence identified by Hughes and Barratt (1999). The quindecapeptide amino acid sequence is a perfect match, which indicates the presence of ZP1 in the human ZP.

ZP1 is well recognized as being a low abundance protein (Bleil and Wassarman, 1980b; Epifano *et al*., 1995; Green, 1997). Using the equivalent of several thousand human ZP, a recent MS/MS study on the mouse confirmed the low abundance of ZP1 in that species (Boja *et al*., 2003). Moreover, semiquantitative data in Figure 1 suggest that human ZP1 is less abundant than ZP2, ZP3 or ZPB. Due to the inherent low abundance of ZP1, combined with the paucity of

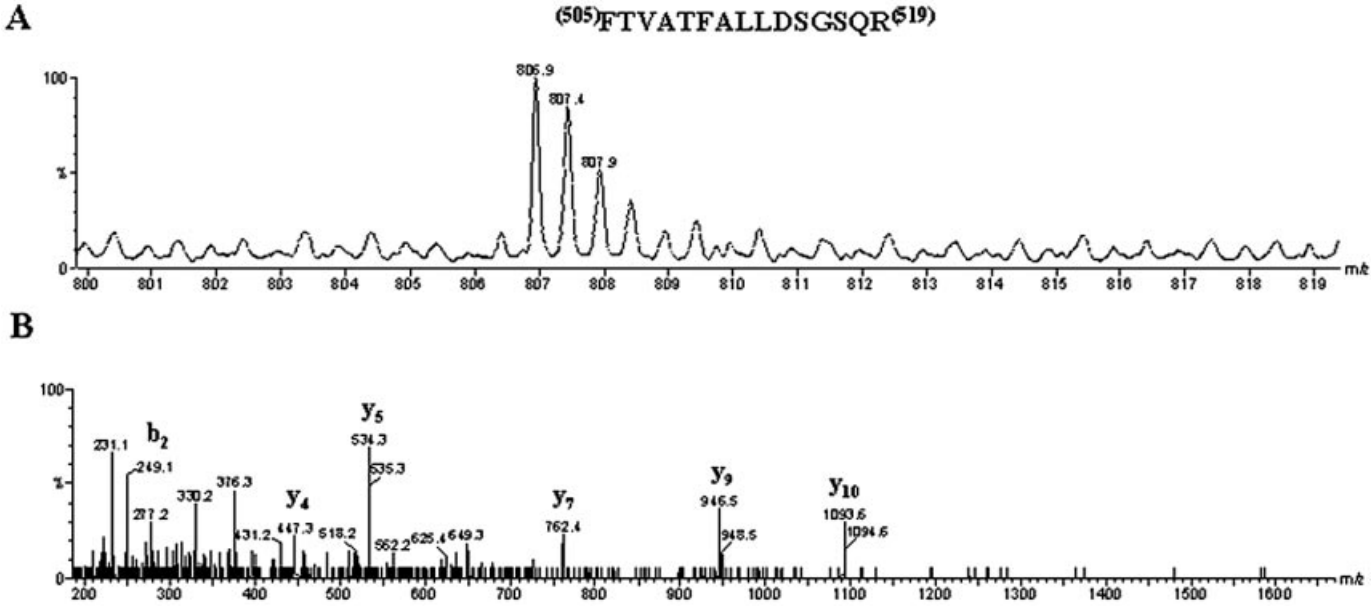

Figure 3. Tandem mass spectrometry (MS/MS) spectra of a C-terminal peptide of MH^+ 1611.88 Da corresponding to the sequence ^{505}FTVATFALDSGSQR^{519}. (**A**) Reconstructed ion chromatogram MS/MS for ZP1 peptide as a 2+ charged ion at *m/z* 806.95 Da and (**B**) collision-induced dissociation (CID) spectrum which confirms the sequence identity of this peptide through b- and y-ions series of peptide fragments.

human oocytes for research purposes, we have been unable to obtain further peptides for ZP1. Additional experiments have been performed using a variety of preparation methods including enzymatic deglycosylation of the ZP prior to the trypsin digestion as described by Boja *et al*. (2003). Although ZP2, ZP3 and ZPB were identified on each occasion, no peptides for ZP1 were obtained.

Existence of mouse ZPB

The current model of the structure of mouse ZP incorporates three glycoproteins, consistent with the detection, to date, of only three mouse ZP genes (ZP1, ZP2 and ZP3). Having established that a fourth ZP gene is present in the human genome (Hughes and Barratt, 1999) and that this gene is expressed (Figure 1) and protein present (Figure 2) in the human ZP, we undertook analysis of the mouse genome to determine whether a ZPB gene is present.

Using a bioinformatics approach, a putative mouse ZPB cDNA sequence was generated by comparison with rat ZPB sequence, coupled with manual identification of splice sites in the genomic sequence. This mapped to chromosome 13, a region of conserved synteny with human chromosome 1 (1q43) where human ZPB is found and rat chromosome 17 (17q12.1) where rat ZPB is found. However, this hypothetical sequence did not encode a continuous open reading frame. Alignment with the rat ZPB cDNA sequence revealed a number of insertions and deletions which resulted in shifts in the open reading frame and the generation of premature termination codons (Figure 4). To confirm the accuracy of the mouse genomic sequence, individual exons of the putative mouse ZPB gene were used to search the trace sequence database (http://www.ncbi.nlm.nih.gov/blast/tracemb). For each exon screened, the sequence was confirmed, with multiple independent trace sequences of both DNA strands (data not shown).

Discussion

This paper presents the first evidence for the existence of four glycoproteins in the human ZP. The exact match of the quindecapeptide with the deduced ZP1 coding sequence (Hughes and Barratt, 1999) in addition to the presence of mRNA transcript for ZP1 in four independent experiments strongly supports the premise that four ZP glycoproteins ZP1, ZP2, ZP3 and ZPB are expressed and present in the human. This study also represents the first protein analysis of the human ZP relying on protein sequencing rather than antibody-based approaches.

The difficulty in obtaining a definitive protein identification of ZP1 is primarily due to the low abundance of ZP1 in the mammalian ZP. For example, in the mouse, levels of ZP1 mRNA were four times lower compared to ZP2 and ZP3 (Epifano *et al*, 1995) and consequently only 56% of the ZP1 polypeptide chain was identified by direct MS/MS, compared to 96 and 100% for ZP2 and ZP3 respectively (Boja *et al*., 2003). However, despite its low abundance, experiments using ZP1 null mice demonstrated that ZP1 is an essential element of the ZP primarily required for the structural integrity of the zona matrix, although not involved directly in sperm binding (Rankin *et al*., 1999).

The demonstration of the existence of four human ZP proteins requires a re-interpretation of the numerous electrophoretic studies on the ZP. Although only three diffuse bands were observed on one-dimensional (1D) electrophoresis, the increased resolution provided by two-dimensional (2D)

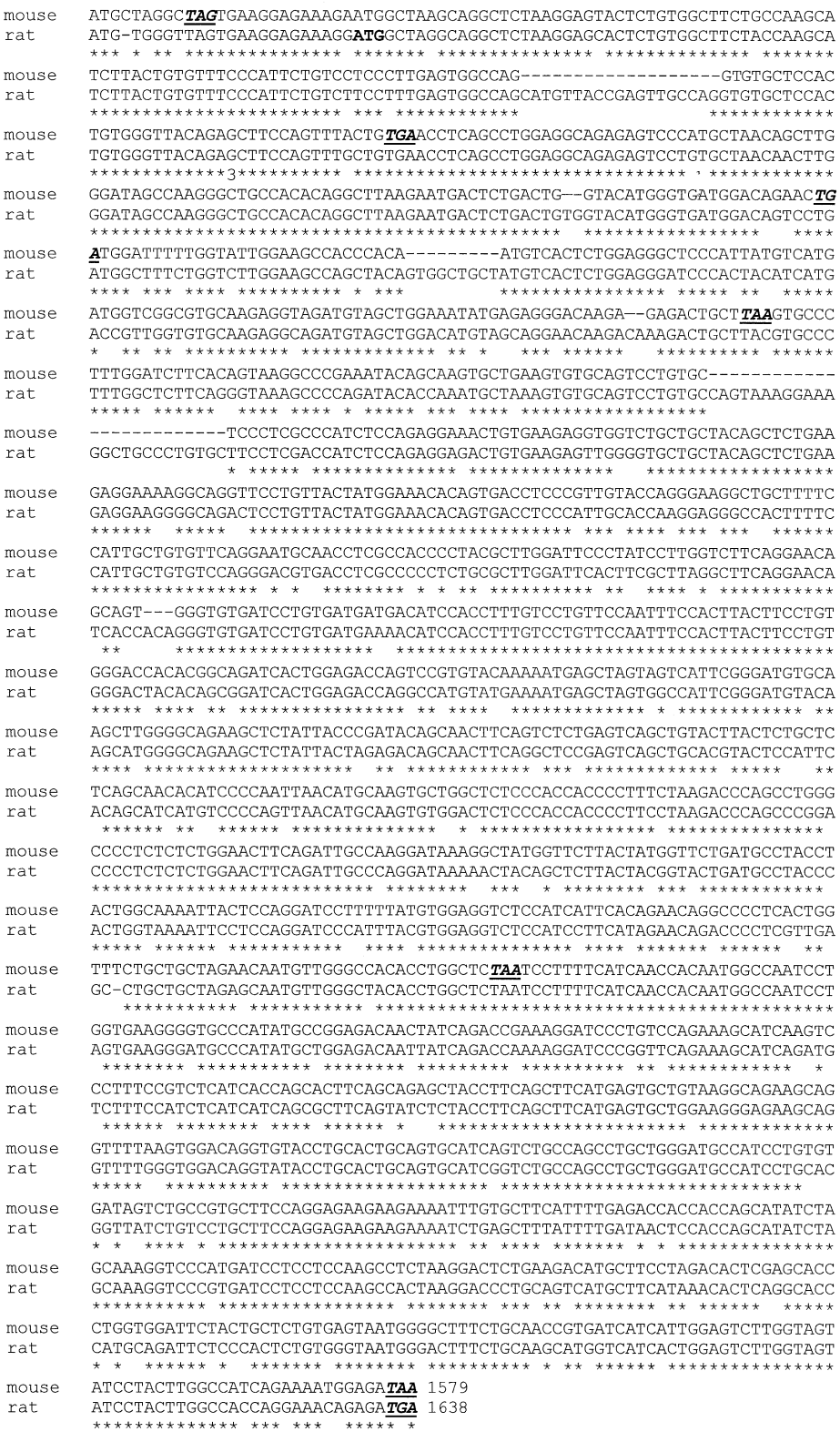

Figure 4. Alignment of rat ZPB mRNA and putative mouse ZPB mRNA produced using CLUSTAL W (Higgins *et al.*, 1994). Predicted termination codons are italicized, underlined and in bold. The published initiation codon for rat ZPB is in bold (NP_758833).

electrophoresis has previously suggested additional levels of complexity to the human ZP (Bercegeay *et al.*, 1995; Moos *et al.*, 1995). This is further complicated by the variable patterns of glycosylation observed. With hindsight, it is apparent that the ZP3-containing band on 1D electrophoresis most likely resolves into two proteins which correspond to ZP3 and ZPB [Yurewicz *et al.*, 1987 (pig); Topper *et al.*, 1997 (cow); Gupta *et al.*, 1998 (human)].

The presence of the ZPB gene in a large number of species (cat, chick, cow, human, macaque, marmoset, pig, possum, rat and rabbit; see Lefièvre *et al*., 2003) raises the question: does the mouse have a ZPB orthologue? Our bioinformatic analysis demonstrates that, like humans and rats, the mouse has four ZP genes. However, comparative sequence analysis reveals that the mouse ZPB gene has acquired a number of changes making it unlikely that functional ZPB protein will be expressed (Figure 4). This *in silico* evidence is supported by recent data using MS analysis which failed to identify ZPB (Boja *et al*., 2003). Numerous peptides from ZP1, ZP2 and ZP3 were identified but the authors did not find any unassigned peptides which could correspond to ZPB.

Knockout experiments, in which murine ZP proteins were replaced by human equivalents (Rankin *et al*., 1998, 2003), showed that, although mouse sperm continued to bind to mouse oocytes engineered to express human ZP2 and ZP3 proteins, human sperm did not bind. One explanation for this result is that mouse oocytes express mouse ZP O-glycans on both mouse and human ZP3 (Dell *et al*., 2003) and that it is these O-glycans that are key to sperm binding (Wassarman, 1999). Another complementary explanation, derived from our results, is that human sperm have evolved to interact with a ZP composed of four, not three, proteins, with ZPB as well as ZP3 being required for sperm zona binding. This explanation is supported by data from other species, e.g. rabbit, bovine, porcine and macaque, which show that ZPB has sperm-binding properties (Prasad *et al*., 1996; Topper *et al*., 1997; Yurewicz *et al*., 1998; Govind *et al*., 2000). Thus, although the mouse ZP may support the hypothesis that the mammalian ZP has three proteins of which ZP3 is the primary sperm receptor, it may fall short as a hypothesis for sperm–zona interaction in other species. Consequently it appears that the model species for the study of mammalian fertilization may only serve as a good model for itself.

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ORIGINAL ARTICLE *Andrology*

The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation

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STUDY QUESTION: What is the prevalence of defects in the Ca^{2+}-signalling pathways mediating hyperactivation (calcium influx and store mobilization) among donors and sub-fertile patients and are they functionally significant, i.e. related to fertilization success at IVF?

SUMMARY ANSWER: This study identifies, for the first time, the prevalence of Ca^{2+} store defects in sperm from research donors, IVF and ICSI patients. It highlights the biological role and importance of Ca^{2+} signalling (Ca^{2+} store mobilization) for fertilization at IVF.

WHAT IS KNOWN ALREADY: Sperm motility and hyperactivation (HA) are important for fertility, mice with sperm incapable of HA are sterile. Recently, there has been significant progress in our knowledge of the factors controlling these events, in particular the generation and regulation of calcium signals. Both pH-regulated membrane Ca^{2+} channels (CatSper) and Ca^{2+} stores (potentially activating store-operated Ca^{2+} channels) have been implicated in controlling HA.

STUDY DESIGN, SIZE, AND DURATION: This was a prospective study examining a panel of 68 donors and 181 sub-fertile patients attending the Assisted Conception Unit, Ninewells Hospital Dundee for IVF and ICSI. Twenty-five of the donors gave a second sample (~4 weeks later) to confirm consistency/reliability of the recorded responses. Ca^{2+} signalling was manipulated using three agonists, NH_4Cl (activates CatSper via pH), progesterone (direct activation of CatSper channels, potentially enhancing mobilization of stored Ca^{2+} by CICR) and 4-aminopyridine (4-AP) (effect on pH equivalent to NH_4Cl and mobilizes stored Ca^{2+}). The broad-spectrum phosphodiesterase inhibitor 3-isobutyl-1-methyxanthine (IBMX), a potent activator of HA was also used for comparison. For patient samples, an aliquot surplus to requirements for IVF/ICSI treatment was examined, allowing direct comparison of Ca^{2+} signalling and motility data with functional competence of the sperm.

MATERIALS, SETTING, METHODS: The donors and sub-fertile patients were screened for HA (using CASA) and changes in intracellular Ca^{2+} were assessed by loading with Fura-2 and measuring fluorescence using a plate reader (FluoStar).

MAIN RESULTS AND THE ROLE OF CHANCE: The relative efficacy of the stimuli in inducing HA was 4-AP >> IBMX > progesterone. NH_4Cl increased $[Ca^{2+}]_i$ similarly to 4-AP and progesterone but did not induce a significant increase in HA. Failure of samples to generate HA (no significant increase in response to stimulation with 4-AP) was seen in just 2% of research donors but occurred in 10% of IVF patients ($P = 0.025$). All donor samples generated a significant $[Ca^{2+}]_i$ increase when stimulated with 4-AP but 3.3% of IVF and 28.6% of ICSI patients failed to respond. Amplitudes of HA and $[Ca^{2+}]_i$ responses to 4-AP were correlated with fertilization rate at IVF ($P = 0.029$; $P = 0.031$, respectively). Progesterone reliably induced $[Ca^{2+}]_i$ responses (97% of donors, 100% of IVF patients) but was significantly less effective than 4-AP in inducing HA. Twenty seven per cent of ICSI patients failed to generate a $[Ca^{2+}]_i$ response to progesterone ($P = 0.035$). Progesterone-induced $[Ca^{2+}]_i$ responses were correlated with fertilization rate at IVF ($P = 0.037$) but induction of HA was not. In donor samples examined on more than one occasion consistent responses for 4-AP-induced $[Ca^{2+}]_i$ ($R^2 = 0.97$) and HA ($R^2 = 0.579$) were obtained. In summary, the data indicate that defects in Ca^{2+} signalling leading to poor HA do occur and that ability to undergo Ca^{2+}

-induced HA affects IVF fertilizing capacity. The data also confirm that release of stored Ca^{2+} is the crucial component of Ca^{2+} signals leading to HA and that Ca^{2+} store defects may therefore underlie HA failure.

LIMITATIONS, REASONS FOR CAUTION: This is an *in vitro* study of sperm function. While the repeatability of the $[Ca^{2+}]_i$ and HA responses in samples from the same donor were confirmed, data for patients were from 1 assessment and thus the robustness of the failed responses in patients' needs to be established. The focus of this study was on using 4AP, which mobilizes stored Ca^{2+} and is a potent inducer of HA. The *n* values for other agonists, especially calcium assessments, are smaller.

WIDER IMPLICATIONS OF THE FINDINGS: Previous studies have shown a significant relationship between basal levels of HA, calcium responses to progesterone and IVF fertilization rates. Here, we have systematically investigated the ability/failure of human sperm to generate Ca^{2+} signals and HA in response to targeted pharmacological challenge and, related defects in these responses to IVF success. $[Ca^{2+}]_i$ signalling is fundamental for sperm motility and data from this study will lead to assessment of the nature of these defects using techniques such as single-cell imaging and patch clamping.

STUDY FUNDING/COMPETING INTEREST(S): Resources from a Wellcome Trust Project Grant (#086470, Publicover and Barratt PI) primarily funded the study. The authors have no competing interests.

Key words: calcium signalling / sperm / male fertility / hyperactivation / sperm motility / IVF.

Introduction

Sperm dysfunction (lacking 'normal' function) has consistently been identified as the single most common cause of male infertility (Hull *et al*., 1985; Irvine, 1998). Men can produce sperm which are dysfunctional even when their semen parameters are 'normal' (Aitken *et al*., 1991). Currently, there are no drugs a man can take, or add to his spermatozoa *in vitro*, to treat sperm dysfunction. The only option is assisted reproductive technology (ART), which comprises a range of treatments, all of which are invasive. The particular treatment selected depends on the severity of the condition, i.e. intrauterine insemination for mild, IVF for moderate and ICSI for men with severe sperm dysfunction. The development of non-invasive, pharmacological treatment alternatives has been severely hampered by our limited understanding of the cellular and molecular workings of the mature spermatozoon (reviews Aitken and Henkel, 2011; Barratt *et al*., 2011). There are, however, some areas where progress has been made. One that has received considerable attention, ignited by the creation and characterization of CatSper knockout mice, concerns the generation and regulation of calcium signals that control sperm motility, in particular hyperactivation (HA) (see below and Publicover *et al*., 2007; Costello *et al*., 2009; Lishko *et al*., 2011; Publicover and Barratt, 2011a, b; Strünker *et al*., 2011; Barratt and Publicover, 2012).

HA is critical to sperm function, playing a key role in the ability of sperm to successfully ascend the female reproductive tract and reach the site of fertilization. For example, HA may facilitate sperm migration through the highly visco-elastic oviductal mucus and enable penetration of the layers surrounding the oocyte (Suarez *et al*., 1991; Suarez and Dai, 1992; Stauss *et al*., 1995; Ren *et al*., 2001; Carlson *et al*., 2003; Quill *et al*., 2003). Additionally, experimental studies show that HA may be required to detach sperm from the oviduct epithelium in animals (Demott and Suarez, 1992; Gwathmey *et al*., 2003) and in humans (Pacey *et al*., 1995). Although data are limited in humans, clinical studies on HA generally suggest that (i) the percentage of hyperactivated sperm correlates with fertilization rate *in vitro* (e.g. Sukcharoen *et al*., 1995) and (ii) there are significant differences in the proportion of hyperactivated cells (spontaneous and in response to physiological or artificial stimulants) between men with normal semen parameters and sub-fertile patients (Burkman, 1984; Tesarik *et al*., 1992; Peedicayil *et al*., 1997; Munire *et al*., 2004).

As the spermatozoon ascends the female tract, its motility must be finely regulated by cues from the female tract and cumulus–oocyte complex, in order that the cell can deploy behaviour appropriate to its environment (Olson *et al*., 2011). The central regulator is Ca^{2+}, elevated $[Ca^{2+}]_i$ being required both for the initiation and maintenance of hyperactivated motility. There are at least two sources of Ca^{2+} that contribute to HA in mammalian sperm: firstly, entry of Ca^{2+} via pH-dependent CatSper channels in the plasma membrane of the flagellar principal piece. Sperm from mice null for CatSper are motile but do not hyperactivate, rendering them unable to migrate to or within the oviduct and unable to fertilize oocytes even by IVF (Ren *et al*., 2001; Carlson *et al*., 2003; Quill *et al*., 2003; Ho *et al*., 2009). In bovine sperm elevation of pHi with NH_4Cl, to activate CatSper, induces HA and similar results have been reported in mouse sperm (Marquez and Suarez, 2007; Chang and Suarez, 2011). Secondly, induction of HA has been reported in bovine, mouse and human cells upon mobilization of calcium stored in the neck/midpiece region (Ho and Suarez, 2001, 2003; Marquez *et al*., 2007; Costello *et al*., 2009). In human sperm thimerosal (which activates intracellular Ca^{2+} channels, releasing stored Ca^{2+}) potently induces HA Alasmari *et al.* (2013) whereas stimulation of CatSper with progesterone (Lishko *et al*., 2011; Strünker *et al*., 2011) or by raising pHi have little effect Alasmari *et al.* (2013). 4-Aminopyridine (4-AP), a particularly potent inducer of HA (Bedu-Addo *et al*., 2008; Gu *et al*., 2004; review Costello *et al*., 2009), both stimulates release of stored Ca^{2+} and raises pHi (Ishida and Honda, 1993; Grimaldi *et al*., 2001; Navarro *et al*., 2007; Bhaskar *et al*., 2008; Chang and Suarez, 2011; Alasmari *et al*., 2013). However, equivalent cytoplasmic alkalinization induced with NH_4Cl or trimethylamine hydrochloride fails to cause HA and pharmacological block of CatSper does not inhibit 4-AP-induced HA Alasmari *et al*., (2013). Therefore it is unlikely that the increase in pHi produced by 4-AP explains its effect and we can conclude that it stimulates human sperm HA primarily or fully through its action on stored Ca^{2+}.

A key question therefore is: do functional defects occur in the sperm Ca^{2+}-signalling apparatus that prevent regulation of HA and

cause sperm dysfunction? To investigate this a panel of donors and sub-fertile patients were screened for HA and changes in intracellular Ca^{2+} in response to targeted agonists namely (i) NH_4Cl (pH-induced activation of flagellar CatSper channels); (ii) progesterone (direct activation of flagellar CatSper channels, potentially enhancing the mobilization of stored Ca^{2+} by calcium-induced calcium release (CICR) Harper *et al*., 2004) and (iii) 4-AP (raises pHi (similarly to NH_4Cl) and mobilizes stored Ca^{2+} in the neck/midpiece of human sperm). 3-Isobutyl-1-methylxanthine (IBMX) was used to stimulate HA via the cAMP pathway which does not induce an immediate calcium influx in human sperm cells (Strunker *et al*., 2011). Additionally, the relationship between HA (spontaneous and induced), intracellular stimulus-induced Ca^{2+} responses and fertilization rates at IVF was investigated. The primary aims of this study were to examine (i) the incidence of defects in the Ca^{2+}-signalling pathways that mediate HA in sperm from donors and from sub-fertile patients; (ii) if these defects (assessed by measuring HA/calcium signalling) were related to IVF success.

Materials and Methods

Reagents

4-AP (Sigma Aldrich, Catalog number 275875-5G, UK), progesterone (Sigma Aldrich, Catalog number P8783-5G, UK), IBMX (Calbiochem, Catalog number 410957, UK) and ammonium chloride (NH_4Cl) (Sigma Aldrich, Catalog number 4316230J, UK) were dissolved in distilled water, ethanol, dimethyl sulphoxide (DMSO) and distilled water, respectively. Aliquots were diluted and added to the sperm suspensions to achieve a final concentration of 2 mM, 3.6 μM, 100 μM and 25 mM, respectively, volumes were chosen so that the maximum concentration of solvent was 1% (v/v). Fura-2 acetoxymethyl ester (Fura-2/AM) (Molecular Probes, Invitrogen, OR, USA) was dissolved in DMSO and used at a final concentration of 1 μM.

Media used for donor samples

Synthetic tubal fluid (STF; based upon Mortimer, 1986) was used as capacitating media (CM) for donor samples. It consisted of 4.7 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 106 mM NaCl, 5.6 mM D-glucose, 1.5 mM NaH_2PO_4, 1 mM Na pyruvate, 41.8 mM Na lactate, 25 mM $NaHCO_3$, 1.33 mM glycine, 0.68 mM glutamine, 0.07 mM taurine, non-essential amino acids (1: 100 dilution in STF) and 30 mg/ml bovine serum albumin. A non-capacitating HEPES-buffered medium (NCM) adapted from the above but lacking in both albumin and bicarbonate (5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 116.4 mM NaCl, 5.6 mM D-glucose, 1.0 mM NaH_2PO_4, 2.7 mM Na pyruvate, 41.8 mM Na lactate and 25 mM HEPES) was also used.

Ethical approval

Written consent was obtained from each patient in accordance with the Human Fertilisation and Embryology Authority (HFEA) Code of Practice (version 8) under local ethical approval (08/S1402/6) from the Tayside Committee of Medical Research Ethics B. Similarly, volunteer sperm donors were recruited in accordance with the HFEA Code of Practice (version 8) under the same ethical approval.

Study subjects

Semen samples were obtained from three groups: 100 semen samples from 68 young healthy research donors (mainly students with no known fertility problems, aged 20–35 and with a normal sperm concentration and motility according to WHO criteria 1999), 181 sub-fertile patients who underwent IVF (170 patients) or ICSI (11 patients) treatment at the Assisted Conception Unit (ACU), Ninewells Hospital, Dundee, Scotland, between April 2009 and August 2011.

Semen samples

Semen samples from donors and patients were collected by masturbation into a sterile plastic container after 2–3 days of sexual abstinence. The samples were used for analysis after liquefaction of the semen at 37°C for approximately 30 min and within 1 h of production. Semen samples obtained from patients were assessed for the semen profile by clinical embryologists. Patients were selected for IVF or ICSI according to clinical indications and semen quality. For the latter, although it was no't always the case, men with approximately 1×10^6 progressively motile cells post-preparation were allocated to IVF and below this limit to ICSI.

With respect to the patient samples, to eliminate inter ejaculate-variation, the surplus of the clinical sample used in the IVF or ICSI treatment process was taken for analysis of HA and where possible intracellular Ca^{2+}. IVF fertilization rates were obtained in order to examine the potential functional relationship between stimulation with agonists known to act on different components of the Ca^{2+}-signalling system and IVF.

Density gradient centrifugation

For the donors, spermatozoa were isolated from seminal plasma by density gradient centrifugation (DGC) using PureSperm (Nidacon, Molndal, Sweden) diluted with NCM. After centrifugation (300*g*, 20 min), the supernatant was discarded and pellet was washed (500*g* for 10 min) resuspended in CM, and incubated for ~2 h at 37°C in a 5% CO_2 incubator. This incubation period in CM was chosen because in general, no further notable change was observed in the percentage of spontaneous/induced HA (see Supplementary data, Fig. S1) or in agonist-stimulated intracellular Ca^{2+} level. In the ACU commercially available media was used for sperm preparation. The spermatozoa were separated from semen by DGC using PureSperm diluted with Cook Sydney IVF Gamete Buffer, a HEPES-buffered solution (Cook Sydney IVF Limited, National Technology Park, Ireland, UK). After centrifugation, the pellet was washed by centrifugation at 500*g* for 10 min in 4 ml of Cook Gamete Buffer. If the samples were assigned for IVF, following centrifugation, the supernatant was discarded and pellet resuspended in Cook Sydney IVF fertilization medium, a bicarbonate-buffered medium similar to STF, containing 25 mM $NaHCO_3$ (Moseley *et al*., 2005; data not presented). If the sample was allocated for ICSI, the cells were washed in Cook Gamete Buffer. Following this, IVF samples were gassed with CO_2 and kept at room temperature (up to 4 h) until 1 hur before the insemination, at which point the sample was incubated at 37°C in a 5% CO_2 incubator. The ICSI samples were kept at room temperature (up to 4 h) until the time of the injection procedure. Following insemination or injection the remaining portion of the sample was analysed for basal and agonist-induced HA and if there was a sufficient yield of sperm available, for intracellular Ca^{2+} (see below). ICSI samples were prepared in the IVF clinic (in Cook Gamete Buffer™ which does not support the completion of capacitation; Moseley *et al*., 2005). These samples were only assessed for intracellular Ca^{2+} and were re-suspended in CM as part of the Fura loading protocol (see below).

Assessment of basal level of HA and sperm motion characteristics by CASA

A Hamilton Thorn CEROS machine (version 12) attached to an external microscope was used to assess motion characteristics. The concentration

of prepared spermatozoa from donors or patients was adjusted between 5 and 25 × 10^6/ml with CM. Samples with a concentration ≤2 × 10^6/ml were generally excluded from the study as the number of cells was insufficient to obtain data comparable to the other samples. The samples were mixed to ensure a homogenous concentration of spermatozoa. Hamilton-Thorn 2X-Cel chambers (20 μm depth) (Dual Sided Sperm Analysis Chamber, Hamilton Thorn Biosciences, Beverly, MA, USA) were pre-warmed at 37°C on a heated stage of an Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan) after which 4 μl of sperm suspension was placed onto each slide chamber and then covered by a pre-warmed cover slip (22 mm × 22 mm). Slides were maintained at 37°C for ∼2 min prior to the start of data acquisition. For HA and motion characteristics were assessed under a negative phase contrast objective (×10) at a final magnification of ×100. Four different samples were assessed for each ejaculate and at least 200 motile cells were counted on randomly selected fields in each sample so that a minimum of approximately 800 motile cells were assessed in total. The percentage of hyperactivated cells was assessed using standard criteria to identify HA, namely VCL ≥150 μm/s, linearity ≤50%, and ALH ≥7 μm (Mortimer *et al.*, 1998).

Assessment of hyperactivated motility in response to different agonists

Agonist stimulation was achieved by adding 1 μl of agonist to 99 μl of sperm suspension, giving final concentrations of 2 mM 4-AP, 100 μM IBMX, 3.6 μM progesterone or 25 mM NH_4Cl. Sperm HA and other kinematic parameters were then assessed as described above.

Twenty-one of the 68 donors produced more than one sample. The basal and 4-AP-induced HA for donors who produced two samples is presented in Supplementary data, Fig. S1. Importantly, the HA response to 4-AP was consistent between the assessments in all 21 donors with 20/21 donors showing a normal significant response in both assessments and 1/21 showing a poor response in both samples (Supplementary data, Fig. S2a).

Fertilization rate at IVF

Oocytes were considered normally fertilized when two pronuclei (2PN) and two distinct or fragmented polar bodies were observed. In IVF, the fertilization rate (FR) was calculated from the number of oocytes normally fertilized divided by the total number of inseminated oocytes. In order to reduce the influence of minimal egg numbers the data used for fertilization rates in this analysis are only those cases where at least four mature eggs were inseminated for IVF ($n = 145$). Fertilization rates where ICSI was the designated treatment were not taken into account as ICSI bypasses any functional requirement needed for a sperm to bind and penetrate the egg vestments. The median female age of IVF patients ($n = 145$) was 34 (31–37 25 and 75th centile, respectively). The median number of eggs recovered in the IVF patients ($n = 145$) was 10.5 (8–14; 25 and 75th centile, respectively).

Measurement of intracellular Ca^{2+}

After sperm preparation (either in the research laboratory for healthy donors or at the IVF laboratory for patients), 500 μl aliquots of sperm suspension (concentration adjusted to ∼8–20 × 10^6/ml with CM) were loaded with Fura-2 by incubating them with 1 μM Fura-2-AM for 12 min at 37°C under 5% CO_2 in the dark (the DMSO concentration was 0.2% (v/v)), then centrifuged at 500*g* for 15 min. The supernatant was removed and the pellet resuspended in 100 μl of medium. Since loading with Fura-2 required a centrifugation step followed by resuspension, samples from all three groups (donors, IVF, ICSI) could be resuspended in the same medium (CM). The intracellular Ca^{2+} response is rapidly developed with capacitation (Baldi *et al.*, 1991; Bedu-Addo *et al.*, 2005). Fluorescence measurements were carried out on a FLUOstar Omega (BMG Labtech Offenburg, Germany) using alternating excitation wavelengths of 340 nm and 380 nm and recording emission at 510 nm. Stimulus-induced increments in the ratio of emission intensities (at 340 and 380 excitation) were used to quantify changes in $[Ca^{2+}]_i$ concentration. Aliquots of 50 μl were pipetted into a 96-well plate and 100 s of control data (20 readings) were acquired (resting level (*R*)). Five microlitres of agonist (4-AP or progesterone) were then added and the response was recorded. Usually, a minimum of ∼2 million cells per well (50 μl) were required for robust results. In a number of cases (primarily those involving ICSI samples) this could not be achieved and no measurement of intracellular Ca^{2+} response was reported.

To examine the consistency of different samples from the same donors, eight donors were tested on two occasions with an interval of at least 1 month between donations. Responses to progesterone were very similar between samples ($R^2 = 0.972$) (Supplementary data, Fig. S2b). One donor who showed a poor response was tested on several different occasions with consistent results (Supplementary data, Fig. S2b).

Definition of failed HA and Ca^{2+} responses among donors and sub-fertile patients

A failed HA response was recorded when agonist stimulation did not induce a significant change in the % of hyperactivated cells compared with control (basal) level (assessed by one-way analysis of variance (ANOVA) and non-parametric ANOVA on ranks Kruskal–Wallis test on four different samples from each ejaculate, $P < 0.05$).

To define failed Ca^{2+} responses a normal range was determined from the distribution of response amplitudes (agonist-induced increments in the 340/380 ratio) in donor populations stimulated with 4-AP and progesterone (Supplementary data, Fig. S3). Upper and lower limits were set to include 99% of the distribution (mean ± 2.58 × SD; see the Statistical analysis section below for normalizing procedure). Using this approach, the cut-off values for a failed Ca^{2+} response were increments in the 340/380 ratio of ≤0.1 upon addition of 4-AP or progesterone.

Statistical analysis

Normality of data was assessed according to the Kolmogorov–Smirnov test. Results are expressed as the mean ± SD (standard deviation), median and range for HA. Statistical comparisons were made using the ANOVA if the data were either originally normally distributed or normalized after transformation by square root. However, some HA and intracellular Ca^{2+} data were not normalized by transformation. Thus, the statistical comparisons for these data were examined by non-parametric ANOVA Kruskal–Wallis one-way ANOVA on ranks. The correlation between HA and intracellular Ca^{2+} in response to agonists (4-AP and progesterone) was examined using Pearson's correlation coefficient. The correlations between HA and intracellular Ca^{2+} in the basal level and in response to agonists with IVF fertilization rates were examined by Spearman's correlation coefficient (because the data were not normalized after transformation) and Pearson's correlation coefficient, respectively.

To define a cut-off value for a failed Ca^{2+} response, the data were log-transformed and cut-off values were calculated based on mean and SD. The differences in proportions of failed responders between the populations (donors and IVF patients) and between agonists were examined using a χ^2 test. $P < 0.05$ was considered significant. All statistical analyses were performed using the SigmaStat 10 statistical package (Systat Software Inc., Chicago, IL, USA).

Results

Induction of HA in donor and IVF patient samples

Of the four agonists used (4-AP, NH_4Cl, progesterone, IBMX), 4-AP was the most potent inducer of HA in sperm from donors, the magnitude of the effect being significantly greater than that of all other agonists ($P < 0.001$; Fig. 1). IBMX was the next most effective agonist, followed by progesterone (Fig. 1). As reported in detail elsewhere Alasmari *et al.* (2013), 25 mM NH_4Cl was not an effective inducer of HA (NS; Fig. 1) though in a minority of samples, primarily those with low basal HA (≤10% HA), there was a detectable response ($P < 0.05$).

The relative efficacy of the four different agonists on IVF patient samples was similar to that seen with donor samples (Fig. 1 compare left and right panels), but for the three effective stimuli (4-AP, IBMX and progesterone) the percentage of hyperactivated cells after treatment was significantly lower in IVF patients ($P < 0.05$) (Fig. 1 and Supplementary data, Fig. S4). The basal level of HA was significantly lower in IVF patients compared with donors ($P < 0.05$).

Intracellular calcium responses to 4-AP and progesterone in donors and patients

Spermatozoa from 37 donors, 68 IVF and 11 ICSI patients were assessed for their $[Ca^{2+}]_i$ responses to 4-AP and progesterone. The 340/380 ratio for fluorescence of fura-2 (R) in resting cells differed between individuals. To facilitate comparison the data from each individual were normalized to the pre-stimulus value (Fig. 2). Descriptive statistics of the raw 340/380 ratio data are presented in Supplementary data, Table SI. The basal Ca^{2+} was significantly lower in spermatozoa from ICSI patients, compared with research donors and IVF patients ($P < 0.05$) (Supplementary data, Table SI).

Both 4-AP and progesterone induced a biphasic elevation of intracellular Ca^{2+} in donor sperm, comprising a $[Ca^{2+}]_i$ transient followed by a sustained phase. However, the shape of these responses was clearly different, the peak ratio increase in the transient induced by progesterone typically being higher than that induced by 4-AP, whereas the $[Ca^{2+}]_i$ increase induced by 4-AP was sustained for longer (Fig. 2; Supplementary data, Fig. S5). Both agonists were effective in raising $[Ca^{2+}]_i$ in sperm from the two patient groups, but whereas responses in IVF patients were similar to those in donor sperm, the magnitude of the response was much smaller in ICSI patients (Fig. 2, $P < 0.05$). There was a significant difference between the ratio at the sustained phase in response to 4AP between donor and ICSI patients ($P < 0.001$) and between IVF and ICSI patients ($P = 0.007$).

Failed HA and Ca^{2+} responses among donors and sub-fertile patients

To further characterize the differences between donor and patient groups we assessed the occurrence of HA and $[Ca^{2+}]_i$ signal 'failures' (as defined in the Materials and Methods section). In response to stimulation with 4-AP only 2% of the donor samples gave a 'failed' HA response, whereas HA failure occurred in ≈10% of the IVF patients ($\chi^2 = 7.9$, $P = 0.025$). Due to practical and technical limitations it was not possible to assess intracellular Ca^{2+} for all patients but of those who could be tested a failed response was recorded in 2/61 (~3%) and 2/7 (28.6%) of IVF and ICSI patients, respectively ($P = 0.048$). In contrast, none of 37 donor responses fell below the threshold defining failure (see Materials and Methods). The two IVF patients who showed a failed intracellular Ca^{2+} response to 4-AP did not hyperactivate in response to 4AP and had poor IVF fertilization rates (39%, 6%).

Stimulation with progesterone induced a significant $[Ca^{2+}]_i$ response in >97% of samples from donors and all IVF patients. However, as with 4-AP, ICSI patients showed a higher failure rate (3/11 men, 27.3%; $P = 0.035$, cf. donors). Failure of significant HA was much more common in response to stimulation with progesterone compared with 4-AP, 51% of donors and 62% of IVF samples failing to respond. In some of the samples where there was no significant induction of HA there were still detectable effects on motility. Changes in one or more kinematic parameters (VCL, ALH or LIN) were observed in 56% (19/34 samples) of the donors who failed to hyperactivate and 40% (23/57) of the IVF patients. Twenty of 65 (31%) of IVF samples screened for both HA and intracellular Ca^{2+} showed a normal Ca^{2+} response to progesterone but no significant change in HA or kinematics as measured by CASA.

In most cases failure of HA was not stimulus-specific, the majority of samples that showed a failed HA response to 4-AP showing no significant response to IBMX, progesterone or NH_4Cl (Supplementary data, Table SII).

Correlation between HA and intracellular $[Ca^{2+}]_i$

To determine whether or not there was a relationship between the magnitude of agonist-induced intracellular Ca^{2+} elevation (absolute increase in 340/380 ratio) and HA (absolute increase % cells), we examined responses in samples from IVF patients to 4-AP ($n = 57$) and progesterone ($n = 65$). There was a significant correlation for responses induced by 4-AP ($R = 0.35$, $P = 0.009$). Figure 3 shows data from four representative IVF patient samples upon stimulation with 4-AP. There was no significant relationship between the Ca^{2+} and HA responses induced by progesterone ($R_s = -0.002$, $P = 0.98$), an observation consistent with the great disparity in $[Ca^{2+}]_i$ and HA responses seen with this agonist.

Relationship between agonist-induced HA, $[Ca^{2+}]_i$ and IVF fertilization rates

To assess the clinical significance of $[Ca^{2+}]_i$ and HA responses, basal and 4-AP-induced $[Ca^{2+}]_i$ and HA in cells from IVF patients were examined in relation to fertilization rates. Analysis of $[Ca^{2+}]_i$ data showed a significant relationship between basal intracellular Ca^{2+} (340/380 ratio before stimulation) and fertilization rates ($R = 0.3$, $P = 0.025$). Additionally, there was a significant relationship between the increment in $[Ca^{2+}]_i$ induced by 4-AP (52 patients) and progesterone (57 patients) and fertilization rates ($R = 0.28$, $P = 0.047$, $R = 0.280$, $P = 0.037$, respectively).

Fertilization rate was significantly related both to the basal level of HA ($R_s = 0.19$; $P = 0.02$) and to the 4-AP-induced increment in HA ($R_s = 0.18$, $P = 0.029$) (Fig. 4A). When the data were separated into four groups according to the fertilization rates achieved, FR1

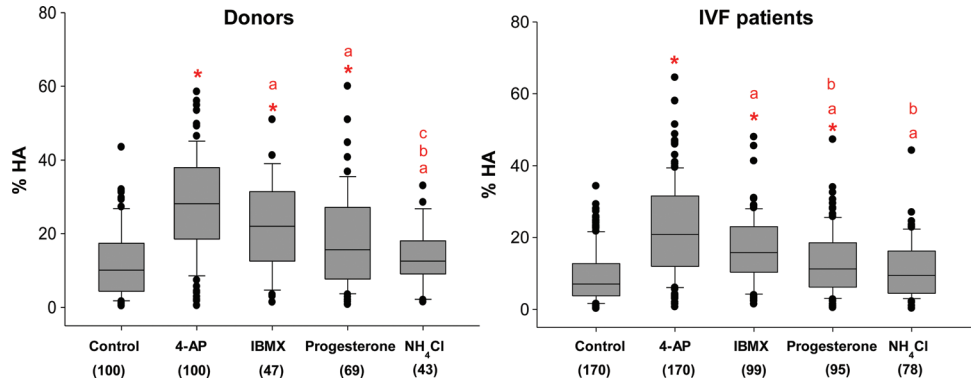

Figure 1 Comparison of four different agonists on HA) in two populations (donors and IVF patients). Box and whisker plots illustrating the data distribution for HA in the baseline (control) and samples treated with 4-AP, IBMX, progesterone and NH_4Cl from donors and IVF patients. The boxes represent the interquartile range and lines within them are the medians. The number in brackets is the sample size. *Highlights that the agonist-induced HA is significantly different to baseline, (**a**) highlights a significant difference between responses to 4-AP and all other agonists (**b**) highlights a significant difference between responses to IBMX compared with progesterone and NH_4Cl and (**c**) highlights a significant difference between responses to progesterone and NH_4Cl. Significance was considered as $P < 0.05$ assessed by one-way ANOVA and non-parametric ANOVA on ranks Kruskal–Wallis test. Not all samples were tested with each of the agonists.

≤25%, FR2 25%–50%, FR3 50–75%, FR4 >75%, it was clear that sperm samples giving the highest fertilization rate were more likely to exhibit a large increment in HA when stimulated with 4-AP (Fig. 4B). Incidence of failure to respond to 4-AP was similarly related to fertilization success. There was no significant relationship between fertilization rates and increment in HA in response to progesterone ($n = 84$) or to the other agonists IBMX ($n = 89$) and NH_4Cl ($n = 68$) (data not shown).

Discussion

The primary aims of this study were to examine the prevalence of defects in Ca^{2+}-signalling pathways mediating HA among donors and sub-fertile patients and the significance of such defects for IVF success. The most effective inducer of HA, in both donors and IVF patients, was 4-AP and this compound was therefore the focus of this study. Failure of HA in response to stimulation with 4-AP was significantly more common in IVF patients than in donors and failure of Ca^{2+} signalling was a common observation in ICSI patients. Importantly, both 4-AP-induced intracellular Ca^{2+} responses and consequent induction of HA were significantly related to IVF fertilization rate. It has been established previously that there are differences in HA (spontaneous and in response to physiological or artificial stimulants) between men with proven fertility and sub-fertile patients (Burkman, 1984; Tesarik *et al.*, 1992; Peedicayil *et al.*, 1997; Munire *et al.*, 2004). The data reported here document, for the first time, the biological significance of responses to direct, targeted manipulation of the Ca^{2+}-signalling apparatus in human spermatozoa.

This study not only employed a large sample size (compared with other clinical studies on HA and $[Ca^{2+}]_i$ signalling) but responses to agonist stimulation were also assessed in samples that were used for IVF treatment, permitting direct comparison of functional responses with fertilization success. In achieving this it was necessary to analyse the IVF samples in keeping with the clinical protocols. We believe this had a minimal effect on the results as (i) the donors and IVF samples were prepared using similar techniques and in comparable media supporting capacitation (Supplementary data, Fig. S6, Moseley *et al.*, 2005) and (ii) though IVF samples were incubated in CM for longer than donor samples, preliminary experiments showed that 4--AP-induced HA was not modified by varying incubation time (2–4 h) under capacitating conditions (Supplementary data, Fig. S1). (iii) Though ICSI samples were prepared and incubated in non-capacitating conditions in the IVF laboratory, transfer to capacitating conditions restores $[Ca^{2+}]_i$ responses within minutes (Bedu-Addo *et al.*, 2005) and as such calcium assessments are robust and allow comparison between donors, IVF and ICSI patients. The robust nature of the data reported here is illustrated by the consistency in agonist-induced $[Ca^{2+}]_i$ and HA responses between ejaculates (Supplementary data, Fig. S2).

Differing potency of the agonists used

4-AP was the most effective inducer of HA in both donor and IVF cells. Cytoplasmic alkalinization by 25 mM NH_4Cl is equivalent to the effect on pHi of 2 mM 4-AP Alasmari *et al.* (2013). NH_4Cl induced Ca^{2+} influx under both capacitating and non-capacitating conditions (data not presented), yet there was negligible effect on HA. These data confirm the pivotal role of stored Ca^{2+} in HA of human sperm and demonstrate that Ca^{2+}-influx induced by elevated pHi alone is not sufficient to induce robust levels of HA (Fig. 1). Progesterone induced an intracellular calcium response in >97% of donors and all IVF patients yet HA was weak. It has been suggested that the large progesterone-induced $[Ca^{2+}]_i$ transient (up to 40 s) is accompanied by a burst of HA in some cells (Gakamsky *et al.*, 2009; Servin-Vences *et al.*, 2012). This brief effect is hard to detect using standard CASA and in our study HA was not significantly different between 1 and 5 min of treatment with progesterone (data not presented). Thus, Ca^{2+} influx (through CatSper) induced by progesterone, similar to cytoplasmic alkalinization, was not sufficient to induce sustained

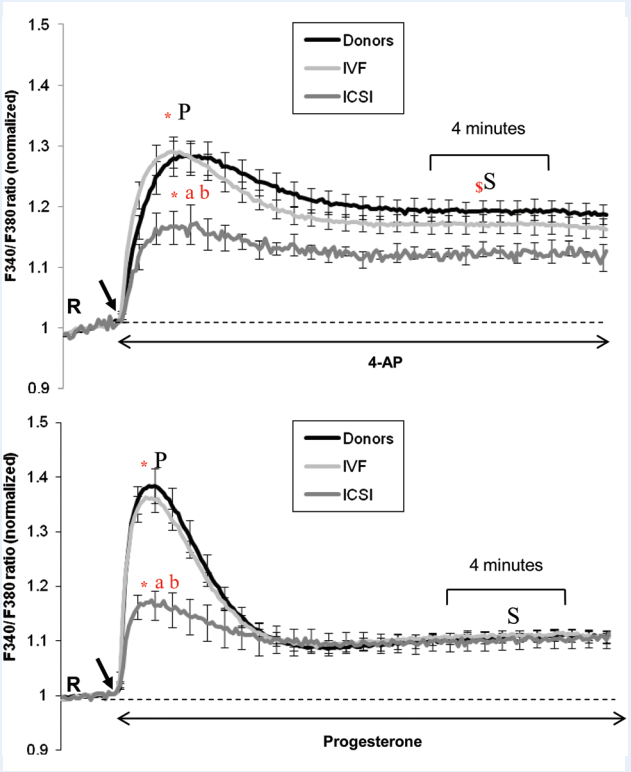

Figure 2 Ca^{2+} ratio in response to 4-AP and progesterone in three populations (donors, IVF and ICSI patients). Intracellular Ca^{2+} responses induced by 4-AP (upper panel) and progesterone (lower panel) in donors (4-AP $n = 36$, progesterone $n = 37$), IVF (4-AP $n = 61$, progesterone $n = 68$) and ICSI patients (4-AP $n = 7$, progesterone $n = 11$). Each trace shows mean of n fluorimetric (population) responses ± SE. Agonists were added (indicated by black arrow) at 100 s after acquisition of 20 readings at resting level (R). The data for each sample were normalized to pre-stimulus (R) level to facilitate comparison. *Significant difference between the ratio at the peak (P) and the initial resting level (R), (**a**) significant difference of ratio at peak between donor and ICSI patients, (**b**) significant difference of ratio at peak between IVF and ICSI patients. $^{\$}$Significant difference between the ratio at the sustained phase (S) between donor and ICSI patients ($P < 0.001$) and between IVF and ICSI patients ($P = 0.007$). There was no significant difference in the sustained response with progesterone between the groups. Significance was considered as $P < 0.05$ assessed non-parametric ANOVA on ranks Kruskal–Wallis test.

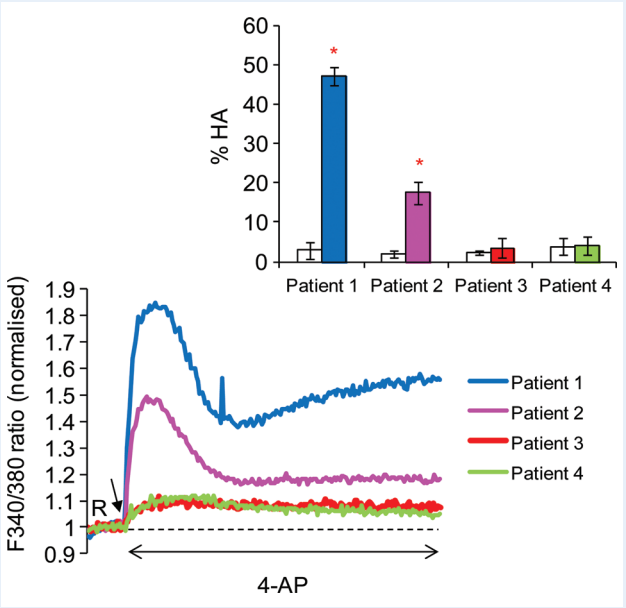

Figure 3 Intracellular Ca^{2+} in response to 4-AP from four IVF patients including two with a failed Ca^{2+} response. Inset shows % HA from the same patients labelled with the same colours; white bars show % HA in the baseline (control) and coloured bars show % HA in samples treated with 4-AP. Patients 3 and 4 showed a failed intracellular Ca^{2+} and HA responses to 4-AP (IVF fertilization rates for patients 3 and 4–39% and 6% respectively). 4-AP was added to suspensions at 100 s after acquisition of 20 readings at resting level (R) indicated by black arrow. The HA data (inset) are the mean ± SD. *Values are significantly different to baseline ($P < 0.05$).

changes in kinetic parameters and consistently reach the threshold criteria to identify HA. While CatSper channels are essential for HA in mice (Ren *et al.*, 2001; Carlson *et al.*, 2003; Quill *et al.*, 2003), their role in humans remains largely unknown (Brenker *et al.*, 2012). Genetic studies have indicated that mutations in human CatSper channels are significant in rare cases of male infertility (Avidan *et al.*, 2003; Avenarius *et al.*, 2009), but HA was not examined. Our observations clearly do not preclude involvement of CatSper in human HA. CatSper may become activated during capacitation, supporting spontaneous HA Alasmari *et al.* (2013) and when strongly activated (as occurs during the progesterone transient) CatSper may trigger activation of Ca^{2+} stores or store-operated channels (Lefievre *et al.*, 2012). It is also possible that more sophisticated sperm function tests may be required to determine the effect of direct CatSper activation such as penetration into a viscous media (Ivic *et al.*, 2002) or more sophisticated analysis of flagella motion—3-dimensional tracking (Su *et al.*, 2012) and high-speed videomicroscopy (Kirkman-Brown and Smith 2011; Curtis *et al.*, 2012).

IBMX was used to stimulate HA by strong activation of the cAMP pathway, which does not induce an immediate Ca^{2+} influx in human sperm cells (Strunker *et al.*, 2011). The majority of the samples from donors and IVF patients responded to IBMX with a significant increase in HA, consistent with other studies on human sperm, which have reported a specific influence of phosphodiesterase inhibitors such as pentoxyfilline on the kinematic parameters defining HA (Tesarik *et al.*, 1992; Kay *et al.*, 1993; Tournaye *et al.*, 1994). These observations are in contrast to other studies on bull sperm which have documented that HA occurred upon elevation of intracellular Ca^{2+} and did not respond to the AC/cAMP/PKA-signalling pathway (Ho *et al.*, 2002; Marquez and Suarez, 2004, 2008). The difference in findings between studies on human and bull sperm may simply reflect species variation in the requirements for HA.

Differences in HA and calcium between donor and patient groups

4-AP-induced HA was correlated with the amplitude of the $[Ca^{2+}]_i$ response, confirming that 4-AP-induced HA was mediated by this

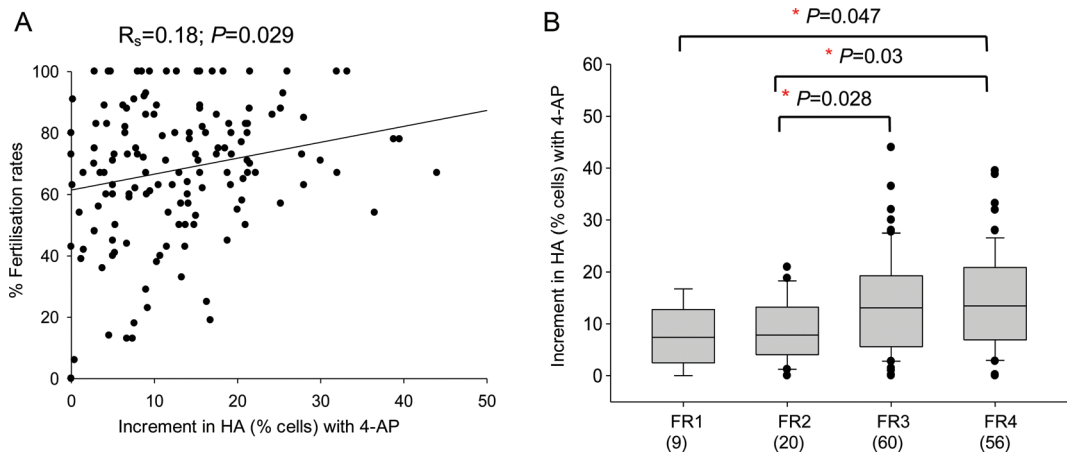

Figure 4 Relationship between increment in HA (% cells) stimulated by 4-AP and IVF fertilization rate. (**A**) 4-AP-induced increment in HA (% cells) was significantly correlated to fertilization rate ($R_s = 0.18$; $P = 0.031$, $n = 145$). (**B**) Expression of these data in four defined groups according to fertilization rate: FR 1 ≤25%, FR2 >25–≤50%, FR3 >50–≤75%, FR4 >75%. Box and whisker plots show the 4-AP-induced increment in HA for the samples from patients in each group. The boxes represent the inter-quartile range and lines within them are the medians. The number in brackets is the sample size. Significance was assessed by one-way ANOVA.

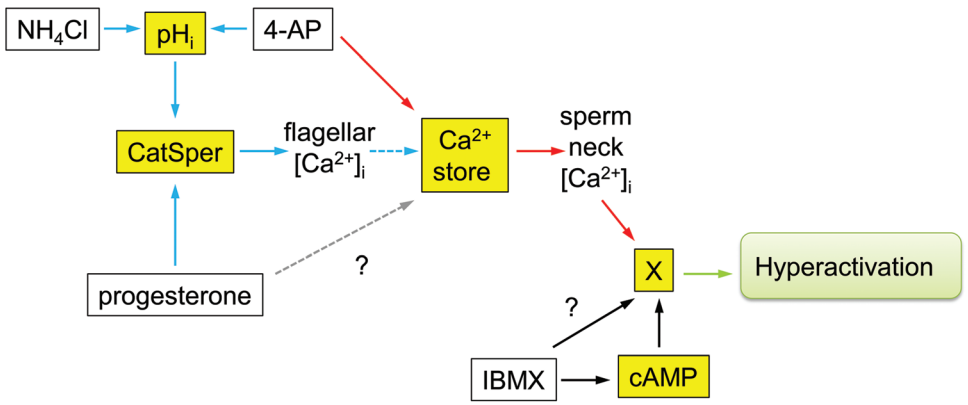

Figure 5 Model of the calcium-signalling cascade in the human spermatozoon: key points in the pathway affected in sub-fertile men (adapted from Barratt and Publicover, 2012). White boxes show compounds used to manipulate pH_i (NH_4Cl, 4AP), stored calcium (4AP) and cAMP (IBMX). Yellow boxes show signalling components activated by these compounds (pHi, Ca^{2+}, cAMP). Blue and red arrows show Ca^{2+}-signalling pathways involving CatSper and the calcium store, respectively. The Ca^{2+}-store pathway may involve activation of SOCs (not shown; Lefievre *et al.*, 2012). Dashed blue arrow shows the mobilization of stored Ca^{2+} downstream of CatSper activation by CICR. This occurs in a minority of the cells, is sensitive to modulation (e.g. by capacitation) and is responsible for CatSper-mediated hyperactivation. Dashed grey arrow shows possible modulation of Ca^{2+}-store mobilization by progesterone through CatSper-independent mechanisms (Sagare-Patil *et al.*, 2012). X represents unknown target mechanism/pathway whereby following mobilization of stored Ca^{2+} hyperactivation is stimulated. ? represents potential pathway subject to further experimentation. The amplitude of the calcium transient (stimulated by both 4AP and progesterone) and the hyperactivation response (to 4AP) were significantly related to IVF fertilization rates suggesting the occurrence of important abnormalities in the Ca^{2+}-signalling pathways mediated by CatSper (blue) and the calcium store (red). The calcium signal (4AP and progesterone) in the ICSI patients was significantly lower than in the donors or the IVF patients (~25%; Fig. 2), providing further evidence of abnormalities in the CatSper functioning/operating complex and calcium store in male infertility. Examination of individual cases demonstrated ~10% of men undergoing IVF had defective calcium hyperactivation. Although the data are limited, a number of these men did not show a hyperactivation response to IBMX (probably through cAMP, black arrows, ?). Previous studies have indicated that, in humans, increases in cAMP are do not lead to changes in $[Ca^{2+}]_i$ (Brenker *et al.*, 2012), thus these men may suffer from a specific defect in hyperactivation as opposed to a Ca^{2+}-signalling deficit (Supplementary data, Table SII).

pathway. The proportion of samples in which 4-AP failed to induce normal HA and $[Ca^{2+}]_i$ responses was significantly greater in IVF samples than in donors showing that malfunction of the Ca^{2+}-signalling pathway activated by 4-AP is likely to be responsible for a proportion of cases of male-factor sub-fertility. Additionally, there was a clear difference in $[Ca^{2+}]_i$ responses between ICSI patients and other

populations (donors and IVF patients) demonstrating increased incidence of $[Ca^{2+}]_i$ abnormalities in the former. Interestingly, the basal Ca^{2+} was significantly lower in spermatozoa from ICSI patients (Supplementary data, Table SI; $P < 0.05$). This suggests that these cells have a reduced ability to initiate the Ca^{2+}-signalling cascade. The reasons for this are unclear but may be due to (i) low expression or abnormalities in CatSper leading to reduction in Ca^{2+} influx across the plasma membrane and consequently poor recruitment of intracellular Ca^{2+} stores and/or (ii) the sperm cytoplasm does not become sufficiently alkalinized to activate CatSper due to abnormality in the expression or regulation of HV1; and/or (iii) defects in internal store channels such as RyRs, IP3R or SOCs.

Samples that gave a failed HA response to 4-AP usually failed to respond to IBMX, progesterone or NH_4Cl. Though the lower efficacy of these stimuli as inducers of HA (particularly progesterone and NH_4Cl) makes interpretation complex, it appears many HA failures involve a signalling node or late stage in a cascade such that alternative stimulation (such as increasing cAMP) does not bypass the lesion (Fig. 5). HA failure may reflect either failure of Ca^{2+} signalling itself or of events downstream of Ca^{2+}, independent of the $[Ca^{2+}]_i$ signal amplitude. Analysis of HA and $[Ca^{2+}]_i$ in 4-AP-stimulated cells showed that the amplitudes of the two responses were significantly correlated and that in those samples where $[Ca^{2+}]_i$ signal failure occurred (≈3%), there was also a failure of HA. Thus it is likely that failed HA in sub fertile men can reflect failure of Ca^{2+} signalling. Potential defects include impaired capacitation, which may affect activity of CatSper channels (Lishko *et al.*, 2011; Strunker *et al.*, 2011), resulting in a low resting $[Ca^{2+}]_i$ and consequent effects on emptying and filling of the internal stores. It has been suggested that Src kinase located in the neck/midpiece and in the post-acrosomal region of human sperm head regulates Ca^{2+} store mobilization during capacitation (Varano *et al.*, 2008). Further investigation of Ca^{2+} store filling and mobilization in sperm is required to clarify this.

Stimulus-induced $[Ca^{2+}]_i$ elevation, HA and fertilization success

Fertilization rate of the IVF samples was correlated with basal HA, a finding that is concordant with data from other studies (Burkman, 1984; Wang *et al.*, 1993; Sukcharoen *et al.*, 1995). More significantly, the ability of 4-AP to raise $[Ca^{2+}]_i$ and to induce HA was correlated significantly with IVF outcomes, providing clear evidence of a biological role of the sperm Ca^{2+}-signalling apparatus (almost certainly store mobilization; see above) in human sperm (Fig. 4). Amplitude of the $[Ca^{2+}]_i$ transient induced by progesterone, probably reflecting activation of CatSper (Lishko *et al.*, 2011; Strünker *et al.*, 2011), was similarly correlated to fertilization rates at IVF, consistent with other studies (Krausz *et al.*, 1996). However, progesterone was not a potent inducer of HA (Fig. 1) and targeted activation of CatSper is not sufficient to induce HA in human sperm Alasmari *et al.* (2013), thus there was no significant correlation between progesterone-induced HA and IVF success. Defective Ca^{2+} responses to progesterone were not seen in IVF patients and occurred in one donor and three ICSI patients. This low incidence of functionally significant CatSper defects, as manifested by failed $[Ca^{2+}]_i$ responses, suggests that such failures are relatively rare. Patch clamping studies will be required to confirm abnormalities in CatSper function (Kirichok and Lishko, 2011; Lefievre *et al.*, 2012).

In summary, this study highlights the biological role and importance of Ca^{2+}-signalling (and neck/midpiece Ca^{2+} store mobilization) for fertilization at IVF and identifies for the first time, the incidence of store defects among donors, IVF and ICSI patients. Further studies are required to investigate the nature of these defects. Such studies will act as a platform for a potential drug-based screening programme to augment/modulate calcium mobilization as a possible rational treatment for sperm dysfunction.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

W.A. performed the sperm function assays, prepared the cells for analysis, analysed the initial data and wrote, the first draft of the manuscript. S.K.O. was involved in the experimental design and interpretation of data. C.L.R.B., S.J.P. and V.K. designed the study and obtaining funding for the experiments. S.M.D.S., V.K. and S.K.O. were involved in the recruiting and consenting of patients. E.F. screened some patients for the HA assay. K.M.W. was involved in the IVF, experimental design and delivery of ART samples. C.L.R.B. and S.J.P. were responsible for and wrote the final drafts of the manuscript. All authors contributed to the editing of the manuscript.

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Conflict of interest

None declared. The Excel spread sheets with all the primary data are available from c.barratt@dundee.ac.uk.

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Paper 19:

Williams HL, Mansell S, Alasmari W, Brown SG, Wilson SM, Sutton KA, Miller MR, Lishko PV, Barratt CL, Publicover SJ, Martins da Silva S. Specific loss of CatSper function is sufficient to compromise fertilizing capacity of human spermatozoa. *Hum Reprod.* 2015 **30**:2737-46.

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human reproduction **ORIGINAL ARTICLE** ***Andrology***

Specific loss of CatSper function is sufficient to compromise fertilizing capacity of human spermatozoa

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STUDY QUESTION: Are significant abnormalities of CatSper function present in IVF patients with normal sperm concentration and motility and if so what is their functional significance for fertilization success?

SUMMARY ANSWER: Sperm with a near absence of CatSper current failed to respond to activation of CatSper by progesterone and there was fertilization failure at IVF.

WHAT IS KNOWN ALREADY: In human spermatozoa, Ca^{2+} influx induced by progesterone is mediated by CatSper, a sperm-specific Ca^{2+} channel. A suboptimal Ca^{2+} influx is significantly associated with, and more prevalent in, men with abnormal semen parameters, and is associated with reduced fertilizing capacity. However, abnormalities in CatSper current can only be assessed directly using electrophysiology. There is only one report of a CatSper-deficient man who showed no progesterone potentiated CatSper current. A CatSper 2 genetic abnormality was present but there was no information on the $[Ca^{2+}]_i$ response to CatSper activation by progesterone. Additionally, the semen samples had indicating significant abnormalities (oligoasthenoteratozoospermia) multiple suboptimal functional responses in the spermatozoon. As such it cannot be concluded that impaired CatSper function alone causes infertility or that CatSper blockade is a potential safe target for contraception.

STUDY DESIGN, SIZE, DURATION: Spermatozoa were obtained from donors and subfertile IVF patients attending a hospital assisted reproductive techniques clinic between January 2013 and December 2014. In total 134 IVF patients, 28 normozoospermic donors and 10 patients recalled due to a history of failed/low fertilization at IVF took part in the study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Samples were primarily screened using the Ca^{2+} influx induced by progesterone and, if cell number was sufficient, samples were also assessed by hyperactivation and penetration into viscous media. A defective Ca^{2+} response to progesterone was defined using the 99% confidence interval from the distribution of response amplitudes in normozoospermic donors. Samples showing a defective Ca^{2+} response were further examined in order to characterize the potential CatSper abnormalities. In men where there was a consistent and robust failure of calcium signalling, a direct assessment of CatSper function was performed using electrophysiology (patch clamping), and a blood sample was obtained for genetic analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 101/102 (99%) IVF patients and 22/23 (96%) donors exhibited a normal Ca^{2+} response. The mean ($\pm$ SD) normalized peak response did not differ between donors and IVF patients (2.57 ± 0.68 [$n = 34$ ejaculates from 23 different donors] versus 2.66 ± 0.68 [$n = 102$ IVF patients], $P = 0.63$). In recall patients, 9/10 (90%) showed a normal Ca^{2+} response. Three men were initially identified with a defective Ca^{2+} influx. However, only one (Patient 1) had a defective response in repeat semen samples.

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Electrophysiology experiments on sperm from Patient 1 showed a near absence of CatSper current and exon screening demonstrated no mutations in the coding regions of the CatSper complex. There was no increase in penetration of viscous media when the spermatozoa were stimulated with progesterone and importantly there was failed fertilization at IVF.

LIMITATIONS, REASONS FOR CAUTION: A key limitation relates to working with a specific functional parameter (Ca^{2+} influx induced by progesterone) in fresh sperm samples from donors and patients that have limited viability. Therefore, for practical, technical and logistical reasons, some men (~22% of IVF patients) could not be screened. As such the incidence of significant Ca^{2+} abnormalities induced by progesterone may be higher than the ~1% observed here. Additionally, we used a strict definition of a defective Ca^{2+} influx such that only substantial abnormalities were selected for further study. Furthermore, electrophysiology was only performed on one patient with a robust and repeatable defective calcium response. This man had negligible CatSper current but more subtle abnormalities (e.g. currents present but significantly smaller) may have been present in men with either normal or below normal Ca^{2+} influx.

WIDER IMPLICATIONS OF THE FINDINGS: These data add significantly to the understanding of the role of CatSper in human sperm function and its impact on male fertility. Remarkably, these findings provide the first direct evidence that CatSper is a suitable and specific target for human male contraception.

STUDY FUNDING/COMPETING INTEREST(S): Initial funding was from NHS Tayside, Infertility Research Trust, TENOVUS, Chief Scientist Office NRS Fellowship, the Wellcome Trust, University of Abertay. The majority of the data were obtained using funding from a MRC project grant (# 4190). The authors declare that there is no conflict of interest.

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Key words: CatSper / male fertility / sperm motility / ion channels / calcium stores / electrophysiology / failed fertilization / contraception / unexplained infertility / sperm dysfunction

Introduction

Whilst sperm dysfunction is the single most common cause of infertility (Hull *et al.*, 1985; Irvine, 1998), currently there is no drug a man can take or that can be added to his sperm *in vitro* to improve fertility (Tardif *et al.*, 2014). A fundamental problem with developing new therapies has been the limited understanding of the physiological workings of the normal and dysfunctional spermatozoon (Barratt, 2010; Aitken and Henkel, 2011; Barratt *et al.*, 2011; Aitken and Nixon, 2013).

However, during the last 8 years major progress in our understanding has been achieved through application of the patch clamp technique (Kirichok *et al.*, 2006). This has included the characterization of the proton (H_V1) and chloride channels (Lishko *et al.*, 2010; Orta *et al.*, 2012) and the identification and characterization of the potassium conductance underlying the regulation of sperm membrane potential (Santi *et al.*, 2010; Mannowetz *et al.*, 2013; Brenker *et al.*, 2014; Mansell *et al.*, 2014). Ca^{2+} signalling is particularly important in the regulation of sperm function (Publicover *et al.*, 2007) and potentially the most significant outcome has been the characterization of the sperm-specific Ca^{2+} channel CatSper (Ren *et al.*, 2001). CatSper-null sperm show no detectable Ca^{2+} current, defective calcium signalling, ATP depletion and abnormalities in motility (including hyperactivation; HA). CatSper knockout mice are consequently infertile (Carlson *et al.*, 2003; Jin *et al.*, 2007).

In humans, the importance of CatSper was emphasized by the demonstration that the rapid influx of calcium into the spermatozoon upon interaction with progesterone (and other substances such as prostaglandins) is via CatSper (Lishko *et al.*, 2011; Strünker *et al.*, 2011; Brenker *et al.*, 2012). Patch clamp studies have revealed that CatSper is weakly voltage sensitive and progesterone strongly potentiates CatSper currents while shifting the channel half activation voltage to a less depolarized potential (Lishko *et al.*, 2011; Strünker *et al.*, 2011). This progesterone induced, CatSper-mediated Ca^{2+} influx stimulates the acrosome reaction, either directly and/or by 'priming' the cell and also regulates motility (Alasmari *et al.*, 2013b; Tamburrino *et al.*, 2014). Several clinical studies have shown that suboptimal $[Ca^{2+}]_i$ signalling in response to progesterone is associated with oligoasthenoteratozoospermia, and with reduced fertilization at IVF (Falsetti *et al.*, 1993; Shimizu *et al.*, 1993; Oehninger *et al.*, 1994; Krausz *et al.*, 1995). The occurrence of poor $[Ca^{2+}]_i$ responses to agonists of CatSper and to mobilization of stored Ca^{2+} is related to IVF fertilization rates (Alasmari *et al.*, 2013a). Release of stored Ca^{2+} is more potent than CatSper activation in inducing HA and secondary release of stored Ca^{2+}, downstream of CatSper activation, is likely to be required for HA (Alasmari *et al.*, 2013a). In summary, the clinical data indicate that defects in $[Ca^{2+}]_i$ signalling do occur and that these affect IVF fertilizing capacity.

Significant functional differences between the spermatozoa of mice and men, including regulation of motility by Ca^{2+}-signalling (Alasmari *et al.*, 2013b), are such that findings cannot simply be applied to humans. Equivalent human studies rely upon the identification of 'natural knockouts', subfertile men with specific lesions of key genes. However, case reports of CatSper genetic abnormalities are rare. Recently, Smith *et al.* (2013) showed that sperm from a CatSper 2 deficient man had no progesterone potentiated CatSper current; consistent with the idea that CatSper is the primary channel responsible for Ca^{2+} influx. However, there was no information on the Ca^{2+} influx induced by progesterone. Further, the semen samples in this study had multiple abnormalities suggesting defects at additional loci and multiple suboptimal functional responses in the spermatozoon (Avidan *et al.*, 2003; Zhang *et al.*, 2007; Avenarius *et al.*, 2009; Bhilawadikar *et al.*, 2013). Though important, these studies do not establish either that the specific cause of infertility in these men was failure of CatSper or that inactivation of CatSper is sufficient to prevent/reduce fertilization in humans. Furthermore, they raise the possibility that loss of CatSper function affects spermatogenesis in humans, which would greatly reduce the potential suitability of CatSper as a male contraceptive target (Ren *et al.*, 2001).

Therefore, the primary objective of this study was to examine whether significant abnormalities of CatSper function can be identified in IVF patients and assess their functional significance.

Materials and Methods

Experimental design

Abnormalities in CatSper current can only directly be assessed using electrophysiology; however, this is a low-throughput technique. Therefore, to identify men with potential CatSper abnormalities for electrophysiology, we devised a combination of screening methods to examine calcium signalling. The primary assay was the Ca^{2+} influx induced by progesterone followed by penetration into viscous media and HA (Alasmari *et al.*, 2013a,b). In total 134 IVF patients, 28 donors and 10 patients recalled due to a history of low/failed fertilization at IVF took part in the study. The focus was examination of Ca^{2+} influx but samples were allocated to the other assays if cell number was sufficient. Samples showing a defective Ca^{2+} influx response were further examined in order to characterize the potential CatSper abnormalities. Direct assessment of CatSper was performed using electrophysiology where a consistent and robust failure of calcium signalling (i.e. repeatable in more than one semen sample) was found (Supplementary Fig. S1).

Patient and donor numbers

Of the 134 IVF patients, 102 were screened for calcium response to progesterone, 66 for 4-aminopyridine (4-AP)-induced HA and 16 for penetration of viscous medium. A total of 111 of these patients fertilized at IVF (average 78.6 ± 22% (1 SD)), while 9 patients had total failed fertilization, 8 had less than 4 oocytes retrieved and for 6 there were no fertilization data available.

Of the 28 donors, 23 provided one or more samples (34 ejaculates in total) for the Ca^{2+} response to progesterone, 35 ejaculates for the 4-AP-induced HA assay and 18 ejaculates for the viscous media penetration test.

All of the 10 recall patients were screened for Ca^{2+} response to progesterone and one was screened for 4-AP-induced HA and viscous medium penetration.

Ethical approval and subjects

Written informed consent was obtained from three groups: (i) patients (subfertile men who underwent assisted reproduction techniques in Ninewells Hospital, Dundee, Scotland); (ii) sperm donors (selected with no known fertility problems and normal semen parameters) (Cooper *et al.*, 2010); (iii) patients with a history of failed/low fertilization at IVF. Men were recruited in accordance with the Human Fertilisation and Embryology Authority Code of Practice (version 8) under local ethical approval (13/ES/0091) from the Tayside Committee of Medical Research Ethics B.

Semen samples

Samples from donors and patients were used as previously described (Tardif *et al.*, 2014). Patients were selected for IVF according to clinical indications and semen quality: e.g. normal sperm concentration and motility (Cooper *et al.*, 2010) and ~ 1×10^6 progressively motile cells post-preparation. The surplus of the clinical sample used in the IVF treatment process was assessed for intracellular Ca^{2+} and where possible HA and penetration into viscous media.

Media and preparation

Commercially available Quinn's Advantage Fertilization Medium (HTF) plus 5% human serum albumin (HSA) and Quinn's Sperm Washing Medium (SW) were used to prepare and capacitate sperm. Non-capacitating HEPES-buffered media (NCM) lacking in both albumin and bicarbonate (4.69 mM KCl, 2.04 mM $CaCl_2$, 0.2 mM $MgSO_4$, 97.8 mM NaCl, 2.78 mM D-Glucose, 0.33 mM Na pyruvate, 21.4 mM lactic acid and 21 mM HEPES, ~280 mOsm/kg H_2O, adjusted to pH 7.4 using 10 M NaOH) was used for sample re-suspension in the FLUOstar.

Spermatozoa were isolated by density gradient centrifugation (DGC) using Percoll/PureSperm diluted with SW for donors/patients respectively. After centrifugation (300*g*, 20 min), the supernatant was discarded and pellet was washed (500*g* for 10 min), resuspended in HTF, and incubated for a minimum of 2 h at 37°C in 5% CO_2.

Agonists and reagents

Ca^{2+} signalling was manipulated using progesterone (Lishko *et al.*, 2011; Alasmari *et al.*, 2013a) and 4-AP) (Bedu-Addo *et al.*, 2008). Reagents were purchased from Sigma Aldrich unless otherwise stated. 4-AP and progesterone were dissolved in distilled water and ethanol, respectively. Fura-2 acetoxymethyl ester (Fura-2/AM) (Molecular Probes, Invitrogen, Oregon, USA) was dissolved in dimethylsulphoxide (final concentration of 1 μM).

Measurement of intracellular Ca^{2+}

Approximately 4 million cells were prepared and assessed as previously described using a FLUOstar microplate reader (BMG Labtech Offenburg, Germany) (Alasmari *et al.*, 2013a) with minor modifications encompassing use of 0.05% Pluronic acid F127 and manganese chloride (9.1 mM). Progesterone-induced increments in the ratio of emission intensities (at 340 and 380 excitation) were used to quantify changes in $[Ca^{2+}]_i$ concentration (Alasmari *et al.*, 2013a,b). After adjustment for background fluorescence normalization was achieved by dividing each ratio value at time point × (Rx) by the mean fluorescence ratio value taken from hundreds of basal fluorescence ratio readings (Rbasal).

Assessment of HA and motility

A Hamilton Thorne CEROS computer aided sperm analysis (CASA) machine (Beverley, MA, USA) was used to assess motility for semen and prepared sperm samples (as described in Supplementary Table SI) (Alasmari *et al.*, 2013a,b). Sperm were treated with 4-AP at a final concentration of 2 mM, and the percentage of hyperactivated cells was assessed (Mortimer *et al.*, 1998).

Penetration into viscous media

This was performed as previously described (Ivic *et al.*, 2002; Alasmari *et al.*, 2013b). Methylcellulose solution was created using a 1% w/v methylcellulose in HTF media supplemented with 5% HSA. Methylcellulose solution was introduced into capillary tubes by capillary action (Vitrotubes™, Rectangle Capillaries 0.4 mm × 400 mm, VitroCom, New Jersey, USA). Prepared sperm were adjusted to ~10–20 × 10^{-6}/ml before 3.6 μM progesterone/vehicle were added to the aliquots. Following 1 h incubation at 37°C and 5% CO_2, cell numbers at 1 and 2 cm were normalized to values from parallel, untreated controls (C/C, V/C and P/C, where C = number of cells under control conditions, V = under vehicle controls and P = after treatment with progesterone).

Definition of failed HA and Ca^{2+} responses among donors and sub-fertile patients

A failed HA response was recorded when agonist stimulation did not induce a significant change in the percentage of hyperactivated cells compared with control (basal) level (assessed by paired two-tailed Student's *t*-test, $P < 0.05$). To define a defective Ca^{2+} response to progesterone, the 99% confidence interval was determined from the distribution of response amplitudes in 34 ejaculates from 23 normozoospermic donors: <0.41 and >4.85 delta response upon addition of progesterone.

Fertilization rate at IVF

Oocytes were considered normally fertilized when two pronuclei (2PN) and two polar bodies were observed. In IVF, the fertilization rate was calculated from the number of oocytes normally fertilized divided by the total number of inseminated oocytes. The fertilization rate was calculated where four or more mature oocytes (metaphase II) were present. Low fertilization was defined where <30% of four or more metaphase II oocytes were normally fertilized.

Sperm preparation for electrophysiology

Sperm were prepared, for electrophysiology using a swim-up procedure into HTF as previously described (Lishko *et al.*, 2013; Mansell *et al.*, 2014). The cells were re-suspended in capacitating media and maintained at 37°C for 4 h (5% CO_2). Capacitated cells were transferred into petri dishes at room temperature containing standard bath solution in order to allow cells to adhere to glass coverslips. Coverslips were transferred into a perfusion chamber and perfused with standard bath solution until whole-cell configuration was achieved. The biophysical properties of individual sperm were recorded under whole-cell conditions (Lishko *et al.*, 2011; Mansell *et al.*, 2014) using borosilicate glass pipettes (10–15 MOhms) filled with Cs^+-based divalent free solution. Gigaohm seals were formed on cytoplasmic droplets or on the midpiece region. Break in was achieved via light suction and 1 ms voltage pulses (450–610 mV). Flagellar beating was observed in all cells selected. In order to record CatSper, currents were recorded in Cs^+-based divalent free extracellular solution evoked by a depolarizing ramp protocol imposed (−80 to 80 mV) over 2 s and membrane potential (Vm) was held at 0 mV between test pulses.

Experimental solutions

All concentrations are in mM. Synthetic human tubular fluid (HTF): NaCl, 97.8; KCl, 4.69; $MgSO_4$; 0.2; $CaCl_2$, 2.04; HEPES, 21; Glucose, 2.78; Na-lactate 21.4; Na-pyruvate, 0.33; pH adjusted to 7.4 with NaOH. Capacitating medium: NaCl, 135; KCl, 5, $MgSO_4$, $CaCl_2$, 2; HEPES, 20; Glucose, 5; Lactic acid, 10; Na-Pyruvate, 1; $NaHCO_3$, 25; fetal bovine serum, 20%; pH adjusted to 7.4 with NaOH. Standard bath solution: NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgSO_4$,1; HEPES, 20; Glucose, 5; Na Pyruvate, 1; Na lactate, 10; pH adjusted to 7.4 with NaOH which brought [Na] to 154 mM. Non-selective cation currents flowing via spermatozoon cation channels (CatSper) were quantified using pipette (Cs-methanesulphonate, 130; HEPES, 40; Tris–HCl, 1; EGTA, 3; EDTA, 2 mM, pH adjusted to 7.4 with CsOH) and bath (Cs-methanesulphonate, 140; HEPES, 40; EGTA, 3; pH adjusted to 7.4 with CsOH) solutions devoid of Ca^{2+} and Mg^{2+} that contained Cs^+ as the principal cation.

Genetic screening for mutations in CatSper Complex loci

Blood samples were obtained from Patient 1 and processed for Exome sequencing (Centrillion Biosciences, Palo Alto, USA). Samples were selected using Agilent SureSelect Human all Exon v5-51Mb kit and yielded 1.83 Gbase, giving a raw coverage of 36X. Coverage for the CatSper Complex loci (CatSper 1-4, CatSperB, CatSperG and CatSperD) was verified. Sequences were mapped to human genome and sequences overlapping the loci of interest (Supplementary Table SII) were extracted and realigned using Geneious version 8.1.4 (http://www.geneious.com) and polymorphisms identified and manually verified.

Statistical analysis

Normality of data was assessed according to the Shapiro–Wilk test. Statistical comparisons for the viscous penetration test and induced HA were made using paired two-tailed Student's *t*-tests or the analysis of variance if the data were either originally normally distributed or normalized after transformation by arcsin or square root. To define a cut-off value for a failed Ca^{2+} response, the data were log transformed and cut-off values were calculated based on mean and SD (Alasmari *et al.*, 2013a). Unpaired *t*-tests were performed on currents obtained from whole-cell patch clamp. $P < 0.05$ was considered significant. The statistical package Prism GraphPad (La Jolla, CA, USA) was used.

Results

In total, 134 IVF patients, 28 donors and 10 patients recalled for assessment due to failed/low fertilization at IVF were included in the study. Patients and donors were screened using one or more of the three techniques depending on sample quality: Ca^{2+} response, HA and the viscous media assay.

Screening of IVF patients and donors for Ca^{2+} influx induced by progesterone

The majority of IVF patients (101/102; 99%) and donors (22/23; 95.7%) exhibited a biphasic $[Ca^{2+}]_i$ response as previously described (Alasmari *et al*, 2013a). The $[Ca^{2+}]_i$ transient amplitude was similar in cells from donors and patients attending for IVF treatment (Fig. 1A; donors = 2.57 ± 0.68 [$n = 34$ ejaculates from 23 different donors], IVF patients = 2.66 ± 0.68 [$n = 102$ patients], $P = 0.63$) but the plateau phase of the $[Ca^{2+}]_i$ response was greater in IVF patients ($P = 0.003$). In total, 9/10 patients with previous low/failed fertilization at IVF showed a normal Ca^{2+} response.

Screening of IVF patients for HA

IVF patient samples had significantly lower basal HA than donors (Fig. 1B and C, $P < 0.05$, IVF $n = 66$, donor $n = 35$). Treatment with 2 mM 4-AP robustly and significantly increased HA for both IVF patients and donors ($P < 0.001$).

Screening of IVF patients in the viscous media assay

Sperm from donors and IVF patients (15/18 donors [83%], 13/16 IVF patients [81%]) responded to progesterone with an increase in cell numbers at 1 cm ($P < 0.05$, Fig. 1D and E).

Identification of defective Ca^{2+} influx induced by progesterone

Three individuals had spermatozoa that were unable to respond to progesterone. One was from the group of 10 patients recalled due to a previous low/failed fertilization at IVF (Patient 1), one was a patient undergoing IVF treatment (Patient 2) and one was a donor (Donor 1). In each case, diagnostic semen analysis was normal (Supplementary Table SI).

Patient 1

Patient 1 had one previous cycle of IVF that resulted in a total fertilization failure for all nine oocytes retrieved. Consequently he was recalled for further analysis and provided a sample for research. He had a subsequent cycle of ICSI where 5/6 oocytes fertilized (83.3% fertilization rate). Female age was 39 years with an anti-Mullerian hormone (AMH) of

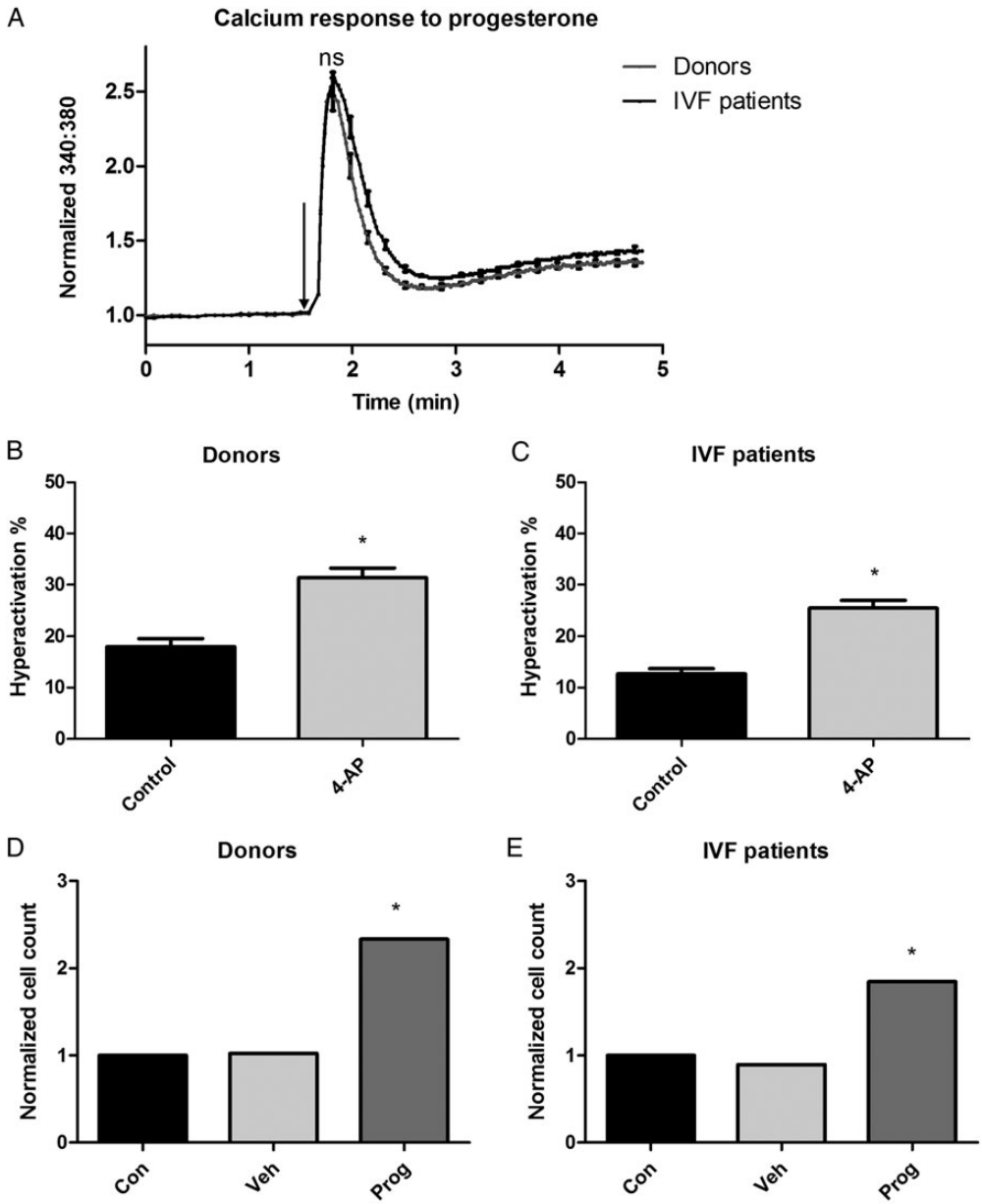

Figure 1 Calcium response to progesterone, hyperactivation in response to 4-AP and penetration into viscous media for spermatozoa from donors and IVF patients. (**A**) Peak calcium response to progesterone (progesterone addition indicated by the arrow) was not significantly different between donors and IVF patients (donor $n = 34$ ejaculates from 23 different donors, IVF $n = 102$), however, the plateau phase response (mean of the last 12 s of recording) was (donor versus IVF peak calcium response to progesterone, $P = 0.003$, Student's unpaired t-test). (**B** and **C**) 4-aminopyridine (4-AP) significantly increased hyperactivation (HA) in spermatozoa from both donors and IVF patients ($P < 0.001$ versus control, Student's paired t-test). HA assay donor $n = 35$, IVF $n = 66$. (**D** and **E**) Penetration into viscous media was higher after stimulation with progesterone for both donors and IVF patients (progesterone versus control, donor $n = 18$, $P < 0.05$; IVF $n = 16$, $P < 0.05$, one-way analysis of variance). Asterisk denotes statistical significance at the $P < 0.05$ level.

10 pmol/l. Semen analysis was normal (concentration of 66 $\times$ 10^6/ml and motility of 60%, Supplementary Table SI).

In the first sample, the spermatozoa were unable to produce a Ca^{2+} influx induced by progesterone (Fig. 2A), though donor sperm tested at the same time and under identical conditions (positive control) gave a normal biphasic response. Furthermore, there was an abnormal response to progesterone in the viscous media assay (Fig. 2D). Stimulation of capacitated cells with 4-AP induced a significant increase in HA ($P < 0.001$, Fig. 2B).

Another sample submitted 7 weeks later showed the same result (Fig. 2A shows the average trace from four assays, two aliquots from the initial sample and two from second) and thus the cells were studied using patch clamp electrophysiology (see below).

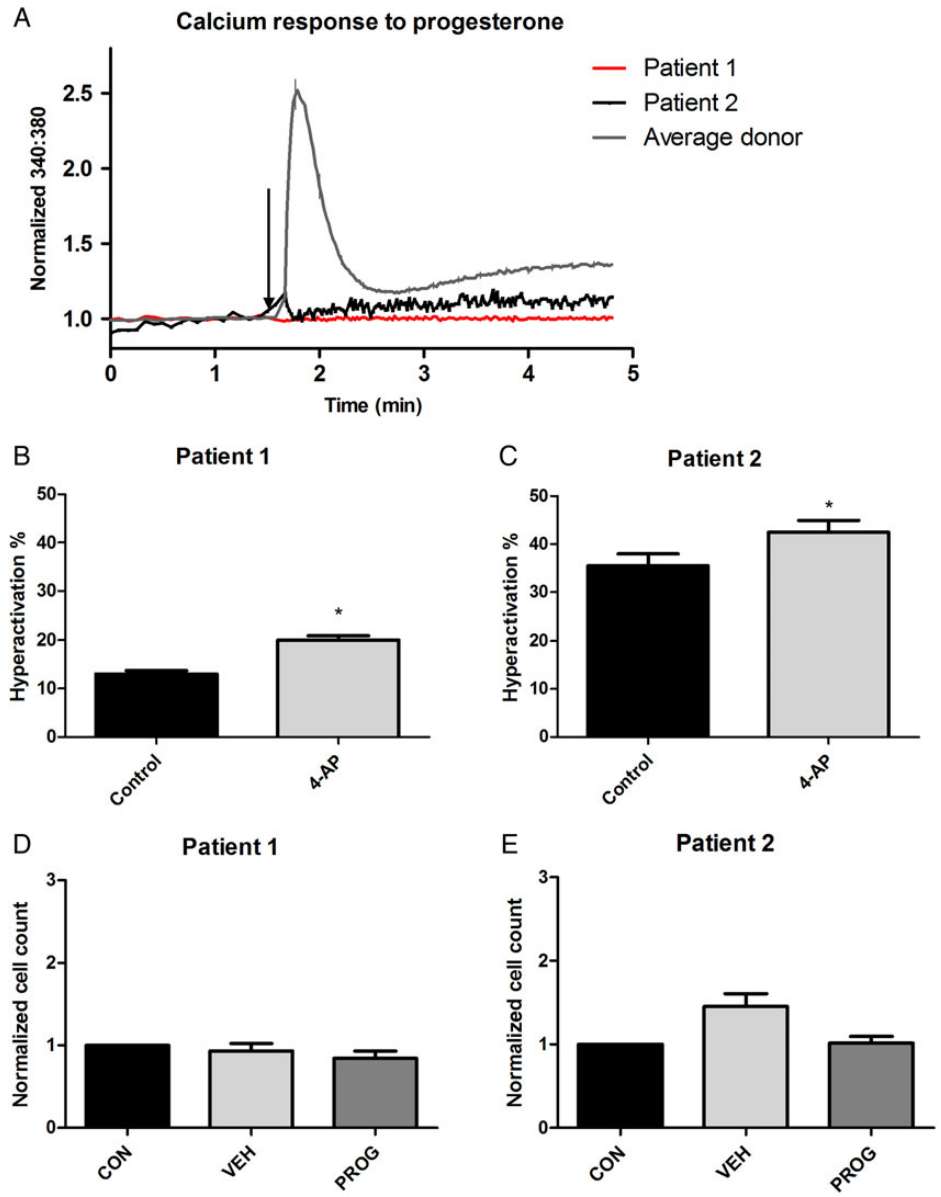

Figure 2 Calcium response to progesterone, hyperactivation in response to 4-AP and penetration into viscous media for spermatozoa from Patients 1 and 2. (**A**) The spermatozoa from Patient 1 and 2 showed no significant calcium response to progesterone (compared with representative average donor trace, as seen in Fig. 1A). Patient 1 trace shows an average of four different aliquots from two ejaculates separated by 7 weeks; Patient 2 trace shows analysis of one sample on the day of IVF treatment. (**B** and **C**) 4-AP induced an increase in HA in the spermatozoa from Patient 1 ($P < 0.001$) and Patient 2 (4-AP versus control, $P = 0.01$, Student's paired *t*-test). (**D** and **E**) Spermatozoa from Patients 1 and 2 showed no significant enhancement in penetration into viscous media when stimulated with progesterone.

Patient 2

On the day of IVF treatment, prepared spermatozoa had a concentration of 20×10^6/ml with a total motility of 87% (Supplementary Table SI). However, as with Patient 1, exposure to progesterone elicited no $[Ca^{2+}]_i$ response or stimulation in the viscous media penetration test (Fig. 2A and E). Additionally, similarly to Patient 1, the spermatozoa were able to significantly hyperactivate when exposed to 2 mM 4-AP ($P = 0.01$) (Fig. 2C). All nine oocytes inseminated by IVF failed to fertilize. Female age was 24 years, with an AMH of 8 pmol/l.

Patient 2 returned for ICSI treatment 7 months later. Three out of five oocytes normally fertilized. Analysis of this sample showed that treatment with progesterone induced both a $[Ca^{2+}]_i$ signal and a significant response in the viscous media penetration test (data not shown). Therefore, Patient 2 was not recalled for further analysis for electrophysiology.

Donor 1

Donor 1 was a healthy young male who had never contributed to a pregnancy. Two semen analyses showed an average sperm concentration of

109 × 10^6/ml with a total motility of 69%. In the initial sample, the sperm were unable to produce a $[Ca^{2+}]_i$ response to progesterone (Supplementary Fig. S2A) and were not able to respond to progesterone in the viscous media assay (Supplementary Fig. S2D): an outcome similar to that of Patient 1 and the first sample provided by Patient 2. However, the spermatozoa were able to produce a significant HA response when treated with 4-AP (Supplementary Fig. S2B, $P = 0.0112$). Donor 1 produced another sample 1 week after the initial sample. Spermatozoa from the second sample showed a clear $[Ca^{2+}]_i$ response when stimulated with progesterone (Supplementary Fig. S2A) and significant HA when stimulated with 4-AP (Supplementary Fig. S2C), but failed to respond to progesterone in the viscous media assay (Supplementary Fig. S2E). Therefore, Donor 1 was not recalled for further study for electrophysiology.

Electrophysiology

CatSper has typically been characterized under monovalent conditions (Lishko *et al.*, 2011; Smith *et al.*, 2013); therefore Cs-based divalent free solutions were used to examine CatSper channel function. CatSper currents ($I_{CatSper}$) were robust in sperm from donors with a maximum outward current of 204.6 ± 17.7 pA/pF (Fig. 3). In contrast, $I_{CatSper}$ was essentially absent in sperm from Patient 1 with the maximum current (4.0 ± 0.4 pA/pF) being significantly lower ($P < 0.01$).

Genetic analysis

In mice all seven members of the CatSper Complex (CatSperα(1-4), CatSperβ, γ & δ) are required for functional expression. Assuming a similar requirement in human would suggest that a genetic lesion in any single gene could result in the absence of CatSper conductance. Exome sequence for Patient 1 was analysed for the presence of single nucleotide polymorphism variants and in/dels. This analysis revealed no evidence for loss of function mutations in any of the CatSper Complex loci (Supplementary Table SII). We further analysed a set of genes which, based on mouse genetics, would lead to fertilization failure without affecting spermatogenesis. Again no loss of function mutation was observed (Supplementary Table SII).

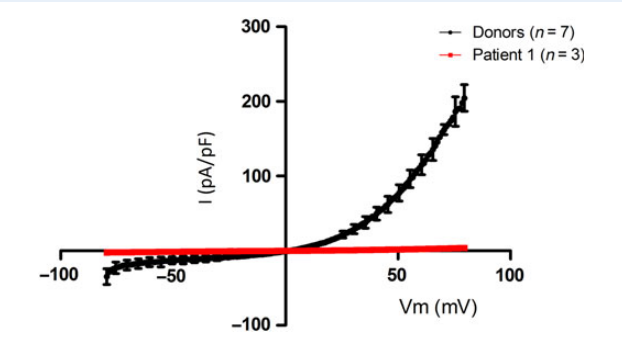

Figure 3 Whole-cell patch clamp recordings from spermatozoa from Donors and Patient 1. CatSper currents are absent from spermatozoa from Patient 1. Current–voltage relationship recorded under Cs-based divalent free conditions from spermatozoa from Patient 1 ($n = 3$). Capacitated donor data are also shown ($n = 7$). Current is measured in picoamps per picofarad, to normalize for variation in capacitance between cells. Error bars represent standard error of the mean (SEM).

Discussion

In previous studies genetic analysis has identified mutations in the genes coding for proteins of the CatSper channel. In humans, these abnormalities occur in association with other phenotypic abnormalities (Avidan *et al.*, 2003; Avenarius *et al.*, 2009; Hildebrand *et al.*, 2010; Jaiswal *et al.*, 2014). Analysis of CatSper currents has been undertaken in only one of these cases (Smith *et al.*, 2013) where there was a 70 kb deletion which spans four loci, CATSPER2, Stereocilin, CKMT1 and KIAA0377 (Avidan *et al.*, 2003). Deletion of these additional loci may explain the observation that, in addition to failure of CatSper currents, semen from this man was grossly abnormal thus making it unclear if the spermatogenesis defect was due to a loss of CATSPER2 or one of the other 3 loci. In the current study we have screened for functional deficiencies consistent with putative defects of CatSper function in men with normal semen parameters attending for IVF treatment. Our data support the conclusion that loss of CatSper conductance is rare, may occur independently of abnormalities associated with spermatogenesis and, importantly, has functional significance.

The data described here and previously (Alasmari *et al.*, 2013a) show that, on average, sperm from IVF patients responded similarly to donors. There was no significant difference between the groups for (i) Ca^{2+} responses to activation of CatSper or (ii) performance in penetration in the viscous media assay (artificial mucus) under control conditions and when stimulated with progesterone. However, samples from three of the men studied (two IVF patients and one donor) combined normal semen parameters with striking CatSper functional abnormalities. Motility parameters, including HA, were normal (and stable for up to 6 h) as was penetration of the sperm into viscous media in the absence of progesterone. However, these sperm failed to respond to activation of CatSper by progesterone (Lishko *et al.*, 2011; Strünker *et al.*, 2011) either with elevation of $[Ca^{2+}]_i$ or a functional response in the viscous media penetration test, which is via activation of CatSper (Alasmari *et al.*, 2013b). Samples from two of these men were also used for IVF and both showed complete failure to fertilize. In contrast, only 14/111 patient samples that had a normal progesterone-induced $[Ca^{2+}]_i$ response (including 9 who previously achieved low/failed fertilization at IVF) failed to fertilize. This is a clear significant difference ($P < 5*10^{-4}$; Chi square contingency test) strongly supporting the concept that functional failure of CatSper (progesterone induced $[Ca^{2+}]_i$) is sufficient to compromise fertility of human sperm, although such defects are rare. Furthermore, the 'stable' loss of CatSper function (see below) was not associated with poor semen characteristics. This is in contrast to the mouse phenotype and suggests that the roles of the CatSper complex in normal physiology differ between mouse and human.

Two of the men whose sperm failed to respond to progesterone (Patient 2 and Donor 1) provided further samples (interval of 1 week for Donor 1 and 7 months for Patient 2) that gave $[Ca^{2+}]_i$ responses upon stimulation with progesterone (e.g. Supplementary Fig. S2c). The assumption is that these men, therefore, have no CatSper genetic lesions. However, sperm from Patient 1 consistently failed to respond to stimulation with progesterone in two samples, and electrophysiological study confirmed the absence of CatSper currents. This suggests the presence of a consistent functional lesion of CatSper in Patient 1 that

was aetiologically distinct to that seen in Patient 2 and Donor 1. The most obvious candidate is a genetic alteration. However, no candidate mutations in the CatSper complexes were observed.

Previous analysis of the impact of CatSper loss has been in patients with deletions in multiple loci. This resulted in diverse defects in the spermatozoa raising the question of whether CatSper function is required for normal spermatogenesis (Avidan *et al.*, 2003; Smith *et al.*, 2013). In contrast, our data suggest that spermatogenesis is normal in men where CatSper function cannot be detected in ejaculated spermatozoa. Together with the observation that loss of CatSper activity may be transient (Patient 2 and Donor 1), this further emphasizes the potential of CatSper interruption as a feasible target for contraception.

The absence of any overt genetic abnormality in Patient 1 together with the transient phenotype in Patient 2 and Donor 1 suggests a defect(s) in testicular and/or epididymal sperm maturation and/or potential processing in the mature cell. However, although impaired localization and/or assembly of the CatSper complex is one explanation for loss of function, detailed proteomic studies combined with high quality co-localization and imaging (Chung *et al.*, 2014) would be required to address this.

In this study, and that of Alasmari, the number of men whose partners had IVF and thus had normal semen profiles (concentration and motility) yet defective Ca^{2+} influx induced by progesterone was small (1% and 0%) respectively. Similarly, in the 10 patients with a history of fertilization failure/low fertilization at IVF only one showed a defective $[Ca^{2+}]_i$ response (Patient 1). Although these findings indicate that CatSper defects are not a common cause of fertilization failure/reduced success, there are caveats to this conclusion. Firstly, since samples from 22% of IVF patients could not be screened (due to practical, technical and/or logistical reasons) the incidence of significant abnormalities might be underestimated. Secondly, the strict definition of a defective Ca^{2+} influx used is such that only substantial abnormal responses were selected for further study. Electrophysiological analysis was performed on Patient 1 with a clear and consistent defect and the sperm from this man showed negligible CatSper current. However, more subtle abnormalities of CatSper function, for example significantly reduced but not absent current, may occur and have some biological significance.

Our studies also shed light on the link between the CatSper complex and the regulation of HA. We demonstrated that 4-AP enhanced HA in sperm from Patient 1, who consistently showed failure of progesterone-induced $[Ca^{2+}]$i elevation and was null for CatSper current. The progesterone-insensitive sample from Patient 2 also responded to 4-AP with an increase in HA. This is consistent with a model for regulation of HA in which the Ca^{2+} stored at the sperm neck is pivotal to HA induction and CatSper-mediated Ca^{2+} influx in the flagellum acts as a trigger for mobilization of this store (Alasmari *et al.*, 2013a,b). Mobilization of stored Ca^{2+} in mouse sperm null for CatSper has also been shown to induce HA (Marquez *et al.*, 2007). Intriguingly, this suggests that pharmacological mobilization of stored Ca^{2+} can bypass failure due to impaired function or regulation of CatSper. Further investigations into the role of calcium response to progesterone and CatSper function, and their relationship to fertilization rate in IVF, should include the use of single-cell Ca^{2+} imaging. This would enable a high resolution investigation into the diverse cellular responses between individuals, and could provide further insight into Ca^{2+} signalling dysfunction.

We conclude that, in IVF patients, a total lack of CatSper current is rare, functionally significant and does not necessarily involve mutation in the CatSper gene complex.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

H.L.W. performed the initial screening on all of the IVF patients, recalling patients and research donors. W.A. performed initial screening and identification of initial recall patients. S.M.d.S., S.M. and H.L.W. were involved in the recruiting, recalling and consenting of patients and donors. S.M. and S.G.B. performed the patch clamping experiments. S.G.B. and S.M. performed the detailed analysis of the electrophysiological data. K.A.S., P.V.L. and M.R.M. were responsible for the genetic analysis. S.M.W., S.J.P., S.G.B. and C.L.R.B. were involved in the design of the study and obtained funding for the experiments. The initial, interim and final manuscript was drafted by C.L.R.B., H.L.W., S.G.B. and S.J.P. All authors contributed to the construction, writing and editing of the manuscript. All authors approved the final manuscript for submission.

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Conflict of interest

None declared.

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Paper 20:

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Evaluation and treatment of familial globozoospermia in five brothers

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Objective: To document the pathology of five siblings with complete globozoospermia and to report the effectiveness of repeated intracytoplasmic sperm injection (ICSI) treatment as therapy.

Design: Case report.

Setting: A tertiary center for assisted reproduction.

Patients(s): Five siblings with globozoospermia.

Intervention(s): Twenty cycles of repeated ICSI treatment. Detailed light and electron microscopy studies were performed on three of the globozoospermic brothers.

Main Outcome Measure(s): Clinical pregnancy and live birth after ICSI treatment.

Result(s): Light and electron microscopy showed that all spermatozoa had round heads and no acrosome. Of 129 metaphase-II oocytes injected, 49 fertilized normally, giving an overall fertilization rate of 38% (range, 0–100%). No pregnancies resulted from 13 cycles in brothers 1–3. Brothers 4 and 5 had three pregnancies after seven ICSI cycles, with one live birth and two first-trimester losses.

Conclusion(s): Despite variable fertilization rates, pregnancies and a live birth can be achieved after repeated ICSI treatment cycles in globozoospermic siblings. No apparent pattern was manifest that reflected the true pathology or determined the outcome of ICSI treatment. (Fertil Steril® 2004;82:1436–9. ©2004 by American Society for Reproductive Medicine.)

Key Words: Infertility, fertilization, globozoospermia, repeated, ICSI, siblings

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Globozoospermia (round-headed spermatozoa) is a well-known but very rare condition. It was previously thought to be a sterilizing pathology, but fertilization and pregnancies can be achieved with intracytoplasmic sperm injection (ICSI); however, the success rates are generally very low (1–3). Because it is a rare condition, relatively little is known about its etiology. Globozoospermia is certainly not a single pathology, given that different men with the condition can show a wide spectrum of effects (4). For example, some men show a very high level of sperm aneuploidy (5), whereas others do not (6).

Some reports describe clear abnormalities in the nucleus, presumably in chromatin packaging (7), though at least one report shows levels of DNA damage and chromatin packaging to be within normal limits (8). Many men with globozoospermia have almost normal sperm concentrations, with a relatively high proportion of motile spermatozoa. However, sperm–egg interaction studies suggest abnormalities in sperm binding to the egg vestments, though decondensation seems to be normal (9). It has been suggested that spermatozoa from globozoospermic men lack the putative oocyte-activating factor, but because repeat ICSI without specific oocyte activation results in conception, the relevance of this is unknown (2). The variety in pathology might reflect the different underlying causes, which might manifest themselves in the varying success rates with ICSI.

In this report, we present a family of 10 siblings in which globozoospermia was a uniquely prevalent phenotype. In this family, two sisters and three brothers were naturally

fertile, but five siblings were infertile because of complete globozoospermia. Our aim was first to document the pathology of the siblings with globozoospermia, thereby making a contribution to the description of this abnormality, and second to report the achievement of pregnancies with repeated ICSI treatment.

CASE REPORT

In this family of 10 siblings, two sisters and three brothers were naturally fertile. Five brothers had primary infertility and were referred for assisted conception at the Farah Hospital, Amman, Jordan, for ICSI. The average duration of infertility was 10.8 years (range, 3–15 years); the average age for male partners was 32.6 years (range, 27–41 years) and for female partners was 28 years (range, 19–36 years). There were no reports of a history of infertility in the immediate family. There were no consanguineous marriages, except that the grandfather and grandmother of the siblings were first-degree cousins. All the married cousins were naturally fertile. None of the men reported any exposure to potentially toxic chemicals, radiation, or other potential environmental factors that might have adversely affect fertility. Local ethical approval from the Farah Hospital was obtained for this study.

On physical examination, both the men and women were found to have normal health. Based on semen analysis (World Health Organization 1999) (10), median semen characteristics were as follows: volume 3.3 mL (range, 1–4 mL), concentration 22 $\times$ 10^6/mL (range, 5–60 $\times$ 10^6/mL), progressive motility 30% (range, 7%–60%), and semen morphology revealed a diagnosis of globozoospermia in all five men. Globozoospermia was diagnosed when all (100%) of the spermatozoa had round heads and the result was confirmed by two different embryologists. A normal peripheral blood leucocytes karyotype (XY) was obtained for four brothers diagnosed with globozoospermia (brothers 1–4) and for the three fertile brothers, as well as for the father of all siblings.

Three of the brothers with globozoospermia agreed to provide a semen sample for research (brothers 1–3); three of their fertile siblings also provided semen samples. The samples were collected and cryopreserved in sperm-freezing media (Medi-Cult, Copenhagen, Denmark) at the Farah Hospital Jordan and transported to The University of Birmingham, United Kingdom for further analysis.

Light Microscopy for the Presence of an Acrosome

The acrosomal status of the spermatozoa was evaluated with an indirect immunofluorescence technique with a monoclonal antibody (designated mAb 18.6), as described previously (11). An acrosome was present in the spermatozoa from the fertile brothers (median, 66%; range, 50%–72% intact acrosome), but no acrosomes were observed in the globozoospermic brothers (brothers 1–3).

Transmission Electron Microscopy

Cryopreserved semen samples were thawed, processed, stained (with 30% methanolic uranyl acetate and Reynold's lead citrate) and then visualized on a JOEL JEM-1200EX electron microscope (MA) (12). Qualitative transmission electron microscopy showed a variety of shapes of spermatozoa in the three globozoospermic brothers, with almost all of the spermatozoa having round heads. Very occasionally, a cell more like an elongating spermatid was observed. No acrosomes were seen, but in a small number of cells, remnants of what seemed to be acrosomal membranes were observed. From this qualitative examination, the pathology seemed to be very similar in each brother. Qualitative transmission electron microscopy of the three samples from the fertile brothers showed no differences from a series of fertile control samples (cryopreserved sperm from donors in our unit).

Controlled Ovarian Stimulation, ICSI, and ET

Ovarian stimulation cycles were carried out with either a short or long protocol (2). Sperm and oocytes were prepared for ICSI according to standardized procedures (2). Fertilized oocytes with two pronuclei (2PN) were classified as normal. Embryos were graded for quality according to four classifications adapted from Dokras et al. (13). Embryo transfers were performed 2 days after oocyte retrieval. Oral progestin was given for luteal-phase support. Pregnancy was confirmed by ultrasonic diagnoses of fetal sac(s) and the presence of fetal heart activity.

A total of 20 ICSI treatment cycles for the five globozoospermic brothers were carried out and are summarized in Table 1. The overall fertilization rate was 38% (range, 0–100%). In two treatment cycles no normal fertilization (2PN) was recorded. Thirty-two percent of the embryos were classified as grade 1 (17 of 47). The overall implantation rate was 6% (3 of 44), with a pregnancy rate of 15% per cycle started (3 of 20).

There seemed to be no clear pattern in fertilization or pregnancy success. For example, in brother 4 a pregnancy occurred in a cycle in which 12 mature oocytes were injected and only 1 oocyte fertilized normally. Brother 5 produced two pregnancies, but only one of them went to term. However, there were no pregnancies from 13 cycles with sperm from brothers 1–3.

DISCUSSION

This case report presents the clinical and pathologic details of a unique family, in which the majority of the male siblings had globozoospermia. This is the only clinical report of treatment outcomes in five siblings with complete globozoospermia. The primary finding is that pregnancies can be

TABLE 1

ICSI treatment for five brothers with complete globozoospermia.

	Cycle order	No. of metaphase II eggs injected	2PN	Fertilization rate (%)	No. of embryos transferred	Pregnancy (fetal heart)	Live birth
Brother 1	1	3	1	33	1	−	−
	2	8	0	0	0	−	−
	3	12	4	33	4	−	−
	4	10	4	40	4	−	−
	5	8	2	25	2	−	−
	6	6	3	50	3	−	−
Brother 2	1	8	6	75	4	−	−
	2	7	2	28	2	−	−
	3	10	3	30	2	−	−
Brother 3	1	2	2	100	2	−	−
	2	3	1	33	1	−	−
	3	1	0	0	0	−	−
	4	1	1	100	1	−	−
Brother 4	1	4	2	50	2	−	−
	2	10	3	30	3	−	−
	3	12	1	8	1	+	[a]
Brother 5	1	9	6	66	5	+	+
	2	2	2	100	2	−	−
	3	6	4	66	3	+	[a]
	4	7	2	28	2	−	−

[a] First-trimester loss.

Kilani. Five brothers with complete globozoospermia. Fertil Steril 2004.

achieved with repeated ICSI treatment cycles, even when failure is observed in the first cycle.

It is important in studies such as this to correctly identify cases of globozoospermia. In our study, globozoospermia was diagnosed when all (100%) of the spermatozoa examined on a wet preparation had round heads. The diagnosis was confirmed by transmission electron microscopy. Unfortunately, multiple samples were not available from every sibling, and therefore we can only comment on the details of the patients studied. We are confident of our diagnosis of globozoospermia by light microscopy in brothers 4 and 5, but of course we do not have transmission electron microscopy findings to determine whether the spectrum of pathology is the same in brothers 4 and 5 as in brothers 1–3.

We used specific staining of the acrosome and electron microscopy to obtain more information about the pathology. There was little if any difference between affected siblings (brothers 1–3), with no acrosomal staining, complete globozoospermia, and in general a small proportion of spermatozoa with less-than-dense nuclear staining. Absence of an acrosome is a very common feature of globozoospermia, but there are varying reports of possible defects in nuclear material (7, 8). There is clearly a wide spectrum of manifestations of globozoospermia, but the family in this study seemed to be homogenous, at least according to the methods we used.

Brothers 1–3 had 13 cycles of ICSI with no pregnancies and thus can be regarded as sterile. However, pregnancies were observed from brothers 4 and 5. Because of the low numbers, we do not know whether this represents a true clinical difference. It is apparent that pregnancies can be achieved with repeat attempts at ICSI, even if failure is observed in the first attempt (Table 1). This supports other observations in case report studies of globozoospermia (2, 3) and underscores the need to offer repeated attempts at ICSI.

To date, there is no clear explanation for the underlying cause(s) of globozoospermia. Although not a frequent observation, globozoospermia has been reported to occur within families, thus suggesting a strong, unidentified genetic component (14–17). However, even in such cases, the siblings might show very significant differences. For example, in a case study of two brothers with globozoospermia, Carrell et al. (17) noted profound differences in cytoplasmic organization, aneuploidy rates, and nuclear protein extracts. In that family, four other brothers were believed to be fertile, thus suggesting a possible environmental effect.

In our case report, no noticeable differences in sperm morphology or ultrastructure between the samples from the brothers were detected with light and electron microscopy. In cases in which the data have been reported, men with globozoospermia have been shown to have a normal karyotype, and thus gross structural chromosomal abnormalities

have been ruled out (14, 17, 18). With no clear explanation for the genetic causes of globozoospermia, the main question is: what caused the globozoospermia in this family?

Currently, we can only speculate as to the cause. There are some examples of mice with severe teratozoospermia, which might provide clues to the genetic origin of globozoospermia. For example, mice lacking the casein kinase II $\alpha 1$ catalytic subunit are infertile and have a sperm morphology similar (but not identical) to that manifested in globozoospermia (19). However, to date no deletions or abnormalities in expression of casein kinase II $\alpha 1$ catalytic subunit in men with globozoospermia have been reported. Several other knock-out mice have recently been reported to demonstrate a phenotype similar to globozoospermia; for example, Golgi-associated PDZ- and coiled-coil motif-containing protein (20), Hrb (21). We await detailed genetic studies to provide insight into the molecular basis of globozoospermia.

In conclusion, despite the variable fertilization success rates, pregnancies and live birth can be achieved with repeated ICSI treatment cycles in globozoospermic siblings. There were no apparent patterns in either fertilization or clinical outcomes that reflected the true pathology or that predicted the outcome of ICSI treatment.

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Paper 21:

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A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility*†

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Objective: To determine the effectiveness of the in vivo administration of vitamin E as treatment for reactive oxygen species–associated male infertility.

Setting: University-based center for reproductive medicine.

Design: Double-blind randomized placebo cross-over controlled trial.

Patients, Participants: Thirty healthy men with high levels of reactive oxygen species generation in semen and a normal female partner.

Interventions: Patients were allocated to two groups according to the blinded randomization. Each patient received either 600 mg/d of vitamin E (Ephynal, 300 mg tablets; F. Hoffman-La Roche Ltd., Basle, Switzerland) (order A) or identical placebo tablets (order B) for 3 months. Then after a 1-month wash-out period the patients were crossed-over to the other treatment.

Main Outcome Measures: Improvement in the in vitro function of the spermatozoa measured by conventional semen analysis, computerized motility assessment, determination of reactive oxygen species generation, binding to the zona pellucida of the unfertilized human oocyte in a competitive zona binding assay, development of hyperactivated motility (both spontaneous and in the presence of 20% of the natural agonist, human follicular fluid) and pregnancy.

Results: Rise in the blood serum vitamin E levels after treatment accompanied by improvement in one of the sperm function tests: the zona binding assay. The zona binding ratio for order A improved from 0.2 (range 0 to 0.5) before treatment to 0.5 (range 0.1 to 1.0) after treatment, the corresponding values for order B were 0.2 (range 0 to 1.0) before treatment and 0.3 (range 0.1 to 0.7) after treatment.

Conclusion: Oral administration of vitamin E significantly improves the in vitro function of human spermatozoa as assessed by the zona binding test. Fertil Steril 1995;64:825–31

Key Words: Male infertility, reactive oxygen species, in vivo treatment, vitamin E, sperm function assessment.

Spermatozoal dysfunction is the single most common cause of infertility (1). It is therefore surprising that only a limited number of therapies are available (2). To date there are few effective in vivo treatments for male infertility with the exception of corticosteroid administration for antisperm antibodies (3). Assisted reproduction techniques such as in vitro fertilization and donor insemination are the most commonly used options.

It is clear that an effective rational treatment can

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be achieved only when the underlying cause of the defect has been defined clearly. Recently, the generation of reactive oxygen species by the human ejaculate was identified as one of the major causes of sperm dysfunction (4, 5). Further evidence in support of these observations was provided by a prospective study of 139 couples (6). This report demonstrated that men with high levels of reactive oxygen species generation had seven times less chance of achieving a pregnancy compared with men with low reactive oxygen species.

Interestingly, in vitro experiments (7, 8) using vitamin E, one of the major membrane protectants against reactive oxygen species and lipid peroxidation (9), have shown a significant protection of the spermatozoa from peroxidative damage, loss of motility and in high concentrations, enhancement of the sperm performance in the hamster egg penetration assay. Although vitamin E has been used extensively in vivo to treat a variety of conditions (10), its potential in treating reactive oxygen species–associated male infertility has not been evaluated critically.

To assess the effect of vitamin E on the in vitro and in vivo function of human spermatozoa and fertility, we performed a randomized double-blind placebo cross-over controlled trial. We report here the oral administration of a high dose of vitamin E (300 mg twice daily) as a treatment of a group of men with a well-recognized, clearly defined cause of sperm dysfunction: high levels of reactive oxygen species generation in semen.

MATERIALS AND METHODS

Trial Design

The study was a randomized double-blind placebo cross-over controlled trial. Vitamin E in the form of α-tocopheryl acetate (Ephynal, 300 mg tablets; F. Hoffman-La Roche Ltd., Basle, Switzerland) and identical placebo tablets were used for the study. The randomization was performed by the manufacturer, and the code was blind for the researchers and patients. The code was broken at the end of the trial. Patients were given either an oral dose of 300 mg vitamin E twice daily for 3 months and then placebo for a further 3 months (order A) or placebo for 3 months followed by vitamin E for 3 months (order B). Three months was chosen to allow for a full spermatogenetic cycle. Between the two arms of the trial there was 1-month wash-out period in both orders. Because there is a paucity of conclusive data on the kinetics of vitamin E in the reproductive tract, 1 month was estimated to be a safe time to avoid carry-over effect. Furthermore, we used appropriate statistical methods to test for a carry-over effect. Oral administration of vitamin E to human subjects at doses as high as 3,200 mg/d has been reported to have very few side effects and to be nontoxic (11).

Patient Selection And Screening Semen Sample Analysis

Subjects for this trial were men attending the University Department of Obstetrics and Gynaecology, Jessop Hospital for Women, Sheffield, United Kingdom, for semen analysis as part of infertility investigations. Standard semen analysis was performed according to the World Health Organization guidelines (12) and included computerized motility assessment (Hamilton Thorn, Beverly, MA) (13). Computerized analysis of sperm motion generates information predictive of in vivo sperm function (13). Reactive oxygen species generation was determined with a simple chemiluminescent method (7), which has been shown to be highly predictive of in vitro sperm function and in vivo fertility (6). High levels of reactive oxygen species generation in the unfractionated ejaculate were defined as being greater than the mean + 2 SD of the fertile population (Kessopoulou E, Tomlinson MJ, Pearson MJ, Barratt CLR, Cooke ID, abstract). Men with detectable antisperm antibodies in their semen, defined as >20% spermatozoa with immunoglobulin (Ig) A and/or IgG surface-bound antibodies (12) and a spermatozoal concentration in the ejaculate $<5 \times 10^6$/mL were excluded from the trial. The latter criterion was being set to allow for adequate numbers of spermatozoa for analysis.

To maximize the chances of in vivo conception, additional acceptance criteria were agreed on for the female partner: [1] tubal patency assessed either by hysterosalpingogram and/or diagnostic laparoscopy and [2] ovulation (midluteal serum P > 5.66 ng/mL [conversion factor to SI unit, 3.180]) either spontaneous or with the use of ovulation-induction drugs (13). The couples were counseled by a clinician (M.J.P., K.K.S.) who explained the trial, in detail and participation in the trial was suggested to the male partner. All patients gave informed consent and were free to withdraw from the trial at any point. The trial was approved by the South Sheffield Ethics Committee, Sheffield, England.

Spermatozoa Function Assessment

Quality assurance for all the spermatozoa tests performed in this study was obtained through a standard internal quality control system applied regularly to the individual test and equipment used

(14, 15). Each patient had four semen analyses, one before commencing on each arm of the trial and one on completion of the arm. Semen parameters were examined as outlined for the screening semen sample.

In addition, the function of spermatozoa isolated on Percoll gradients (Sigma Chemical Company Ltd., Dorset, United Kingdom) (16) was examined using advanced tests (17) to determine whether the treatment had any effect on the in vitro fertilizing ability of the spermatozoa. This was the main aim of our study because the duration of treatment was not sufficient to allow for a large number of pregnancies to occur (2).

The sperm function tests were as follows: binding to the ZP of the unfertilized human oocyte in a competitive zona binding assay and development of hyperactivated motility (both spontaneous and in the presence of 20% of the natural agonist, human follicular fluid [FF]); a detailed description of the protocols has been published (15, 18). The results of the zona binding test are expressed as a ratio of patient to control spermatozoa bound to the ZP of an unfertilized, nonviable human oocyte; the mean ratio for at least three zonae per test was calculated. These tests have been shown to be of predictive value of IVF success (17, 19). Internal controls for each test were run on a daily basis and were performed using spermatozoa from semen donors of proven fertility. Test and control spermatozoa were treated in the same way. Determination of reactive oxygen species was performed in the unfractionated ejaculate (7) and the Percoll generated fractions (16).

The levels of vitamin E were assessed in patients' serum samples as an indication of body vitamin E status. Venous blood was collected into uncoated glass tubes and centrifuged for 10 minutes at 800 $\times g$. Seminal plasma samples were obtained by centrifuging an aliquot of semen (approximately 300 μL) within 1 hour of production for 10 minutes at 800 $\times g$. The supernatants of both fluids were collected in a dark room to prevent photodegradation of the vitamin E content. The samples were stored at -70°C until assayed (<2 years); previous studies have shown the vitamin E content to be stable under such conditions (20). Vitamin E levels in both fluids was assessed by high performance liquid chromatography (21). Vitamin E concentration in serum was expressed as μmol α-tocopherol/mmol cholesterol to correct for fluctuations in the transport capacity of serum for this vitamin.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) package for Windows, release 6.0 (22). The Mann-Whitney and Wilcoxon ranked nonparametric tests were used to examine any significant differences between groups and treatments (23). The level of significance was $P < 0.05$. The data presented in the tables are the baseline observations and treatment versus placebo differences for each of the two orders. Power analysis was performed to determine the numbers needed in the trial to observe significant improvement in the in vitro and in vivo performance of human spermatozoa.

RESULTS

Thirty patients completed the trial over a period of 2 years. These were allocated randomly as follows: order A, vitamin E to placebo, (n = 15 patients) and order B, placebo to vitamin E (n = 15 patients). The clinical characteristics of the patients were for order A (order B in parentheses): median male age 32 (31) years (range, 26 to 49 [26 to 45]), median female age 34 (28) years (range, 25 to 37 [24 to 34]), and median length of infertility 48 (30) months (range, 12 to 96 [12 to 76]). Primary infertility was a feature of 11 women for both orders while the remaining 4 women of both orders were experiencing secondary infertility. There were no statistically significant differences for these characteristics between the two orders at the point of entry in the trial.

The pretreatment semen sample parameters for the two study orders are displayed in Table 1. There were no significant differences in any of these parameters between the two orders. Overall the patients' samples were characterized by low spermatozoa concentration and motility, although there was a wide range for these characteristics.

Motile spermatozoa were recovered for 108 of 120 (90%) semen samples provided for the trial and for these the advanced sperm function tests could be performed. Analysis of the first semen sample revealed that spermatozoa from samples with high reactive oxygen species generation are characterized by low percentages of spontaneous and induced hyperactivation and a low zona binding ratio (Table 2).

With the single exception of the outcome of the zona binding assay, no significant carry-over or order-treatment interaction effect could be observed in all the parameters examined (Tables 1 to 3). Interestingly a highly significant improvement in the outcome of the zona binding test was observed after treatment for both orders ($P = 0.004$) (Table 2). However, a carry-over effect could be demonstrated for this parameter and in this the two study orders behaved in a different mode ($P = 0.02$), i.e., the carry-over effect was evident for order A ($P = 0.004$) in

Table 1 Semen Parameters of Men in the Vitamin E Trial*

Variable	Order A† (n = 15)		Order B‡ (n = 15)	
	Baseline level	Treatment difference	Placebo difference	Baseline level	Treatment difference	Placebo difference
Semen volume (mL)	2.8 (0.6 to 5.0)	0 (−1 to 1.6)	0 (−2.5 to 0.8)	2.5 (1.3 to 9.5)	0 (−3.5 to 3.5)	−0.5 (−2.5 to 1.5)
Sperm concentration ($\times 10^6$/mL)	37 (8 to 107)	−15 (−58 to 59)	0 (−37 to 160)	22 (5 to 96)	2.7 (−42 to 69)	−4.3 (−26 to 95)
Motility (%)	38 (0 to 83)	7 (−27 to 34)	7 (−33 to 36)	40 (8 to 70)	5 (−35 to 65)	3 (−24 to 36)
VAP (μm/s)§	41 (0 to 61)	−2 (−15 to 25)	−3 (−42 to 9)	42 (31 to 65)	0 (−6 to 13)	0 (−12 to 13)
VCL (μm/s)‖	58 (0 to 76)	−3 (−15 to 30)	−1 (−52 to 12)	52 (40 to 92)	0 (−10 to 17)	1 (−10 to 17)
Morphology						
Ideal forms (%)	37 (12 to 53)	−4 (−21 to 15)	−8 (−32 to 14)	27 (8 to 69)	−6 (−27 to 8)	−2 (−20 to 27)
Head defects (%)	49 (29 to 75)	10 (−9 to 17)	10 (−9 to 37)	68 (21 to 89)	8 (−10 to 22)	2 (−21 to 24)
Midpiece defects (%)	21 (9 to 34)	8 (−2 to 24)	0 (−15 to 25)	23 (9 to 55)	5 (−8 to 19)	5 (−11 to 12)
Tail defects (%)	9 (2 to 32)	3 (−12 to 18)	−1 (−13 to 9)	11 (9 to 43)	−1 (−23 to 14)	0 (−39 to 10)

* Values are median with minimum to maximum values in parentheses. The treatment effect comparison was not significant for any of the variables.

† Three months vitamin E followed by 1 month wash-out period and then 3 months placebo.

‡ Three months placebo followed by 1 month wash-out period and then 3 months vitamin E.

§ Average path velocity.

‖ Curvilinear velocity.

which patients were administered vitamin E first, whereas no carry-over could be demonstrated for order B ($P = 0.3$). There was hence a significant treatment-order interaction ($P = 0.02$). These data are presented in Table 2 as differences between treatment and placebo periods for the two orders.

No significant differences could be demonstrated either in the levels of reactive oxygen species generation before and after treatment for the two study orders (Table 3).

Vitamin E concentration in serum, expressed in μmol α-tocopherol/mmol cholesterol, also was elevated significantly after treatment ($P = 0.04$) (Table 4), and in this the two orders behaved the same. In contrast there was no rise in seminal plasma vitamin E concentrations with treatment (Table 4).

Three pregnancies were achieved in this study, all resulting in live births (two singleton and one twin). One pregnancy was in the vitamin E arm of order A and the other two in order B, one in each of the treatment arms, respectively. There were no complaints of discomfort in the vitamin E arms in either order, although three patients complained of "gastric acidity" in the placebo arm (1 in order A, 2 in order B).

DISCUSSION

This is the first double-blind randomized placebo-controlled trial to document the in vivo administration of vitamin E to treat males with reactive oxygen species–associated infertility. We conclude that therapy with this powerful antioxidant can be a potential treatment for a well-defined group of infertile men, on the basis of the improvement of the performance of the spermatozoa in the zona binding test after oral vitamin E administration. In a previous study high reactive oxygen species generation was

Table 2 Advanced Sperm Function Tests of Men in the Vitamin E Trial*

Variable	Order A† (n = 15)			Order B‡ (n = 15)			Treatment effect significance level
	Baseline level	Treatment difference	Placebo difference	Baseline level	Treatment difference	Placebo difference	
HA (%)§	5 (0 to 28)	0 (−13 to 24)	1 (−14 to 15)	5 (0 to 24)	0 (−13 to 12)	0 (−12 to 11)	NS‖
FF (%)¶	8 (0 to 44)	7 (−17 to 18)	−1 (−15 to 12)	1 (0 to 25)	5.3 (−7 to 15)	2 (−9 to 19)	0.06
Zona binding test ratio	0.2 (0 to 0.5)	0.2 (−0.1 to 0.6)	−0.2 (−0.5 to 0.1)	0.2 (0 to 1)	0.2 (0.1 to 0.9)	0 (−0.2 to 0.5)	0.004

* Values are median with minimum to maximum values in parentheses.

† Three months vitamin E followed by 1-month wash-out period and then 3 months placebo.

‡ Three months placebo followed by 1-month wash-out period and then 3 months vitamin E.

§ HA, spontaneous hyperactivation.

¶ FF, human FF–induced hyperactivation.

‖ Not significant.

Table 3 Levels of Reactive Oxygen Species Generation in Semen Samples of Men in the Vitamin E Trial*

Level of reactive oxygen species	Order A (n = 15)†			Order B (n = 15)‡		
	Baseline level	Treatment difference	Placebo difference	Baseline level	Treatment difference	Placebo difference
40/80% Percoll ($mV \times 10^3$)	25 (1 to 805)	−8 (−574 to 227)	1 (−79 to 62)	6 (1 to 81)	1 (−140 to 216)	1 (−124 to 415)
80% Percoll ($mV \times 10^3$)	1 (1 to 9)	−1 (−8 to 5)	17 (−1 to 6)	1 (1 to 3)	1 (−16 to 16)	1 (−34 to 68)
×3 centrifugation ($mV \times 10^3$)	20 (1 to 3,800)	−17 (−3,785 to 275)	6 (−2 to 39)	3 (1 to 145)	−1 (−10 to 240)	−5 (−170 to 175)

* Values are median with minimum to maximum values in parentheses. The treatment effect comparison was not significant for any level of reactive oxygen species generation.

† Three months vitamin E followed by 1-month wash-out period and then 3 months placebo.

‡ Three months placebo followed by 1-month wash-out period and then 3 months vitamin E.

determined to be a feature of >25% of an "infertile" population (5). Indeed, in our clinic the incidence of high reactive oxygen species generation is 19% (Kessopoulou E, unpublished data).

Thirty subjects finished the course of treatment with vitamin E to date and three pregnancies were achieved in the trial. We are aware that the trial size is small; however, recruitment of suitable patients with well-defined clinical characteristics and the requirement for large numbers of spermatozoa for the extensive in vitro sperm function tests meant that recruitment to the trial was difficult. Despite this, the highly significant improvement in the outcome of the zona binding test justifies the design and pursuit of further studies. The first step should be the use of IVF to test the after treatment performance of spermatozoa after in vivo administration of vitamin E. Successful fertilization with IVF as the treatment end point would eliminate the need for other sperm function tests and hence a larger number of patients will be eligible for treatment because the criterion of $>5 \times 10^6$ spermatozoa/mL of semen will not be necessary. Men with these characteristics form 14% of the total "infertile" male population in our clinic. In addition, by relaxing the criteria for females a significant number of patients would be suitable for such a study. If reactive oxygen species are determined before IVF, patients can then be offered this tested treatment, and the first IVF cycle can be commenced in 3 months to allow for the regime to be effective.

We hope that our study will serve as a pilot for a larger multicenter trial organized between infertility centers. The statistical methods that we used indicated that 1,670 patients would have to be recruited for a significant improvement in the in vivo conception rate to be observed. This results from two pregnancies being reported in the treatment and one in the placebo arm of the trial. It is possible that the small number of patients and the relatively short treatment time were insufficient to allow larger number of pregnancies to be achieved. The large number of patients needed for an in vivo study may be reduced if interim data analysis is performed at different time intervals. Furthermore, this does not invalidate the effectiveness of vitamin E as a treatment because power analysis of the results clearly has shown that 88 patients are required for an 80%

Table 4 Blood Serum and Seminal Plasma Vitamin E Levels of Men in the Vitamin E Trial*

Variable	Order A (n = 15)†			Order B (n = 15)‡			Treatment effect significance level
	Baseline level	Treatment difference	Placebo difference	Baseline level	Treatment difference	Placebo difference	
Blood serum vitamin E§	4.2 (0.8 to 12.7)	0.9 (−0.28 to 9)	0.5 (−4.3 to 2.3)	5.4 (1.7 to 7.3)	1.1 (−3.4 to 7.4)	0.3 (−1.86 to 11.9)	0.04
Seminal plasma vitamin E‖	0.4 (0.2 to 0.9)	0 (−0.2 to 0.3)	0 (−0.7 to 0.6)	0.5 (0.2 to 2)	0 (−1.4 to 1)	0 (−0.4 to 1)	NS¶

* Values are median with minimum to maximum values in parentheses.

† Three months vitamin E followed by 1-month wash-out period and then 3 months placebo.

‡ Three months placebo followed by 1-month wash-out period and then 3 months vitamin E.

§ μmol α-tocopherol/mmol cholesterol.

‖ μmol α-tocopherol.

¶ Not significant.

overall improvement of the zona binding test (23). This may have significant implications for the treatment of these patients using IVF rather than relying on in vivo conception.

Our study is the first to determine the in vitro function of human spermatozoa from samples with high reactive oxygen species, using accepted sperm function tests. Previous reports, which examined the effect of reactive oxygen species on spermatozoa function, were based mainly on the use of in vitro–generated reactive oxygen species. The data generated in this study provide the first evidence that spermatozoa from samples with high reactive oxygen species generation are unable to develop hyperactivated motility either spontaneously or in the presence of the natural agonist FF. Furthermore, such spermatozoa have a lower ability to bind to the ZP of the unfertilized human oocyte. This report constitutes the first evidence that uses the zona binding test; this has been shown repeatedly to be predictive of the in vitro function of human spermatozoa (17). Peroxidative-induced damage to the spermatozoa receptors responsible for binding to the ZP, similar to the damage demonstrated for the rat cortical brain cell (24), could be a possible explanation. This hypothesis warrants further investigation and should become a topic of research when more is understood about the nature of spermatozoa-oocyte interactions.

Interestingly, no significant effect could be demonstrated in the conventional semen analysis parameters (Table 1). The same was true for the remaining function tests although FF-induced hyperactivation approached significance (Table 2). This is in contrast to in vitro studies that demonstrated a protective effect of vitamin E on the motility of the spermatozoa (7, 8); however, this effect was observed with vitamin E levels far higher than the ones determined in the seminal plasma of the subjects in this study (<1 μmol versus 10 mmol).

Furthermore, despite the observed improvement in the performance of the spermatozoa in the zona binding test, the reactive oxygen species levels detected remained unchanged. This can be explained easily if the mode of action of vitamin E is considered together with the method used to determine reactive oxygen species generation (6). The chemiluminescent assay used in the present and the prospective study of Aitken and his colleagues (9) measures both intracellular as well as extracellular reactive oxygen species. Because vitamin E is a chain-breaking and not a scavenging antioxidant, it would be expected to offer protection to membrane components without influencing the reactive oxygen species generation. This is in accordance with the data generated in our study.

The single report of ad hoc oral administration of vitamin E (300 mg/d) to males, none of whom had reactive oxygen species–related infertility, found no significant improvement in any of the conventional semen analysis parameters, despite a significant increase in the seminal plasma vitamin E levels in the treatment group in comparison to the placebo ($P = 0.02$) (25). Although in that study the administration of vitamin E was empirical, it is notable that the ineffectiveness of the treatment was suggested as being due to the small sample size ($n = 15$) and to the low concentration of vitamin E achieved in seminal plasma. These levels are low compared with the levels shown to inhibit lipid peroxidation in vitro (0.1 to 10 mmol) (7). In our study, although the concentration of vitamin E was elevated in blood serum, no significant rise in seminal plasma was detected. This could be indicative of vitamin E oxidation before ejaculation or could be attributed to the fact that the seminal plasma concentrations of vitamin E do not necessarily reflect the levels the spermatozoa encounter in the testis nor the levels in the sperm membrane. Although those data would be more convincing, such experiments are fraught by the lack of available testicular tissue or low sperm numbers in semen.

As we stressed in the introduction, in order to identify an effective treatment for male infertility, the underlying cause has to be recognized. We believe that the generation of reactive oxygen species by the human ejaculate may be a significant cause of male infertility and that the in vivo administration of the antioxidant vitamin E may treat this condition successfully. Our study has therefore suggested a simple and inexpensive potential treatment that needs further rigorous evaluation.

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Paper 22:

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human reproduction

POSITION REPORT *Andrology*

Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications—a position report

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This article reports the results of the most recent in a series of EHSRE workshops designed to synthesize the current state of the field in Andrology and provide recommendations for future work (for details see Appendix). Its focus is on methods for detecting sperm DNA damage and potential application of new knowledge about sperm chromatin organization, vulnerability and repair to improve the diagnosis and treatment of clinical infertility associated with that damage. Equally important is the use and reliability of these tests to identify the extent to which environmental contaminants or pharmaceutical agents may contribute to the incidence of sperm DNA damage and male fertility problems. A working group (for workshop details, see Appendix) under the auspices of ESHRE met in May 2009 to assess the current knowledgebase and suggest future basic and clinical research directions. This document presents a synthesis of the working group's understanding of the recent literature and collective discussions on the current state of knowledge of sperm chromatin structure and function during fertilization. It highlights the biological, assay and clinical uncertainties that require further research and ends with a series of 5 key recommendations.

Key words: sperm DNA damage / sperm chromatin / male infertilty / ART

Background and rationale

Traditionally, the diagnosis of male infertility is based upon microscopic assessment and analysis of sperm concentration, motility and morphology as routine indicators of human semen quality. These indicators provide fundamental information about sperm production upon which clinicians base their initial diagnosis. As emphasized in previous ESHRE reports, it is imperative that semen analysis be performed to the highest standards. To this end ESHRE's special interest group in Andrology (SIGA) has been instrumental in providing formal training programmes and external quality control schemes. However, even with appropriate quality assurance, traditional semen parameters provide a limited degree of prognostic and diagnostic information and their predictive power is highest primarily at the

lower ranges of the spectrum (Lefièvre *et al*., 2007; Lewis, 2007). Sperm production is only part of the story. Sperm chromatin and DNA integrity is essential to ensure that the fertilizing sperm can support normal embryonic development of the zygote. To better inform treatment pathways and, more importantly, to ensure a generation of healthy children from assisted reproductive technologies (ART), we urgently require tests of sperm function, including the normalcy of sperm DNA, that provide high quality and robust diagnostic and prognostic information.

Improved tools for the diagnosis of male infertility clearly benefit the clinician's ability to treat an infertile couple seeking help. In addition, these tools are important in a broader social context. Infertility is no longer solely a personal problem; it has become a public health issue. In developed countries such as the European Union, birth rates have been declining at an unprecedented rate over the past half century. The extent to which birth rates may be impacted by contemporary life style factors, or by exposures to environmental contaminants or pharmaceuticals remains to be determined, but is of growing concern. Environmental epidemiology studies, which measure differences in health outcomes between groups of individuals exposed to environmental contaminants and unexposed controls, are beginning to address this question. Valid indicators of sperm quality and fertility are essential for these epidemiology studies to achieve their objectives. Equally important is the potential demographic and economic impact of the increased use of ART to treat subfertility. Depending on the country, up to 3.9% of EU births are currently from ART (Nyboe Andersen *et al*., 2009). Therefore, the use of ART may result in significant demographic and economic impacts. Part of the latest population policy strategy takes this into consideration (European Parliament, 2008; Ziebe and Devroey, 2008).

Significant progress has been made towards the development of reliable tests for sperm chromatin integrity and DNA damage. A landmark paper by Evenson *et al*. (1980) suggested that assessment of DNA integrity in sperm may be a useful and potentially independent marker of fertility for both animals and men. Clinical data followed, demonstrating higher levels of chromatin damage in men with severe semen defects (Sun *et al*., 1997) and the potential negative impact of high levels of sperm DNA damage on both natural (Evenson *et al*., 1999; Spanò *et al*., 2000) and ART conception (Larson *et al*., 2000). Subsequently, the assessment of DNA damage in the male germ line and the study of its consequences have received considerable attention. However, key questions, particularly regarding clinical significance, remain to be answered (Collins *et al*., 2008).

In animals, where DNA damage can be experimentally induced in the paternal germ line by, for example, chemotherapeutic agents, clear and strong associations have been shown between damage to the paternal genome and embryo development including effects on the new born and subsequent generations (Auroux *et al*., 1990; Hales *et al*., 1992; Fernandez-Gonzalez *et al*., 2008; Delbès *et al*., 2009). These experiments are not feasible in humans, but provide clear warnings of the potential impact of for example cancer treatments in men for the next and subsequent generations. With long-term follow-up on children born as a result of ART still in its infancy, it is essential that we are able to appreciate and translate findings from animals to humans to understand the clinical implications of using spermatozoa with highly damaged DNA in ART. Progress in this area will be dependent on fundamental improvements in our understanding of the causes and consequences of DNA damage in the male germ line and the development of robust diagnostic tests that can be incorporated into the clinical assessment of male infertility patients.

Biological uncertainties: the mysteries unfolding

The nature of the germ cell and sperm chromatin

In developing better methods for assessing clinical sperm samples for use in ART or as indicators in environmental epidemiology studies, a series of assays have been designed that evaluate the integrity of sperm DNA. These include, but are not limited to, the TUNEL assay, the sperm chromatin structure assay (SCSA), the Comet Assay and the Sperm Chromatin Dispersion Assay (for details, see section: How do we assess sperm DNA damage?) These assays are already in clinical use in some infertility centres for two primary reasons. The first is to attempt to predict future ART outcome or explain previous failure. The second is to be able to detect sperm with DNA that is sufficiently damaged as to result in transmission of genetic defects to the embryo and subsequent offspring. To optimize ART, especially ICSI, clinicians would like to be able to choose sperm with intact DNA. As conducted at present, these DNA integrity assays provide an assessment of the distribution of cells in a given ejaculate. However, these assays also destroy the cells and so cannot be used to identify or select an individual intact sperm for use in ICSI. Moreover, for most of these assays, we do not have a good understanding of what kinds of lesions they are measuring. Before they become routine clinical assays, we need to comprehend what these assays are telling us about the integrity of the male genome. This will come from a thorough knowledge of the sperm chromatin structure and how it is packaged.

Most DNA in mature human sperm is bound to protamines, as somatic histones are replaced during spermiogenesis. However, biochemical analyses of human sperm proteins indicate retention of some histones resulting in a nuclear protein composition that is about 90% protamine and 10% histone. We have a fairly clear understanding of how the protamines package human sperm DNA. The protamine-bound DNA is coiled into tightly compacted toroids that contain about 50 kb of DNA (Conwell *et al*., 2003; Hud *et al*., 1993), although the histone-bound DNA is believed to be organized into nucleosomal chromatin (Pittoggi *et al*., 1999; Zalenskaya *et al*., 2000; Wykes and Krawetz, 2003; van der Heijden *et al*., 2006; Hammoud *et al*., 2009). Sperm DNA is so well protected that, unlike somatic cell chromatin, it is resistant to nucleases (Sotolongo *et al*., 2003) and sonication (Tateno *et al*., 2000). The precise packaging of the histone portion of sperm chromatin is not well understood. We are only now beginning to appreciate how this histone bound DNA is distributed throughout the chromatin, but we still do not know the size of the histone bound DNA segments. Recent evidence suggests that histone bound DNA in sperm cells is associated with gene families that are important for cell differentiation and early embryo patterning (Hammoud *et al*., 2009).

How are the current assays expected to interact with sperm DNA that is associated with protamine versus histone fractions? Reagents used in the TUNEL assay, for example, would not be expected to be able to access the DNA packaged and stabilized by protamines into toroids. This is because it uses the action of the enzyme terminal deoxynucleotidyl transferase (TdT) and if protamine bound chromatin is resistant to nucleases, it would be expected to be resistant to other enzymes as well. However, improperly stabilized protamine and histone bound DNA would be expected to be accessible to most of the assays currently in use for sperm DNA assessment. TdT can access DNA breaks in nucleosomal DNA, so the TUNEL assay would be expected to reveal this type of chromatin structure. The SCSA partially denatures sperm with acid which would preferentially extract histones rather than protamines (Ballachey *et al*., 1987; Larson-Cook *et al*., 2003). Therefore, the SCSA would also be expected to identify single stranded DNA in histone bound sperm chromatin. However, SCSA would also measure single-stranded DNA in the protamine bound DNA if the stabilization of the protamine-DNA complex have been compromised (Dias *et al*., 2006) Finally, most protocols for the comet assay involve high salt extraction in the presence of a reducing reagent which removes both protamines and histones (Tomsu *et al*., 2002; McVicar *et al*., 2004). The Comet assay therefore probably detects chromatin breaks in both types of chromatin with equal efficiency. A more detailed discussion of these points can be found elsewhere (Shaman and Ward, 2006). The questions that we need to address include (i) how can we specifically assess the DNA damage in histone bound versus protamine bound DNA in sperm chromatin (ii) how meaningful is this distinction for clinical prognosis and (iii) how can we assess the damage to the whole genome as opposed to specific fractions?

Related to these questions is our limited understanding of how the sperm chromatin is formed during spermiogenesis. We know that protamines largely replace the histones during sperm nuclear condensation and that during this process the sperm DNA is unwound by topoisomerases and other proteins through the normal and necessary induction of strand breaks (Boissonneault, 2002; Kwan *et al*., 2003). It is clear that when this packaging is not complete, DNA single and double strand breaks appear in the fully mature sperm (Marcon and Boissonneault, 2004). Furthermore, the protamine deposition can also be incomplete, resulting in ratios of histone to protamine and of protamines 1 to protamines 2 that differ from normal. Both types of defects in spermiogenesis are associated with subfertility or infertility (Aoki *et al*., 2006a, b; Oliva, 2006). Yet, we do not know how topoisomerase cleaves sperm DNA during spermiogenesis or how the protamines interact to form sperm chromatin toroids.

These are just some examples of the uncertainties that cloud our understanding of how abnormalities in sperm chromatin structure may be used to guide clinical decisions. A host of additional aspects of sperm DNA packaging are also emerging as potential indicators of prognosis, including chromosome position within the sperm nucleus (Zalenskaya and Zalensky, 2004), the presence of mature mRNAs (Ostermeier *et al*., 2002, 2004) and newly described pi and microRNAs in sperm chromatin (Li *et al*., 2001; Martins and Krawetz, 2005; Carrell, 2008). Currently, we know a lot about how sperm chromatin packaging differs from somatic cell packaging, but we do not understand the specifics of how that packaging occurs. This will be important for a real understanding of what current sperm DNA assays are measuring.

Changes in sperm chromatin during sperm transport from the testis to the oocyte: the role of zinc

Zinc is incorporated into the sperm nucleus during spermiogenesis. The chromatin contains around 8 mmol Zn^{2+}/kg which equates to one zinc ion for every 10 base pairs of the DNA, equalling one turn of the DNA-protamine helix (Kvist *et al*., 1985). However, the role of zinc in sperm chromatin structure and function is only partly understood. The chromatin of more than 90% of human spermatozoa can be experimentally decondensed *in vitro* by exposure of freshly ejaculated sperm to the anionic detergent SDS together with the divalent cation chelating EDTA (Björndahl and Kvist, 1985; Kvist *et al*., 1988). This observation suggests a potential rapid mechanism for decondensation of the sperm chromatin that relies on Zn^{2+} depletion and interruption of macromolecules. Thus, at ejaculation, human sperm exhibit zinc-dependent chromatin stability. However, upon *in vitro* culture, human sperm become more resistant to decondensation and subsequently require disulfide bond reduction to enable unpackaging of the chromatin. This change is enhanced when zinc is withdrawn from sperm *in vitro*, and can, to a large extent, be counteracted by storing sperm in a buffer containing Zn^{2+}. Although the role of zinc—to bind thiols—is simple, the consequences of this organization are complex based upon the following observations. (i) Zinc primarily contributes to rapidly reversible chromatin stability. (ii) If zinc is lost, it will not contribute to a sufficient stabilization of the chromatin, leaving the DNA more accessible and therefore more vulnerable to factors that might degrade it (endogenous enzymes or exogenous chemicals). (iii) Extraction of zinc can elicit chromatin decondensation if repulsion of macromolecules (DNA–protein filaments) is induced simultaneously, e.g. by phosphorylation or in experimental studies by the action of detergents like SDS (Kvist *et al*., 1987). (iv) If macromolecules are not repelling the chromatin threads, the lack of zinc can allow the formation of disulphide bridges (S–S dependent chromatin stability).

What happens to the sperm chromatin during ejaculation, liquefaction and after liquefaction during further processing for ART? The zinc content of the sperm head and the type of chromatin stability are influenced by the composition of the surrounding 'seminal fluid' which comprises a mixture of various secretions that vary during ejaculation, liquefaction and after ejaculation. Normally spermatozoa are expelled in the first ejaculatory expulsions suspended in the zinc-rich prostatic fluid and the zinc-chelating seminal vesicular fluid is expulsed in later fractions. Upon mixture during and after liquefaction, spermatozoa are thus exposed to a series of differing environments that can have a marked influence on chromatin stability (Björndahl and Kvist, 2003). Of clinical importance is that in some men the emptying of prostatic fluid is delayed and sperm are expelled in primarily zinc-chelating vesicular fluid, leading to extraction of zinc from the sperm chromatin (Björndahl *et al*., 1991). Several animal studies have reported that experimentally induced zinc deficiency affects sperm chromatin structure (Evenson *et al*., 1980). However, studies in humans are sparse and the biological effects of a zinc depleted diet are yet to be fully explored.

Mechanisms of DNA damage in male germ cells and spermatozoa

One of the potential mechanisms often cited as a cause of DNA damage in the male germ line is abortive apoptosis. The general idea behind this assertion is that as male germ cells metamorphose into highly differentiated spermatozoa, they progressively loose their capacity to undergo programmed cell death in the form of apoptosis. Since these cells are transcriptionally and translationally silent, it could not be otherwise. Thus, instead of engaging in a complete apoptotic response leading to cell death, differentiating haploid germ cells are thought to undergo a restricted form of this process leading to DNA fragmentation in the nucleus whereas retaining the capacity to differentiate into mature functional spermatozoa that may still be capable of fertilization (Sakkas *et al*., 2004). Clearly, haploid germ cells are capable of activating a process that resembles apoptosis in some respects because both caspase activation and phosphatidylserine exteriorization have been observed in human spermatozoa (Weng *et al*., 2002). It is possible that by expressing apoptotic markers on their surface, senescent spermatozoa ensure that their ultimate phagocytosis in the female tract will be silent and not associated with a full-blown inflammatory response (Kurosaka *et al*., 2003).

This default senescence pathway may resemble the intrinsic apoptotic cascade in many respects but in one important detail, it is very different. In an archetypal somatic cell, stimulation of the intrinsic apoptotic pathway leads to the sudden appearance of endonucleases that are either released from the mitochondria (such as endonuclease G) or activated in the cytosol (caspase-activated deoxyribonuclease) and then they move into the nucleus to cleave the intra-nucleosomal DNA. However, in spermatozoa the physical separation of the mitochondria and cytoplasmic space from the sperm nucleus means that such mechanisms cannot be operative. As a result, the claim that 'apoptosis' is a significant cause of DNA damage in human spermatozoa might not be true for cells entering this process as mature gametes. However, sperm mitochondria represent a major source of reactive oxygen species (ROS) in these cells (Koppers *et al*., 2008), that can become activated during the intrinsic apoptotic pathway. The levels of DNA damage recorded in both unselected donors and patients attending an assisted conception clinic were highly correlated with the appearance of a marker for oxidative DNA damage, 8-hydroxy, 2′-deoxyguanosine (8OHdG; De Iuliis *et al*., 2009). Although these data could be interpreted in several different ways, one plausible explanation is that some DNA damage in the male germ line is the result of a programmed senescence pathway, characterized by the activation of mitochondrial ROS formation, oxidative DNA base damage and unresolved DNA strand breakage (Aitken and De Iuliis, 2009).

If this is the case, then a key question is not what induces spermatozoa to undergo this restricted form of apoptosis—because this is their default condition. The real question is what prevents these cells from entering this pathway. The answer to this lies in defining pro-survival factors that will prevent spermatozoa from initiating apoptosis. There are undoubtedly many potential pro-survival factors for spermatozoa in the male and female reproductive tracts; their ultimate characterization is likely to help in the development of *in vitro* culture media that will preserve the functionality and genetic integrity of gametes used for assisted conception.

The nature of the DNA damage exhibited by human spermatozoa

In the literature, most authors simply talk of generic DNA damage without reference to any particular form of molecular lesion. The ability of the TUNEL and Comet assays to detect DNA damage in the male germ line clearly means that both single and double strand DNA breaks are prominent features of these cells (Irvine *et al*., 2000; Van Kooij *et al*., 2004, Enciso *et al*., 2009). In addition, damaged sperm chromatin is known to contain base adducts. The major DNA adducts found in human sperm DNA are 8OHdG and two ethenonucleosides (1,N6-ethenoadenosine and 1,N6-ethenoguanosine). Although the former is a direct consequence of oxidative attacks on sperm DNA, the latter probably arise from exposure to 4-hydroxy-2-nonenal, a major product of lipid peroxidation (Badouard *et al*., 2008). These findings, taken in conjunction with data revealing a high correlation between DNA damage and 8OHdG expression (De Iuliis *et al*., 2009) suggest that the former is commonly the product of oxidative stress originating as a consequence of the apoptotic mechanism described above, infiltrating leukocytes, redox-cycling xenobiotics or failed antioxidant defence systems. An interesting example of the latter, albeit in animals, is the powerful antioxidant protective environment in the epididymis which when disrupted, for example by deletion of glutathione peroxidase 5, can lead to abnormal levels of DNA compaction accompanied by increased miscarriage rates (Chabory *et al*., 2009).

Another type of damage that has recently come to light is DNA cross-linking. In the tightly compacted chromatin that characterizes the mature sperm nucleus, opportunities for DNA–DNA or DNA–protein cross-linking are significantly greater than in the dispersed interphase nuclei of somatic cells. This susceptibility to cross-linking phenomena has been recognized for many years (Qiu *et al*., 1995). Recently, in an analysis of the impact of estrogens on sperm chromatin structure, catechol estrogens were shown to form dimers that then covalently cross-linked the DNA so that it became completely resistant to the decondensation protocols employed in a Comet assay including treatment with reducing agents, detergents and broad spectrum proteases (Bennetts *et al*., 2008). This susceptibility of sperm chromatin to cross-linking was subsequently confirmed using epigallocatechin gallate, the major antioxidant present in green tea (Bennetts *et al*., 2008). Importantly, severely cross-linked chromatin is commonly encountered in populations of defective human spermatozoa (Windt *et al*., 1994) although the molecular basis of such super-stabilization is still unknown. Finally two of the assays most commonly used to detect DNA damage in human spermatozoa assess the stability of sperm chromatin under severely acid (SCSA assay) or alkaline (alkaline Comet assay) assay conditions. The full nature of how these pH-exposures affect the sperm chromatin is not known with certainty at the present time.

Sperm epigenetics

One area of rapidly advancing interest is sperm epigenetics and the role that epigenetic marks in sperm chromatin may play in regulating development of the embryo (Biermann and Steger, 2007; Carrell, 2008). Epigenetics refers to methods other than DNA coding changes (polymorphisms, mutations, deletions, etc.) that can alter or regulate the expression of genes (Nanassy and Carrell, 2008;

Trasler, 2009). Presently, it appears that the major epigenetic regulators in sperm are DNA methylation, retention of pi and micro RNA's and binding of large portions of the genome with protamines, and post-translational modification mainly N-tail chemical modifications to retained histones of the sperm genome (Emery and Carrell, 2006). It is increasingly clear that sperm epigenetics play a role in the function of the paternal DNA following fertilization and is therefore potentially an integral component of the possible effects of DNA damage on sperm function (Hammoud *et al*., 2009).

During late spermiogenesis, 85–95% of histones are replaced in human sperm through a multi-step process (Oliva, 2006). First, the histones undergo hyperacetylation then are replaced by testes-specific variants of the histones, followed by their replacement with transition proteins. The transition proteins are then rapidly replaced by two small, basic molecules termed protamine 1 and 2 (P1, P2). P1 and P2 are normally expressed in a 1:1 ratio in human sperm, and result in a much tighter packaging of the sperm DNA, resulting in a compaction of the nucleus and cessation of gene expression (Carrell *et al*., 2008). Altered expression of protamines is associated with diminished sperm quality and reduced embryogenesis quality in couples undergoing IVF/ICSI (Aoki *et al*., 2006a, b; Oliva, 2006).

The retained histones may present another possible source of epigenetic regulation of the sperm genome. Prior studies evaluating specific regions of the genome have indicated that histone retention may not be random (Gardiner-Garden *et al*., 1998, Li *et al*., 2008). Recent studies have further evaluated the potential role of the retained histones on a genome-wide basis and shown that retained histones are not randomly distributed throughout the genome. Rather they are preferentially retained in the promoter regions of genes required during embryogenesis, micro RNAs and imprinted genes (Hammoud *et al*., 2009). Each of these classes of genes is of interest from a developmental perspective, portending a possible role in early embryogenesis. To address this, the distribution of modified variants of the histones throughout the sperm genome has been analysed. It was found that H3K4Me2 and H3K4me3, which are gene 'activating' marks, were preferentially retained in developmental genes, some of which were also bivalently marked with H3K27me3, a 'silencing' mark. This bivalent marking is similar to the bivalent 'poising' of similar genes in embryonic stem cells (Hammoud *et al*., 2009). Further analysis of the genome-wide methylation of sperm DNA demonstrated that the regions of DNA bound to activating histone modifications were generally demethylated, a further activation signal. These novel findings are striking because they indicate that sperm genes may be packaged and epigenetically modified in a manner that is necessary for poising of the genes for early embryonic expression.

The potential implications of epigenetic gene poising in sperm are numerous, including the possible effects of aberrant spermiogenesis on epigenetic marking. Presently, the data indicate that oligozoospermic males have an increased incidence of abnormal methylation of CpGs in imprinted genes (Marques *et al*., 2004, 2008; Filipponi and Feil, 2009). Recent data have shown that altered methylation is found in some, but not all, of imprinted genes in the sperm of men with abnormal protamine replacement (Hammoud *et al*., in press). The potential link between general sperm DNA damage and epigenetic alterations is not yet understood, but may be very important. Such a link may help to explain the relationship between DNA damage and normal embryonic development.

Human studies of ambient chemical exposures and sperm DNA damage

Toxicology studies expose test species to toxicants of concern and determine associated health outcomes. The toxicology literature indicates that certain environmental contaminants can, at least at experimental doses, induce sperm DNA fragmentation (Evenson and Wixon, 2005) and/or induce oxidative stress in the testes (Aitken and Roman, 2007). Although at least 70 000 chemicals are used in commerce, and at least 200 exogenous chemicals can be measured in most people at any given time, very few chemicals have been evaluated specifically for their sperm DNA damaging potential. Reproductive and multigenerational test protocols specified by federal and international agencies do not include a specific test for sperm chromatin integrity. Nevertheless, numerous epidemiologic and occupational studies have shown that exposure to at least five types of environmental chemicals, known to be ubiquitous in today's modern environment, can be associated with sperm oxidative stress or sperm DNA damage. It is important to note that epidemiology studies demonstrate associations between exposures and outcomes but do not provide definitive information about cause.

Pesticides

By their nature, pesticides and/or their metabolites are biologically active and ubiquitous in the environment due to wide scale commercial and private use. The impact of pesticides on sperm DNA damage has been evaluated in at least seven studies in the last 15 years, with four reporting positive associations (Perry, 2008). Fenvalerate exposure was associated with higher % comet tail DNA and olive tail moment (Bian *et al*., 2004) and organophosphate exposure detected as urinary metabolites in sprayers was significantly associated with DNA fragmentation index measured using SCSA (Sanchez-Pena *et al*., 2004). In the only environmental (versus occupational) exposure studies to date evaluating pesticides and DNA damage, higher levels of urinary chlorpyrifos, carbaryl and pyrethroid metabolites were associated with a higher percentage of comet tail DNA using the neutral comet assay (Meeker *et al*., 2004, 2008).

Phthalates

Phthalate esters are used in food packaging, personal care products and plastics. Therefore, environmental exposures are hard to avoid in today's world. In a sample of 379 men from subfertile couples attending an infertility clinic, urinary levels of two specific phthalate metabolites monoethyl phthalate and mono-(2-ethylhexyl) phthalate were associated with an increased percentage of comet tail DNA using the neutral comet assay (Hauser *et al*., 2007). The levels of urinary phthalates among this sample were similar to levels found in the US general population.

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs), a class of persistent organic pollutants resulting from industrial production of transformers and electrical capacitors, are highly persistent and have known endocrine disrupting properties. Although the use of PCBs was banned in the mid-1970s, environmental residues accumulate in fats and human exposure continues, largely by consumption of contaminated fish and other food. The impacts of PCBs on sperm DNA integrity using the SCSA have

been recently demonstrated in epidemiologic studies of European and Inuit populations (Spano *et al*., 2005; Long *et al*., 2007) and in Swedish fishermen (Rignell-Hydbom *et al*., 2005).

Metals

Metals are common in industrial processing and are distributed widely in air, water and soil. Two epidemiologic studies have suggested that non-essential metals can cause sperm DNA damage. Oxidative DNA damage in sperm measured using 8OHdG was correlated with cadmium in seminal plasma in 56 non-smoking study participants in China (Xu *et al*., 2003). Increased DNA fragmentation in sperm measured using SCSA was correlated with blood lead levels in 80 battery plant workers in Taiwan (Hsu *et al*., 2009).

Air pollution

High levels of air pollution resulting from coal combustion was associated with increased sperm DNA fragmentation measured using SCSA in a longitudinal study of 36 men from the Czech Republic (Rubes *et al*., 2005). The components of the air pollution were not identified but blood metals were not elevated in this group and subsequent studies implicated reactive intermediates of carcinogenic polyaromatic hydrocarbons present in the particulate fraction of the air pollution (Rubes *et al*., 2007).

Although studies of each of these contaminants are limited, the accumulated epidemiologic evidence to date suggests exposures in our everyday environment can adversely affect the genetic integrity of the spermatozoon. However, the array of findings raises a number of questions about the underlying mechanisms through which environmental agents affect sperm production, structure and integrity. The future of this work relies largely on the field's ability to standardize methods of exposure and outcomes and its ability to work across clinical, epidemiologic and basic science disciplines to ask the right questions.

Among the environmental exposure studies to date, methods vary widely with respect to how exposures are defined and how sperm DNA damage is measured. Sound replication of research findings will require both valid and reliable exposure assessment measures, including evidence that the contaminant actually reaches the testis or comes into contact with sperm and validated methods for determining sperm DNA damage. In this regard, methods for detecting sperm DNA adducts that can be attributed to sources would be particularly informative. Epidemiologists are relying on clinical and basic scientists to establish the validity and precision of sperm DNA damage assays and to clarify what is actually being measured (for example, stability versus chromatin integrity). Once an assay makes its way into the reproductive biology literature, environmental epidemiologists may be quick to adopt its use as a meaningful outcome for reproductive health studies. Similarly, epidemiologic studies are designed to test exposure and outcome associations; however, there is reliance on basic science and toxicologic studies for insight into biologically plausible mechanisms that drive exposure-response relationships. In a reciprocal manner, basic scientists look to the epidemiologic data to determine whether associations they see in the lab are observed among humans in the general population or in clinical settings. Epidemiological studies seek to establish associations in large, unbiased and statistically meaningful samples of people. Together, well designed epidemiology and toxicology studies can inform clinicians about risks to sperm integrity posed by environmental contaminants and help them to advise their patients about avoiding such exposures.

Sperm DNA repair in oocytes, epigenetics and the potential consequences of faulty repair

Between the moment the spermatozoon fuses with the secondary oocyte and the first cleavage division (a period of 16–20 h in the mouse and 18–27 h in humans), the highly condensed sperm nucleus unfolds, exchanging protamines for maternal histones and assumes the interphase chromatin configuration in the male pronucleus that is necessary for the semiconservative DNA replication in preparation for the first mitotic division of embryonic development. The period of pronuclear formation in the zygote is unique in a number of features.

Sperm derived nucleosomal chromatin contributes to paternal zygotic chromatin. Using a heterologous ICSI system with human sperm and mouse oocytes, paternal nucleosomes marked by the replication dependent histone isoforms H3.1/3.2 were present in the G1 stage male pronuclei (Van Der Heijden *et al*., 2008). Additionally, examination of human multi-pronuclear zygotes showed these replication variants in paternal chromatin prior to DNA replication. This suggests that the epigenetic program originating from the paternal chromatin has the potential to be transmitted into further embryonic development (see section: Sperm epigenetics).

At least in the mouse, many post-translational histone modifications are asymmetrically present between the male and female pronucleus. Particularly in early G1 phase there are clear differences among histone N tail Lysine methylations (Van Der Heijden *et al*., 2005). Although not as extensively researched, the same pattern is visible in humans (Van Der Heijden *et al*., 2009). This phenomenon is known as epigenetic asymmetry and demonstrates the very different nature of chromatin between the sexes, as a consequence of gamete specialization, although this does not necessarily hold at the level of each gene.

Double strand DNA breaks that are induced during the nucleosome to protamine exchange in elongating spermatids may lead to residual breaks in functioning spermatozoa, although this remains to be determined. Also, and especially in the epididymis, DNA damage could originate from external factors, notably ROS. In the mouse, it has been observed that repair of DNA breaks induced by irradiation is incomplete at the first cleavage division, as the level of chromosome aberrations could be influenced by repair inhibitors at the zygote stage (Matsuda *et al*., 1989). Hence, integrity of the paternal genome also depends on the capacity of the oocyte to recognize DNA damage and repair it.

DNA repair before zygotic S-phase

Humans exhibit a relatively high load of reciprocal translocations (Bonduelle *et al*., 2002), which are mainly of paternal origin. Because a DNA double strand break is a prerequisite for a reciprocal translocation to arise, one deduces that these must be repaired during the zygotic cell cycle. The decondensation of paternal chromatin after gamete fusion is followed by a phase of recondensation, which in the mouse can be monitored by detection of Histone3 Serine10ph (H3S10ph). In the recondensation stage, 1 h after sperm fusion,

double strand breaks can be visualized by chromatin domains positive for phosphorylation of Serine 139 of H2AX (called gamma H2AX). Spontaneously, gammaH2AX foci are visible at this stage in low numbers and only in the paternal chromatin. However, using one genetic source of sperm and four maternal oocyte genotypes, the frequency of DNA single strand breaks was strongly dependent on the maternal genotype. Mice with either the BALB/c hypomorphic DNA.PKcs allele or the very low activity scid DNA.PKcs allele, had the highest averages of around three breaks per male complement, which was about three times the frequency found in B6/CBA hybrid oocytes (Derijck *et al.*, 2008). The kinase DNA.PKcs is the key enzyme of the Non-Homologous End Joining (NHEJ) double strand DNA repair pathway. Subsequently, sperm were irradiated with a standard dose of 3 Gy a dose that damages sperm DNA. Sperm DNA damage translated into additional gamma H2AX foci that in number were highly correlated with the frequencies reported above, and so reflect DNA.PKcs activity. This experiment demonstrated that in the absence of normal DNA.PKcs alleles in oocytes, the capacity to repair DNA double strand breaks, either from a damaged sperm or from a post fusion paternal chromatin remodelling mechanical defect, is affected (Derijck *et al.*, 2008).

Findings in mice have been partly confirmed in human-mouse heterologous ICSI experiments. When sperm samples from oligo-astheno-teratozoospermic men were compared with normozoospermic donors, the fraction of nuclei without damage was lower (Derijck *et al.*, 2007). Again, these findings relate to a fraction of breaks showing slower repair that can be both of prefertilization origin or produced post-fertilization by an interaction between the male chromatin and the female chromatin remodelling environment on the road to pronucleus formation. The observation that motile ejaculated sperm with normal morphology can generate abnormal gammaH2AX signalling patterns up to complete fragmentation supports the contention made earlier that sperm with normal appearance may nevertheless carry DNA damage.

Cytogenetic analysis of the male and female chromosome complements at the first cell cycle has been used to demonstrate the mutagenic effect of sperm irradiation. Abnormalities in first metaphase spreads revealed primarily chromosome type aberrations (inversions, translocations, fragments, etc.). These abnormalities result when the DNA repair takes place before S-phase such that the rearranged chromosomes and chromosomal fragments are faithfully copied. In such studies scid oocytes (NHEJ defect oocytes) exhibited about twice the number of chromosome abnormalities than controls (Derijck *et al.*, 2008), again implying that DNA double strand break repair is executed before S-phase.

DNA repair in the zygotic S-phase

During S-phase, when the replication fork encounters a single strand break, or more likely a base modification that requires excision repair, a double strand break is created in the replication fork. These sites can also be visualized by gammaH2AX as well as by a number of repair proteins among which is RAD51, a key player of the homologous recombination repair (HRR) pathway. A possible consequence of a stalled replication fork is a non-homologous chromatid exchange (a quadriradial) that at zygotic mitosis segregates into a normal chromosome complement and a reciprocal translocation. RAD51 has been visualized in the pronuclei from the beginning of the S-phase until chromosome contraction at mitotic metaphase (Derijck *et al.*, 2008).

In mice with zygotes derived from genetically handicapped oocytes, especially in the HRR pathway (by the RAD54, RAD54B knock-out mutations) but also in the NHEJ pathway, the zygotes were extremely sensitive to the mutagen 4NQO (4-Nitroquinoline 1-oxide, a UV mimic agent). Low doses of 4NQO resulted in chromatid exchanges especially in the male pronucleus whereas higher doses caused a blockage at the pronucleus stage. These and other experiments using irradiation of zygotes before entering the S-phase showed that for all genotypes but especially for HRR mutants, the male pronuclei contain higher amount of RAD51 foci than female ones. This would indicate that the combination of damaged sperm and oocytes with suboptimal DNA repair (a possible scenario for suboptimal oocytes after ovulation induction) favours reciprocal translocation induction in the paternal chromosome complement.

Summarising, it is clear that we are only just obtaining an insight into the complexities of the DNA repair mechanisms in the early zygote. The data overwhelmingly suggest that an extra repair effort from the oocyte is required for damaged male chromatin. In compromised oocytes this may not be available.

Assay uncertainties: refinements on-going

How do we assess sperm DNA damage?

The methods discussed here are adaptations of techniques originally developed and validated for investigation of DNA in somatic cells. A crucial question is whether these adaptations are sufficient to enable reagents to get access to the compacted sperm DNA without inducing damage? Furthermore, it is essential that a method aimed at revealing sperm DNA disorders can compensate for the varying changes in the sperm chromatin structure occurring *in vitro* after ejaculation (Kvist *et al.*, 1988).

The methods designed to detect sperm DNA disorders primarily include (i) Specific detection of free DNA ends ('nicks') by enzymatic incorporation of marked nucleotides (TUNEL). (ii) Detection of DNA fragments by gel embedded single-cell electrophoresis after extended lysis of all nuclear proteins binding DNA (Comet) under neutral (detects double strand) and alkaline (double strand and single strand breaks) conditions. (iii) Detection of green fluorescence as a measure of intact double stranded DNA [Acridine Orange (AO) tests] combined with the detection of red fluorescence as a measure of acid induced denaturation which occurs preferentially at sites of pre existing DNA strand breaks.

An important issue with all these methods is whether the treatments used to prepare the sperm may themselves induce DNA damage. For example, in the comet assay it is typically necessary to include thiols, which are strongly reducing, to elute protamines attaching to and hindering fragmented DNA from migrating as a comet. Does this protamine S–S reduction induce DNA damage, possibly secondary to release of physical constraints on the DNA?

With respect to the AO test using flow cytometry, the red emitted fluorescence may not be limited to pre-existing DNA damage. The assay uses acid treatment to denature damaged DNA which then reacts with AO and fluoresces red. It is possible that sperm with

inherently lower structural stability may be more accessible to the acid and more prone to acid induced DNA denaturation, even in the absence of pre-existing strand breakage. An example is that caput spermatozoa with condensed but unstabilized chromatin were far more sensitive to acid induced DNA-denaturation than mature cauda stallion spermatozoa (Dias *et al*., 2006). Thus, there is a need to further understand how the variability and changes in the degree and type of chromatin stabilization influence the outcome of the AO test to control for potential false negative and false positive results. This need is illustrated by the observations that (i) a high DNA fragmentation index (DFI %) among spermatozoa in raw semen was related to low success after intrauterine insemination (IUI) (Bungum *et al*., 2007), but that (ii) the prepared sperm populations that were actually used for the insemination all had low (4–6%) and normal DFI% (Bungum *et al*., 2008). Thus the 'negative impact' of originating from an ejaculate with >30% DFI is associated with the selected sperm population but hidden to the investigator as a 'falsely' normal value for DFI. For the diagnostic value for the individual patient further methodological work is needed to distinguish whether (i) a high DFI means increased vulnerability to acid and other exposures or true DNA damage that can be transmitted to future generations and (ii) a low DFI means a false negative result or no DNA damage.

To adapt the comet assay for spermatozoa, the compact status of the sperm chromatin must again be broken down using procedures that may also induce damage to the DNA. There are no standardized protocols for these procedures, but two methods have emerged: alkaline and neutral conditions. There are few studies relating this assay to clinical fertility status. Therefore, clinically robust cut-off levels remain to be established. As with the TUNEL assay, the accessibility of the DNA can be a limiting factor which needs to be addressed.

Interpretation of any test may be confounded by the presence of dead cells when these tests are performed on unprocessed semen. Dead cells contain fragmented DNA and may bias the overall results. As with all assays examining the quality of semen, a key consideration is the number of cells examined. Counting higher numbers of cells allows greater accuracy (WHO 1999) and as such any assay based on counting a low number of cells will have significantly wide confidence limits. An advantage of flow cytometry, which can be used for TUNEL and SCSA, is that large number of cells can be assessed thereby providing greater accuracy (as long as an account is made for the influence of non-sperm and dead cells).

Other tests revealing DNA accessibility

Chromomycin A3 is a compound that can bind to DNA and fluoresce. Binding to DNA is competitive with protamines and CMA3 fluorescence has therefore been interpreted as evidence for poor protamination (Bianchi *et al*., 1993). CMA3 fluorescence is certainly evidence of CMA3 binding to DNA, but this could also be due to increased access to sperm DNA due to assay procedures that may also cause a loosening between DNA and protamines (relative deprotamination). An alternative interpretation would therefore be that sperm with a high degree of CMA3 fluorescence possess DNA that is more easily accessed (and thus, more susceptible to injury), as a result of the relative deprotamination of the chromatin.

The aniline blue test is based on the detection of lysine residues with aniline blue as a measure of an excess of histones remaining bound to the sperm DNA (Dadoune *et al*., 1988).

Should DNA tests be based on the whole semen population or the subpopulation prepared for clinical use?

Whether DNA damage tests should be performed before or after sperm preparation depends on the purpose of the investigation. If the aim is to predict the potential for ART success, there is some controversy depending on the tests used. If the SCSA assay is employed, studies suggest that whole semen is more predictive (Larson *et al*., 2000; Bungum *et al*., 2007, 2008) whereas the TUNEL assay has been shown to be discriminative for clinical pregnancy using either raw semen or cohorts of spermatozoa prepared by density centrifugation for clinical use (Duran *et al*., 2002; Borini *et al*., 2006).

Whether we measure DNA from sperm taken from whole semen or isolated subpopulations, we cannot expect one single parameter to provide an absolute criterion for fertility or infertility. As for assessment of the diagnostic and prognostic value of traditional semen analysis, our expectations of sperm DNA testing are excessive. A successful ART outcome will also depend on many other traits of sperm quality e.g. capacity to fuse with the oolemma as well as the influences of the oocytes, uterine receptivity and maternal immune system competence.

The usefulness of animal studies

Animal studies are very important to our understanding of the basic biological mechanisms and the consequences of disruption of key processes. These can range from basic fertility studies, where experiments for example in cattle allow multiple inseminations and thus provide valuable preliminary information on potential diagnostic and prognostic assays (Ballachey *et al*., 1987; Amann and Hammerstedt, 2003), to developing critical models that offer the ability to perform multi-generational analyses of the consequences of ART. For ART, interpretation of animal studies depends upon an understanding of the differences in animal and human ICSI/IVF. We highlight only a few examples to illustrate how important such studies can be for predicting the future consequences of human ART. As the first example, injecting oocytes with spermatozoa with demonstrated DNA lesions as measured by SCSA has caused multigenerational effects (Fernandez-Gonzalez *et al*., 2008). This highlights possible long-term consequences of using sperm with compromised DNA integrity. Two further studies indicate the usefulness of animal models for identifying important areas of research in human ART. In the first, infertile Hook/Hook mice were used to obtain sperm for ICSI (Ward, 2005). These mice produce severely deformed spermatozoa, yet when these sperm were used for ICSI, apparently normal mice were born. More importantly, the severity of the morphological deformations did not increase in three successive generations. A second study however suggested a potential unexpected problem with ART (Collier *et al*., 2009). The clearance of steroids by the placenta was very different in offspring generated by ICSI compared with those generated by normal mating. This points to a potentially important problem that could be monitored in human pregnancies.

The relative ease with which animal studies in ART can be performed compared with the same investigations in human patients suggests an obvious need for the expansion of this particular type of research. The dilemma that the field faces is that it is ethically not possible to perform many of the necessary experiments to ensure that ART is completely safe, yet we have an obligation to do some assessment. Animal models can provide a step in this direction.

Clinical uncertainties: the urgency for a robust clinical test

The study of sperm DNA damage is highly relevant in the era of ART, particularly ICSI, because (i) these technologies bypass the barriers of natural selection, (ii) subfertile men possess substantially more sperm DNA damage than do fertile men and (iii) experimentally, sperm DNA damage has been shown to impact negatively on ICSI embryo development, pregnancy rates and offspring health (Ahmadi and Ng, 1999, Fernandez-Gonzalez *et al*., 2008; Zini *et al*., 2008).

There is good evidence to show that infertile men possess substantially more sperm DNA damage than do fertile men although a small percentage of spermatozoa from fertile men also possess detectable levels of DNA damage (Kodama *et al*., 1997; Evenson *et al*., 1999, Spanò *et al*., 2000; Zini *et al*., 2001). The etiology of sperm DNA damage is multi-factorial and may be due to primary testicular or secondary (e.g. environmental) factors. Ultimately, sperm DNA damage is believed to be the result of aberrant protamine expression, excessive ROS generation and abortive apoptosis during spermatogenesis (de Yebra *et al*., 1993; Carrell and Liu, 2001; Sakkas *et al*., 2003, Aitken *et al*., 2009; see also sections: Mechanisms of DNA damage in male germ cells and spermatozoa and The nature of the DNA damage exhibited by human spermatozoa).

Primary testicular factors that may disrupt spermatogenesis and spermiogenesis and subsequently lead to sperm DNA damage include ageing, congenital abnormities (cryptorchidism), genetic defects and idiopathic abnormalities. Extrinsic factors that may cause sperm DNA damage include drugs (e.g. chemotherapy), cigarette smoking, genital tract inflammation, testicular hyperthermia and varicoceles.

Epidemiologic studies of healthy men have demonstrated that the level of sperm DNA damage increases with advancing age (Spano *et al*., 1998; Wyrobek *et al*., 2006). Studies of infertile men have also generally shown that sperm DNA damage increases with advancing age (Moskovtsev *et al*., 2006; Vagnini *et al*. 2007; Winkle *et al*., 2009). These findings are in keeping with the age-dependent decline in semen parameters (Sartorius and Nieschlag, 2009).

Men with cancer (e.g. Hodgkin's lymphoma and testicular cancer) typically have poor semen quality and sperm DNA damage even prior to cancer-specific therapy (O'Flaherty *et al*., 2008). They may then experience further testicular damage with the cancer therapy (chemotherapy, radiation; Fossa *et al*., 1997; Morris, 2002). The recovery of spermatogenesis may occur months to years after therapy, but evidence of sperm DNA damage may often persist beyond that period (Fossa *et al*., 1997). Patients who are scheduled to undergo definitive cancer therapy (surgery, chemotherapy and/or radiation) are strongly encouraged to cryopreserve sperm for future use (Lee *et al*., 2006).

Cigarette smoking is associated with lower sperm counts and motility, and an increase in abnormal sperm forms and sperm DNA damage (Spano *et al*., 1998; Potts *et al*., 1999). It is postulated that smoking increases leukocyte-derived ROS production with subsequent adverse effects on mature sperm (Potts *et al*., 1999). Smokers should be counselled to stop smoking.

Post-testicular genital tract infection and inflammation (e.g. epididymo-orchitis, prostatitis) can cause leukocytospermia, and have been associated with increased levels of semen ROS and subsequent sperm DNA damage (Spano *et al*., 1998; Erenpreiss *et al*., 2002). Treatment of these genital tract infections with antibiotics may improve sperm DNA damage (Moskovtsev *et al*., 2009).

Testicular hyperthermia has been shown to cause sperm DNA damage and an increase in the histone to protamine ratio in experimental studies (Sailer *et al*., 1997, Banks *et al*., 2005). Clinically, there is limited evidence to demonstrate a relationship between hyperthermia and sperm DNA damage, however, an association between hyperthermia and reduced male fertility potential has been reported (Thonneau *et al*., 1998; Evenson *et al*., 2000). Certain behaviours (e.g. hot baths, saunas) and occupations (e.g. welders, bakers, prolonged driving) are associated with increased scrotal temperatures (Jung *et al*., 2002) and these may cause sperm DNA damage. Although men should be counselled to minimize any activity that can increase scrotal temperature, there is only limited evidence to show that this will specifically reduce sperm DNA damage.

Varicoceles have been associated with sperm DNA damage and the damage has been related to levels of oxidative stress in the semen of these infertile men (Saleh *et al*., 2003). However, it is unlikely that sperm DNA damage is specific to men with varicocele as men without varicocele (e.g. idiopathic infertility) can also possess high levels of DNA damage (Zini *et al*., 2001). Three studies have shown that varicoceletomy may reduce the levels of sperm DNA fragmentation (measured by SCSA or TUNEL assay). However, these studies are all small, uncontrolled, retrospective in design and have only tested one post-operative sperm sample (Zini *et al*., 2005; Sakamoto *et al*., 2008; Werthman *et al*., 2008). As such, the beneficial effect of varicocelectomy remains to be verified with larger, prospective studies.

It has been proposed that sperm DNA damage may be caused by seminal oxidative stress resulting from antioxidant deficiency, although the evidence in this respect is largely indirect. As such, a number of investigators have evaluated the effects of dietary antioxidant supplementation on sperm DNA integrity. Most of these clinical studies have evaluated men with high levels of sperm DNA damage. In these men, treatment with antioxidant supplements is generally associated with reduced levels of sperm DNA damage and/or improved fertility potential (Kodama *et al*., 1997; Greco *et al*., 2005a, b; Menezo *et al*., 2007; Tremellen *et al*., 2007; Gil-Villa *et al*., 2008). However, these dietary antioxidant studies were small and have not evaluated the mechanism of action of antioxidants: the only end-point that was measured is the integrity of the sperm DNA or pregnancy rate. Moreover, most studies evaluated the effects of a short treatment course (with no long-term follow-up), were not randomized and failed to include a placebo-control group. As such, vitamin supplements (antioxidants) can be offered to men with the understanding that limited clinical outcome data are not yet available to support their use.

Several clinical studies have examined the relationships between sperm DNA damage and reproductive outcomes in the context of natural and ART pregnancies. on the basis of a systematic review and meta-analysis of these studies, sperm DNA damage was found to be associated with lower natural, IUI and IVF pregnancy rates, but not with ICSI pregnancy rates (Collins *et al*., 2008; Zini and Sigman, 2009). Interestingly, the literature strongly suggests that sperm DNA damage is associated with an increased risk of pregnancy loss in those couples undergoing IVF or ICSI (Zini *et al*., 2008). Surprisingly there are no data relating sperm DNA damage to late fetal development or post-natal health in humans (Zini *et al*., 2008). Nonetheless, the true clinical utility of sperm DNA damage assays remains to be firmly established as the available clinical studies are generally small, heterogeneous and poorly designed, and many do not control for female factors (Collins *et al*., 2008; Zini and Sigman, 2009).

While current data suggest that impaired sperm DNA integrity may have the greatest effect (and hence, greatest clinical utility) on IUI pregnancy rates and on pregnancy loss following IVF and ICSI, larger (adequately powered), properly designed and controlled prospective studies are absolutely required to confirm these results. An evaluation of the impact of sperm DNA damage on late fetal and post-natal health is also vital.

Summary and recommendations

Recommendation 1: Fundamental research is urgently required

Significant and fundamental questions remain to be answered as part of a detailed understanding of the basic structure of chromatin and its repackaging during spermatogenesis, sperm maturation, ejaculation and during unpackaging in the oocyte. Although we do know how sperm chromatin packaging differs from that of somatic cell chromatin, we do not understand the specifics of how that packaging occurs. We are at the beginning of our understanding and uncertain where in the lifecycle of the cell the DNA damage originates, and uncertain of the causes (e.g. oxidative in nature) or the nature of the damage (e.g. single and/or double strand breaks and/or DNA cross linking). Other basic questions remain unanswered: does the origin and nature of the damage suggest less/more severe consequences? How does the oocyte recognize and repair the damage? Is there a threshold of repair? Is there a degree of damage beyond the oocyte's ability to repair? Additionally, exciting areas are now emerging such as the presence of histones, mature mRNAs and newly described microRNAs in sperm chromatin. Fundamental research is absolutely required to address the key questions.

Recommendation 2: Standardization of clinical assays

There are clear differences in protocols assessing DNA integrity and surprisingly a general lack of awareness of adequate controls both in clinical and epidemiological research. Due to the lack of standardization in methods, it is difficult to determine whether variations in findings are real (related to biology) or due to differences in method. Presently, none of the protocols appear to address the problem of different types of chromatin stabilization. Standardized methods that allow comparison of results from different laboratories are urgently required. For a correct interpretation of data, it is also essential to understand and control for sperm chromatin changes occurring after ejaculation and to distinguish between genuine DNA damage and artifacts due to lack of reagent access to DNA.

Only with standardized protocols and appropriate external quality control (EQA) is it reasonable to implement findings worldwide. For clinically useful cut-off limits, it is also a requirement that the parameter can distinguish between affected and unaffected individuals. To evaluate that, correlations are not adequate—predictive values, likelihood ratios as well as odds' ratios should be calculated to validate the usefulness of a certain parameter. ROC curves can be used to identify possible cut-off levels. Properly controlled studies with sufficiently high numbers of participating patients are more likely to be multicentre studies, which of course require standardized methods and EQA.

An EQA scheme should be developed, similar to that for Basic Semen Analysis run by the ESHRE Special Interest Group in Andrology.

Recommendation 3: Animal models

Models using animal studies should be developed to make predictions for long-term clinical outcomes of ART. Animal models offer the ability to perform multi-generational analyses of the consequences of ART, and to identify potentially hazardous molecular and biological affects of ART. We have highlighted several examples to illustrate how important animal studies can be for predicting the future consequences of human ART. The relative ease with which animal studies in ART can be performed compared with the same investigations in human patients suggests an obvious need for the expansion of this particular type of research.

Recommendation 4: High quality clinical data is urgently required

While current data suggest that impaired sperm DNA integrity may have the greatest effect (and hence, greatest clinical utility) on IUI pregnancy rates and on pregnancy loss following IVF and ICSI, significantly larger (adequately powered), properly designed and controlled prospective studies are absolutely required to confirm these results. Future studies should also aim to assess the relationships between sperm DNA damage and late reproductive outcomes (pregnancy loss, delivery rate and neonatal health) in view of the worrisome post-natal effects observed in animal studies. Sound clinical strategies for sperm DNA testing and subsequent counselling of couples seeking infertility therapies can only be developed after such studies have been undertaken. It is likely that to perform these trials large multicentre studies will be required.

Recommendation 5: Long-term follow-up of art children

It is essential that long-term comprehensive follow-up studies on ART children are performed to ascertain the safety of the procedures we are currently using. To date, there is a paucity of such studies. In general those available have limited power and do not provide comprehensive outcomes. We recommend that follow-up studies of children are performed as a matter of urgency and should include, where

appropriate, cross linking of databases. It is likely that with increasing developments in ART such follow-up studies will need to be continually updated and will involve international consortia.

The requirement for dedicated funding for research in reproductive medicine and infertility

We have identified real progress and presented further fascinating challenges. However, a fundamental impediment to advancement over the past three decades has been the absence of reproductive medicine and infertility research as a strategic priority for national governments and agencies. This has resulted in a paucity of funding. A prerequisite of achieving the above recommendations is for national and international agencies to realize the importance of both basic and clinical research in this area and, to deliver substantial long-term financial support. We anticipate that identifying areas for future research is an essential starting point in meeting this challenge.

Authors' Roles

Following extensive and detailed discussions at the consensus workshop, key areas to be addressed were identified (biological, assay and clinical uncertainties). Each author wrote a section related to their expertise addressing the above area(s). All contributed equally to the final drafting, editing and presentation of the paper and take equal responsibility. C.L.R.B. coordinated the construction of the manuscript and is the first author. All other authors are listed alphabetically.

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Appendix: Workshop details

The workshop was developed by ESHRE Special Interest Group (SIG) 'Andrology' as a ESHRE Campus course entitled 'Sperm DNA: organization, protection and vulnerability—from basic science to clinical application'. The meeting took take place in the Karolinska Institute Stockholm (Sweden) and was divided into two workshops: (i) a consensus workshop on 19th–20th May 2009 for invited speakers only and (ii) an ESHRE campus course on 21st–22nd May 2009 which was an open meeting. The meeting was initiated, organized and coordinated by Lars Björndahl and Ulrik Kvist. The speakers were invited, by EHSRE, based on their expertise in the area.

Previous ESHRE workshops in Andrology have been published as ESHRE Consensus Workshop on advanced diagnostic andrology techniques. ESHRE (European Society of Human Reproduction and Embryology) andrology special interest group. *Hum Reprod* 1996;**11**:1463–1479 and ESHRE Guidelines on the application of CASA techology in the analysis of spermatozoa. ESHRE andrology special interest group. European society for human reporduction in Embryology. *Hum Reprod* 1998;**13**:142–145.

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human reproduction update

The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance—challenges and future research opportunities

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BACKGROUND: Herein, we describe the consensus guideline methodology, summarize the evidence-based recommendations we provided to the World Health Organization (WHO) for their consideration in the development of global guidance and present a narrative review of the diagnosis of male infertility as related to the eight prioritized (problem or population (P), intervention (I), comparison (C) and outcome(s) (O) (PICO)) questions. Additionally, we discuss the challenges and research gaps identified during the synthesis of this evidence.

OBJECTIVE AND RATIONALE: The aim of this paper is to present an evidence-based approach for the diagnosis of male infertility as related to the eight prioritized PICO questions.

SEARCH METHODS: Collating the evidence to support providing recommendations involved a collaborative process as developed by WHO, namely: identification of priority questions and critical outcomes; retrieval of up-to-date evidence and existing guidelines; assessment and synthesis of the evidence; and the formulation of draft recommendations to be used for reaching consensus with a wide range of global stakeholders. For each draft recommendation the quality of the supporting evidence was then graded and assessed for consideration during a WHO consensus.

OUTCOMES: Evidence was synthesized and recommendations were drafted to address the diagnosis of male infertility specifically encompassing the following: What is the prevalence of male infertility and what proportion of infertility is attributable to the male? Is it necessary for all infertile men to undergo a thorough evaluation? What is the clinical (ART/non ART) value of traditional semen parameters? What key male lifestyle factors impact on fertility (focusing on obesity, heat and tobacco smoking)? Do supplementary oral antioxidants or herbal therapies significantly influence fertility outcomes for infertile men? What are the evidence-based criteria for genetic screening of infertile men? How does a history of neoplasia and related treatments in the male impact on (his and his partner's) reproductive health and fertility options? And lastly, what is the impact of varicocele on male fertility and does correction of varicocele improve semen parameters and/or fertility?

WIDER IMPLICATIONS: This evidence synthesis analysis has been conducted in a manner to be considered for global applicability for the diagnosis of male infertility.

Key words: male infertility / spermatozoa / genetics / Y deletions / cystic fibrosis transmembrane conductance regulator / semen analysis / varicocele / evidence-based guideline / cancer

Introduction

In 2012, the World Health Organization (WHO) held a meeting of experts to scope the field of fertility care in order to develop comprehensive guidelines on infertility. Six Evidence Synthesis Groups (ESG) were established. One group was the WHO ESG on Male Infertility: Diagnosis. Following the initial meeting, key PICO (problem or population (P), intervention (I), comparison (C) and outcome(s) (O)) questions were developed and agreed upon. This included working through the WHO GDG (Guideline Development Group) Committee, as well as through web-based surveys, and through outreach to developing country scholars taking an on-line Geneva Foundation for Medical Education and Research- American Society for Reproductive Medicine- (GFMER-ASRM) WHO evidence-based infertility course. For each PICO question, a systematic analysis of the literature was performed according to the 'WHO handbook for guideline development' (WHO, 2014). A preliminary analysis of the data was presented to the WHO/GDG Steering Committee Working Experts Consultation in December 2014 during which modifications were made to various components of a few of the PICO questions, and additional PICOs were also identified. A comprehensive document including draft recommendations was presented to the WHO/GDG Steering Committee Meeting for Guidelines and Nomenclatures in September 2015 (Fig. 1). This manuscript provides a narrative review of the evidence synthesized by the ESG that helped to generate the recommendations (Table I), provides an update of the evidence as recommended through expert review, defines some of the challenges in addressing these questions and discusses current research gaps. It concludes by presenting future research opportunities and outlines how these may be realized.

Methods

The key PICO questions

The following eight topics were pre-determined and identified by WHO, and were later formulated into PICO questions for systematic analysis, as follows:

- What is the prevalence of male infertility and what proportion of infertility in the couple is attributable to the male?
- Is it necessary for all infertile men to undergo a thorough evaluation?
- What is the clinical (ART/non ART) value of traditional semen parameters?
- What key male lifestyle factors impact on fertility (focusing on obesity, heat and tobacco smoking)?

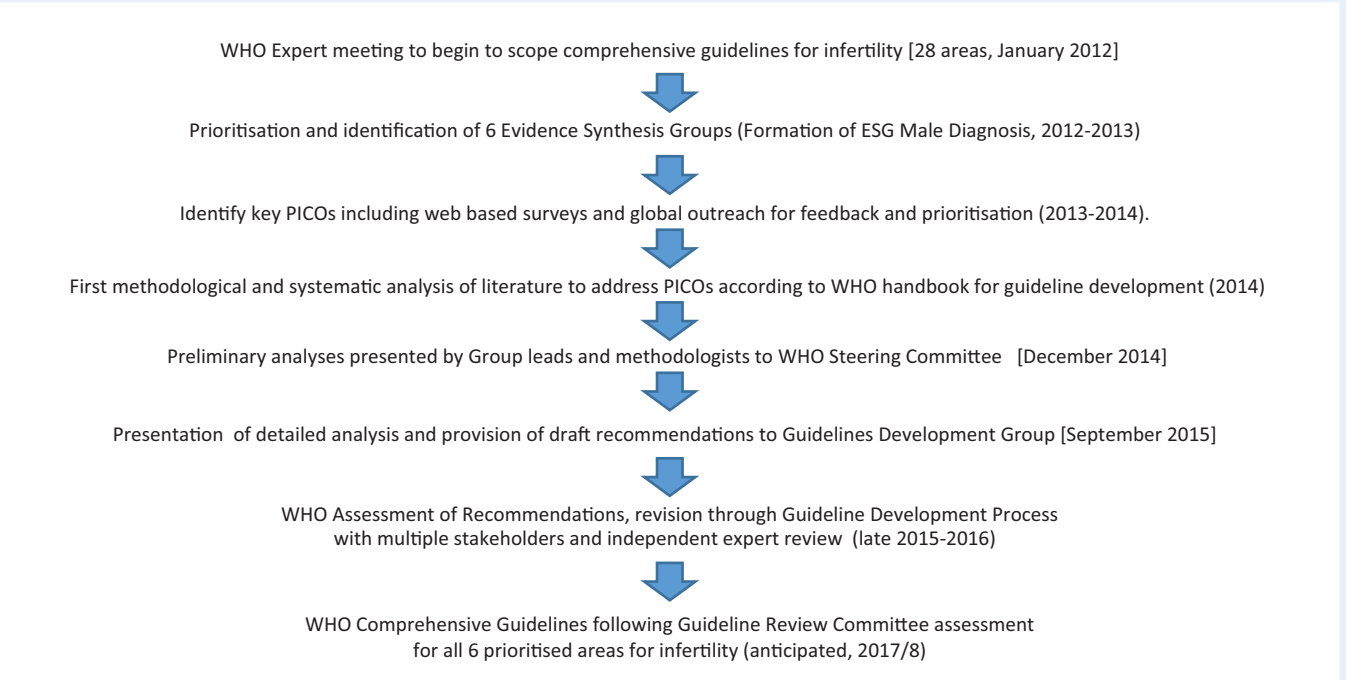

Figure 1 Outline flowchart of WHO methodology for ESG Male Diagnosis. Flowchart outlining the WHO process for obtaining the evidence, and formulating and presenting recommendations for male infertility (Diagnosis). This includes stages and methods for synthesis of evidence according to WHO process. Dates in square bracket reflect specific meetings at WHO in Geneva. PICO: problem or population (P), intervention (I), comparison (C) and outcome(s) (O). WHO, World Health Organization; ESG, Evidence Synthesis Group.

- Do supplementary oral antioxidants or herbal therapies significantly influence fertility outcomes for infertile men?
- What are the evidence-based criteria for genetic screening of infertile men?
- How does a history of neoplasia and related treatments in the male impact on (his and his partner's) reproductive health and fertility options?
- What is the impact of varicocele on male fertility and does correction of varicocele improve semen parameters and/or fertility?

Outline evidence synthesis methodology

The methodology used to support the provision of the recommendations was outlined by the WHO (2014) namely: identification of priority questions and critical outcomes; retrieval of up-to-date evidence and existing guidelines; assessment and synthesis of the evidence; and formulation of draft recommendations to be used for reaching a consensus with a wide range of global stakeholders. For each recommendation the quality of the supporting evidence would then be graded (very low, low, moderate and high) for consideration for the consensus. For example, a rating of high quality of evidence means that further research is very unlikely to change our confidence in the estimate of the effect. Conversely a rating of very low quality of evidence means that any effect is very uncertain.

We qualified the strength of our recommendations (as strong or weak) based upon consideration of the quality of the evidence. These recommendations were then later assessed through the WHO guideline development processes that are based upon other factors including values and preferences of stakeholders, the magnitude of effect, the balance of benefits versus harm, resource used and the feasibility of implementation.

Overall, wherever possible, original literature, and data from recently published systematic and Cochrane reviews were used. However, due to the diagnostic nature of the questions, a significant amount of the literature addressing them does not lend itself to high evidence level RCTs. Therefore, in addition, key guidelines and professional committee opinions were examined, in order to assist in developing a broader and more comprehensive evidence base, as well as in the construction of recommendations, and in particular: ASRM—Diagnostic evaluation of the infertile male: a committee opinion (2015) (ASRM, 2015a); American Urological Association [AUA] Best Practice Statements (updated 2010 and 2011); EAU Guidelines on Male Infertility (Jungwirth *et al.*, 2015); and the Society for Male Reproduction and Urology (2014) Report on varicocele and infertility.

Data were presented in summary form and descriptively, in tables or narratively in the evidence reviews for each PICO question. Where appropriate, meta-analyses were conducted. The GRADE framework was applied to the body of evidence for each outcome within each PICO. The WHO then used the worksheets to summarize the volume and quality of the evidence supporting the recommendations as well as to outline the values, preferences and judgements made about the strength of recommendations. The uniqueness of the WHO process was that these balanced worksheets were also to be used to note considerations especially for low- and middle-income countries or settings, and to be able to record the reasons for changes made to the default strength of the recommendations.

The principles or best practice guidance need to be consensus-based and are also intended to underscore the importance of respect for reproductive rights and dignity as recipients of care, and the need to maintain high ethical and safety standards in clinical practice. These principles, in addition to the strategies for implementation, monitoring and evaluation, are expected to guide end-users in the process of adapting and implementing any recommendation provided by the WHO to consider for a range of global contexts and settings.

Table I Male Factor Infertility Diagnosis: Summary Recommendations.

Clinical questions	RECOMMENDATIONS through assessment of developed PICO question and associated evidence analysis	Strength of the evidence
1. What is the prevalence of male infertility and what proportion of infertility is attributable to the male?	It is not possible to determine an unbiased prevalence of male infertility in the general population.	Very low
2. Is it necessary for all infertile men to undergo a thorough evaluation?	The initial evaluation for male factor infertility should include a PE performed by an examiner with appropriate training and expertise, a reproductive history and at least one properly performed (high quality) semen analyses. A full evaluation by a urologist or other specialist in male reproduction should be done if the initial screening evaluation demonstrates an abnormal PE, an abnormal male reproductive or sexual history, or an abnormal semen analysis is found. Further evaluation of the male partner should also be considered in couples with unexplained infertility and in couples in whom there is a treated female factor and persistent infertility	Moderate
3. What is the clinical (ART/non ART) value of traditional semen parameters?	Assessment of a combination of several ejaculate parameters is a better predictor of fertility success than a single parameter	High
	Analysis of a single ejaculate is sufficient to determine the most appropriate investigation and treatment pathway although semen analysis could be repeated if one or more abnormalities is found	High
4. What key male lifestyle factors impact on fertility?	Evidence supports a detrimental effect of obesity on many aspects of health; evidence is conflicting about a potential effect on reproductive function. Males presenting for fertility evaluation should be counseled about weight-loss strategies when the BMI and waist circumference data demonstrate obesity and especially morbid obesity.	Moderate
	There is insufficient evidence to conclude that exposure to heat, be it occupational or as a result of clothing or body position, affect semen quality and/or male fertility	Very low
	There is some evidence to suggest a negative effect of cigarette (tobacco) smoking on semen quality but not all studies report this. However, as smoking has an adverse effect on general health and wellbeing it is recommended that men trying for a pregnancy should abstain from smoking	Moderate
5. Do supplementary oral antioxidants or herbal therapies significantly influence fertility outcomes for infertile men?	There are insufficient data to recommend the use of supplemental antioxidant therapies for the treatment of men with abnormal semen parameters and/or male infertility	Low
	There are insufficient data to recommend the use of herbal therapies for the treatment of men with abnormal semen parameters and/or male infertility	Very low
6. What are the evidence-based criteria for genetic screening of infertile men?	Karyotype testing should be performed on all males with severe oligozoospermia ($<5\times10^6$/ml) or NOA prior to any therapeutic procedure	High
	YCMD testing should be performed on all males with severe oligozoospermia prior to a therapeutic procedure or NOA prior to any therapeutic procedure	High
	Appropriate *CFTR* mutation analysis should be offered to all males with CBAVD or CF	High
7. How does a history of neoplasia and related treatments in the male impact (his and his partner's) reproductive health and fertility options?	Every male cancer patient should be provided with information about the impact of his cancer treatment on spermatogenesis and the option of sperm banking	Moderate
	Patients should be advised to use contraception if they do not wish to procreate even after prolonged periods of azoospermia following radiotherapy, as recovery is possible	Low
	Male cancer patients should be informed that pregnancy outcomes in partners of male cancer survivors are good but a slightly higher risk of congenital anomalies in their offspring cannot be excluded	Low

Continued

Table I *Continued*

Clinical questions	RECOMMENDATIONS through assessment of developed PICO question and associated evidence analysis	Strength of the evidence
8. What is the impact of varicocele on male fertility and does correction of varicocele improve semen parameters and/or fertility?	Good Practice Point: Treatment of a clinically palpable varicocele may be offered to the male partner of an infertile couple when there is evidence of abnormal semen parameters and minimal/no identified female factor, including consideration of age and ovarian reserve	Very low
	Good Practice Point: IVF with or without ICSI may be considered the primary treatment option when such treatment is required to treat a female factor, regardless of the presence of varicocele and abnormal semen parameters	Very low
	Good Practice Point: The treating physician's experience and expertise, including evaluation of both partners, together with the options available, should determine the approach to varicocele treatment	Very low

PICO, problem or population (P), intervention (I), comparison (C) and outcome(s) (O); CBAVD, Congenital Bilateral Absence of the Vas Deferens; PE, physical examination; YCMD, Y chromosome microdeletion; NOA, non-obstructive azoospermia.

The evidence based and detailed analysis, with GRADE tables where possible for each of the prioritized PICO questions, were commissioned by and provided by the first author to the WHO in support of their guideline processes. A WHO assessment of our evidence-based outcomes was then undertaken with many stakeholders who evaluate other factors including values and preferences of stakeholders, the magnitude of effect, and the balance of benefits versus harms, resource use and the feasibility of implementation to better assure global applicability. As required by WHO, following these outcomes, additional independent expert review would be conducted (2016 and early 2017). Once completed, the WHO will be publishing their expert and stakeholder consensus-driven guidelines together with the detailed evidence base (evidence tables, detailed search strategies, balanced worksheets etc.) and related products (Fig. 1).

The present manuscript provides a narrative of the evidence. It particularly focuses on areas where evidence is controversial, of poorer quality and more challenging to obtain. It discusses what is missing from the analysis and critically provides a discussion of potential research gaps. It is not the purpose of this manuscript to reproduce the original documents submitted to the WHO. As it was necessary to undergo a global prioritization method to identify answerable PICO questions, there inevitably are a number of questions in the diagnosis of male infertility not addressed by the WHO ESG and thus absent from this manuscript. For example, the effect of paternal age, alcohol and environment on male fertility (those interested can consult for example: Age—Eisenberg & Meldrum, 2017; Nybo-Andersen and Urjoj, 2017; Johnson *et al.*, 2015; Ramasamy *et al.*, 2015; Alcohol—Karmon *et al.*, 2017; Jensen *et al.*, 2014; Oil and natural gas extraction—Balise *et al.*, 2016; Bisphenol A—Mínguez-Alarcón *et al.* 2016; Outdoor air pollution—Lafuente *et al.*, 2016).

Summary of outcomes and narrative review as related to the eight prioritized PICO questions

The specific recommendations formulated for the presentation to the WHO on the diagnosis of male infertility are included in the text along with an assessment of the quality of the supporting evidence (Table I) and strengths of recommendations based upon our evidence synthesis. The final recommendations will only result following an independent expert review of our work and review by stakeholder societies, following assessment through the Guidelines Review Committee of WHO.

What is the prevalence of male infertility and what proportion of infertility is attributable to the male?

This is a simple and fundamental question. It is critical to know the prevalence of a disease in order to provide resources, estimate impact, make effective health economic arguments, present rational research questions and manage patients. Investigators studying other diseases often have the incidence of the disease well established in a variety of different populations. However, for male infertility this remarkably simple question is surprisingly very difficult to answer.

The most recent publication presents a population prevalence estimate of infertility amongst 15 162 men and women in the UK (Datta *et al.*, 2016). This was a cross-sectional survey asking if the participants had ever had a time, lasting 12 months or longer, when they and their partner were trying for a pregnancy but it did not happen. One in eight women (12.5%, 95% CI 11.7–13.1) and one in ten men (10.1%, 95% CI 9.2–11.1) answered yes to this question and thus had experience of infertility. This type of study needs repeating in a number of different geographical regions.

Addressing the prevalence of male infertility is a challenging one. For example, a difficulty arises from a lack of continuity in the definitions of infertility. Generally, infertility is defined as failure of a 'couple' to become pregnant despite 12 or more months of unprotected intercourse. However, some studies such as Hull *et al.* (1985) include in their definition couples who become pregnant but miscarry. Other studies such as Anderson *et al.* (2009) and Gurunath *et al.* (2011) include those who seek medical advice in order to be able to make a partner pregnant. There is no current method to capture men as individuals or in same-sex relationships, who may desire a biological child through ART, and who may be found to be infertile. Furthermore, there is also generally a lack of differentiation between

primary and secondary infertility in the heterosexual male, and the relative rates of primary and secondary infertility vary significantly between studies, especially when comparing clinic-based and population-based studies (see Malekshah *et al.*, 2011 versus Klemetti *et al.*, 2010).

The varying definitions used for male infertility and the fact that men are not always evaluated (Pastuszak *et al.*, 2016) can result in misleading study conclusions. Mehta *et al.* (2016) recently documented many of these obstacles when trying to obtain an accurate assessment of the prevalence of male infertility in the USA. Consequently, it is not surprising that there are currently no rigorous systematic reviews or meta-analyses on the epidemiology of male infertility. Agarwal *et al.* have attempted to pursue this type of review (Agarwal *et al.*, 2015) but due to a paucity of high-quality comparable studies they were unable to make robust conclusions. There are significant variations in the variables assessed between studies, including, but not limited to the age of the participants, the participants themselves (individual males, females or couples), the method of data collection and the outcomes measured. These caveats create inconsistencies in the study results, and consequently, studies of male infertility can generally be divided into two categories: those that seek to determine the prevalence or incidence of the experience of infertility amongst men, or those which focus on the proportion of total infertility that is attributable to the male factor. For example, it is insufficient to simply ask men if they experienced infertility because this information does not give a true representation of male factor infertility (as their partner could be the cause of the infertility). On the other hand, clinical studies of diagnosed male factor infertility itself often suffer from small sample size (Geelhoed *et al.*, 2002) or a biased population—those that consult—which could skew the data. Not all couples experiencing infertility choose to consult a physician and, of couples that do consult, not all will have experienced greater than 12 months of infertility. For example, Louis *et al.* (2013) reported a higher prevalence of infertility than Anderson *et al.* (2009) using the same study population, because the latter's definition of infertility was restricted to men who had gone for a consultation. Similarly, van Roode *et al.* (2015) reported a higher prevalence of infertility amongst 38-year-old men (18.3% compared to 14.4%) when they expanded their definition of infertility to include those who had sought medical help to generate a pregnancy. The selection of subject populations is often inherently biased. And in the case of assessing male infertility, for example, studies performed on specific populations, such as military recruits, may not reflect the general population. Large-scale studies of the prevalence of infertility generally focus on women's experience of infertility as reported in demographic surveys which are based upon contraceptive usage (e.g. Gurunath *et al.*, 2011, Mascarenhas *et al.*, 2012) and few large-scale studies are able to gather data on men. Van Roode *et al.* (2015) reported a large difference in the diagnosis of fertility problems when asking women in a survey compared to asking men.

Current studies of male infertility often employ cross-sectional population study designs (e.g. Datta *et al.*, 2016 above), or are observational studies of those men who present to infertility clinics. Only one prospective birth cohort study was identified (van Roode *et al.*, 2015). A further limitation of the available literature is that some studies are relatively old, such as Hull *et al.* (1985) and Thonneau *et al.* (1991); studies which have not been updated in a quarter of a century. There may also be a geographical variation in the incidence of male infertility—one study in France suggested that male factor alone accounted for 20% of total infertility (Thonneau *et al.*, 1991) whilst a study in Western Siberia put the figure at 6.4% (Philippov *et al.*, 1998). It is unclear whether these represent true geographical variations or simply differences in methodology.

Suffice it to say that, based on current evidence, few reliable conclusions can be drawn about the epidemiology of male infertility. Several studies have suggested that male factor infertility is the single most common diagnosis among heterosexual couples who struggle to become pregnant but definitions and diagnosis of male factor vary and several other studies report that female factor infertility is more prevalent. Nevertheless, all of these studies highlight the need for further research.

One simplistic and frequently used approach to assess male infertility has been to examine semen parameters in men of the general population and determine the frequency of semen abnormalities against standard ranges (Cooper *et al.*, 2010, Virtanen *et al.*, 2017). A plethora of studies have done this and also used this information in an attempt to address changes in semen quality over time (Virtanen *et al.*, 2017). The advantage of these studies is that they can provide comparable data but only if first the populations are well characterized and second the laboratory methods used to determine semen quality are robust and consistent across study sites (cf. Björndahl *et al.*, 2016). However, the primary disadvantage of this approach is that semen parameters alone are not equivalent to defining infertility/fertility (MacLeod, 1950; Guzick *et al.*, 2001; Cooper *et al.*, 2010). As such, the focus of the current analysis was on the proportion of heterosexual men who experience a delay (extended time) in inducing a pregnancy (Fig. 2).

In summary, we strongly recommend, owing to the very low quality of evidence, that it is not currently possible to determine an unbiased prevalence of male infertility within the global, regional or national populations, including neglected individual populations. Additionally, it is not currently possible to determine what proportion of infertility in heterosexual couples is attributable to the male partner (Table I).

A number of topics were identified for future research. There is a need for large population-based studies to determine the prevalence of male infertility in the general population of males whether in a relationship or not. Ideally, large population-based cohort studies conducted in a number of different geographical regions must be carried out with consistent definitions of infertility and comparable clinical study designs.

Is it necessary for all infertile men to undergo a thorough evaluation?

Medical conditions in the male may be causative of the infertility (such as hypogonadotropic, hypogonadism or bilateral cryptorchidism) or associated with the infertility (testis tumour in male with normal semen analysis). The rationale for evaluating the male and the extent of that evaluation depends on the goals of the evaluation. Several medical best practice statements (AUA, 2011; ASRM, 2015a) suggest that the goals of the evaluation of the male are to identify: conditions that can be corrected; conditions that are irreversible for which ART will be needed using the male partner's sperm; irreversible conditions for which the male partner's sperm will not be available or appropriate and may require consideration of donor sperm

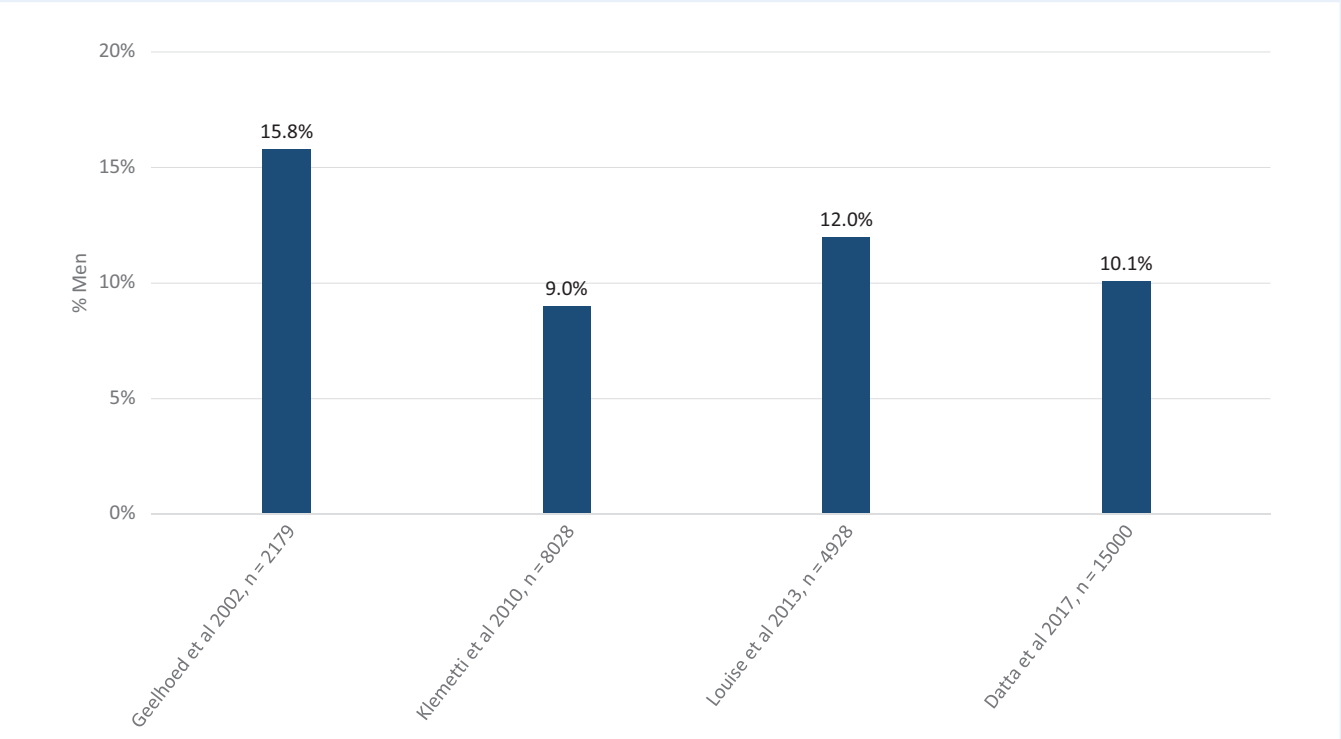

Figure 2 Prevalence of male infertility. Prevalence of male infertility in surveys of general populations. Male infertility was generally defined as men reporting experience of infertility (generally >12 months in duration).

or adoption; serious medical conditions that may be causing or present with male infertility and that could affect the health of the male and require medical treatment; and genetic causes of male infertility that could affect the success of treatment or the health of offspring if ART is utilized.

Evaluations of populations of infertile men have identified patients in each of these categories emphasizing the need for evaluation of the male (Nieschlag and Behre, 2001; Tournaye *et al.*, 2016; Olesen *et al.*, 2017; Punab *et al.*, 2017, Pastuszak *et al.*, 2016). An initial evaluation of the male consists of three primary components: history, physical examination (PE) and semen analyses. There is general agreement about the importance of obtaining a reproductive history (including a sexual history) and semen analyses. The ASRM (ASRM, 2015a) suggest that the reproductive history should include: coital frequency and timing; duration of infertility and previous fertility; childhood illnesses and developmental history; systemic medical illnesses (such as diabetes mellitus and upper respiratory diseases); previous surgery; medications and allergies; sexual history (including sexually transmitted infections); and exposures to gonadotoxins (including environmental and chemical toxins and heat). A discussion point, however, is the need, timing of investigations, and indications for PE; and it is in this area where the current guidance appears to be inconsistent. Practice statements by the AUA and ASRM recommend an initial evaluation of all males of infertile couples that consists of a detailed reproductive history and semen analyses. For example, the ASRM states 'At a minimum, the initial screening evaluation of the male partner of an infertile couple should include a reproductive history and analysis of at least one semen sample' (ASRM, 2015a). The ASRM then recommends that those men with risk factors in their reproductive history or abnormal semen parameters should be referred to a male reproductive specialist for a more thorough evaluation that includes a PE. Notably, both organizations (ASRM and AUA) recommend consideration of a full male evaluation (including PE) in those couples with unexplained infertility or those that remain infertile after correction of female factors. The European Association of Urology (EAU) states 'A medical history and PE are standard assessments in all men' (Jungwirth *et al.*, 2015). Others have suggested a full evaluation of all men in infertile relationships (Honig *et al.*, 1994; Kolettis and Sabanegh, 2001; Olesen *et al.*, 2017; Punab *et al.*, 2017), which includes a PE.

The question is: Which diagnostic strategy is optimal? A significant number of identified male factors are associated with abnormalities found by semen analysis. However, the aetiology remains to be robustly quantified, especially in light of causes of infertility such as birth defects, acquired forms of infertility, infection, inability to have an erection or ejaculation, various syndromes as well as metabolic and endocrine disorders. Sexual dysfunction may be associated with normal semen parameters but can be identified through a sexual history. Additionally, there are a number of genetic syndromes that predispose or cause male infertility some of which are associated with abnormal semen parameters. An initial evaluation of the male consisting of a reproductive history, a simple PE and semen analysis would potentially identify the majority of these cases, however, these evaluations must also be sensitive to different cultural practices and different regional aetiologies, e.g. HIV, genital tuberculosis (TB) in TB endemic areas, lifestyle, environmental and occupational hazards. Significant medical conditions have been reported in 1.1–6% of men presenting for infertility evaluations and a number of these men have

abnormal semen parameters. Importantly, however, there are limited data on the incidence of significant medical conditions that predispose men to infertility. One older data series reported that 0.16% of men had significant medical conditions but normal semen parameters indicating that conditions will be missed by limiting a male assessment to a reproductive history and semen analysis (Honig *et al.*, 1994).

Consequently, there are several approaches that can be utilized consisting of the following possibilities. One approach is that all sub-fertile men should have an initial evaluation with history, PE by an examiner with appropriate training and expertise, and a semen analysis. Importantly, this will pick up conditions missed by excluding a PE and is consistent with the EAU guidelines (Jungwirth *et al.*, 2015). Alternatively, initial evaluation consists of detailed reproductive history and semen analyses; in this scenario, only if either is abnormal does the male undergo a more thorough history and a PE. However, men with significant medical conditions will be missed by this approach. On balance, there are, on a global scale, a number of advantages and few disadvantages to include a PE performed by an examiner with appropriate training and expertise as part of this initial evaluation. For example, in low- to middle-income countries where visits to infertility health professionals will be more restricted due to factors such as geography and costs, it is less likely that a man will return to the clinic even if the results of a semen analysis are abnormal.

Interestingly, both ASRM and EAU ASRM do not recommend endocrine testing as a primary first line investigation. For example, the ASRM (2015a) suggest endocrine testing in men with abnormal semen parameters (particularly when the sperm concentration $1 < 10$ million/ml), impaired sexual function or clinical findings that suggest a specific endocrinopathy. And, as ASRM state, some experts think that all infertile men merit an endocrine evaluation (Ventimiglia *et al.*, 2016; Olesen *et al.*, 2017). What is important is that the key recommendations are verified in different populations to establish how robust they are, and if required such recommendations are amended. Interestingly, Ventimiglia *et al.* have recently examined the ASRM indications for endocrine assessments in a cross-sectional study of 1056 infertile men to predict hypogonadism. Using the same database, the authors developed a logistic regression-based nomogram including testis volume measured during the physical exam, BMI and azoospermia to predict total testosterone levels of <3 ng/dl. Although, their nomogram had a higher predictive accuracy (68%) than ASRM's guidelines (58%), they concluded, based on their statistical analyses, that their nomogram also was not reliable enough to predict hypogonadism. These examples emphasize the importance of validating recommendations in a variety of populations.

In summary, we strongly recommend based on a moderate quality of evidence that:

- The initial evaluation for male factor infertility should include a PE performed by an examiner with appropriate training and expertise, a reproductive history, and at least one properly performed (high quality) semen analyses.
- A full evaluation by an urologist or other specialist in male reproduction should be carried out if the initial screening evaluation demonstrates an abnormal PE, an abnormal male reproductive or sexual history, or an abnormal semen analysis is found.
- Further evaluation of the male partner should also be considered in couples with unexplained infertility and in couples in whom there is a treated female factor and persistent infertility (Fig. 3, Table I).

There are significant areas for future research. For example, what constitutes the most appropriate minimal PE and does this provide significant additional information for male health in general, does performing a PE improve male engagement in diagnosis and treatment, what is the cost-effectiveness of doing a PE on all men attending an infertility clinic instead of doing a PE only after abnormal findings in semen analysis and/or an abnormal reproductive and sexual history, can a globally validated questionnaire/topic list for reproductive and sexual history be used (encompassing low-income settings) to identify individuals at risk for male infertility, and Is the outcome better with earlier diagnosis and is treatment less expensive with earlier diagnosis?

What is the clinical (ART/non ART) value of traditional semen parameters?

Specifically, we posed two clinical questions. This first question was whether the predictive value of semen analysis for reproductive outcome is better using a combination of several parameters compared to a single semen analysis parameter, and the second was whether an evaluation of a single ejaculate versus two ejaculates is sufficient for referral to infertility investigation and treatment.

A fundamental challenge in the analysis of the literature is the quality of the laboratory testing which is often performed using suboptimal methods (Keel, 2004; Björndahl *et al.*, 2016). This is illustrated by data presented from three recent national quality control programmes in Belgium Germany and Italy (Mallidis *et al.*, 2012; Filimberti *et al.*, 2013; Punjabi *et al.*, 2016) documenting that, in general, a number of laboratories do not adhere to WHO methods for semen analysis. Lack of adherence to recommended and appropriately standardized methods significantly undermines the potential diagnostic value. Effective strategies to address this issue remain a subject of significant debate (e.g. Carrell and De Jonge, 2016).

The analysis of studies to address this PICO question was limited to those published after 1 January 2000, based on two key assumptions. First, there has been a very slow increase in compliance with WHO recommendations for semen analysis. The fourth edition was published in 1999 and few publications before that bear evidence of significant and clear compliance with the recommended techniques (see Tomlinson *et al.*, 1999). Second, evaluation of the prognostic value of semen analysis is dependent on the quality of the clinical interventions available. It is, therefore, likely that 'historic' data for ART success are not relevant for treatments available in recent years as the ART techniques have significantly improved (Wade *et al.*, 2015).

Another major challenge which has been recognized for over 70 years is the substantial overlap in the distribution of semen analysis results in fertile men and those from men in infertile couples (MacLeod, 1950; Guzick *et al.*, 2001; Cooper *et al.*, 2010). This means that comparing a patient's semen parameters to the distribution of results for fertile men is not in itself sufficient to determine whether or not the patient is fertile or infertile (Björndahl, 2011). Additionally, semen analysis is only part of the investigation of the man and a number of other attributes contribute to his fertility potential.

With regard to the first question namely, Is the predictive value of semen analysis for reproductive outcome better using a combination of several parameters compared to a single semen analysis parameter?

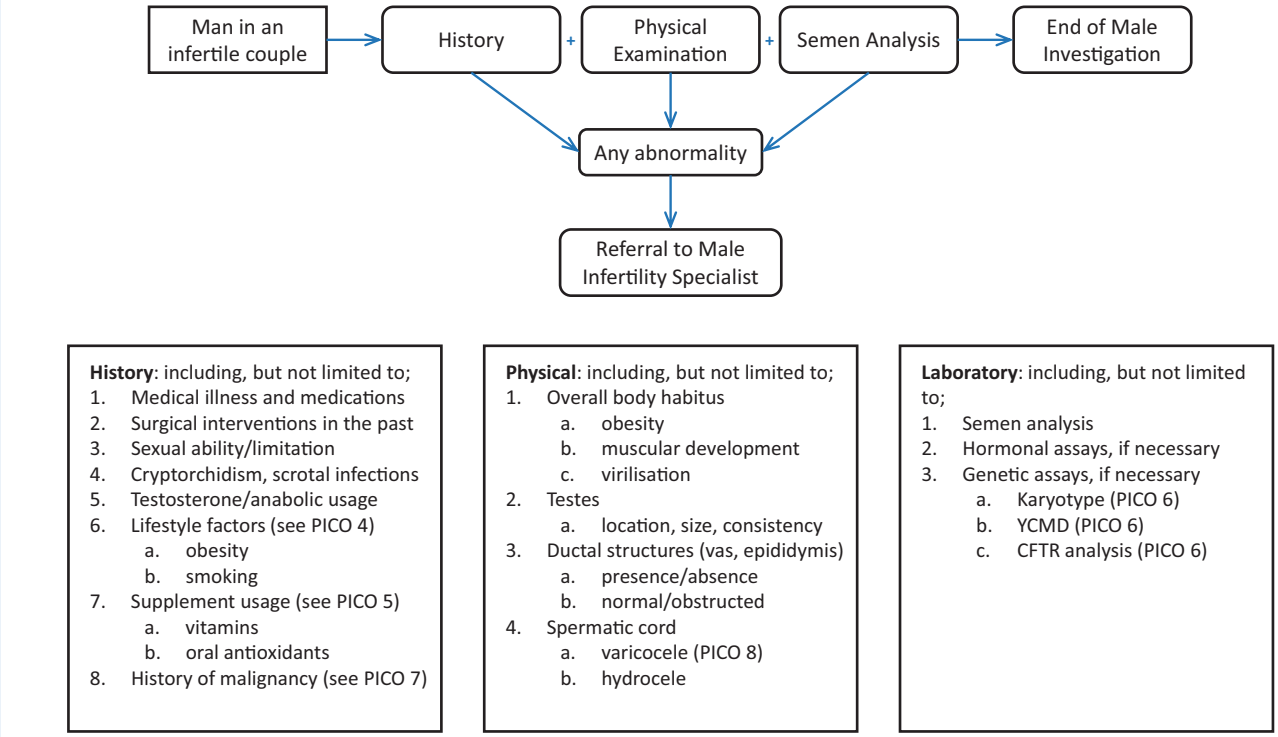

Figure 3 Flowchart summary of algorithm for diagnosis of male infertility. As detailed in section PICO 2 (Is it necessary for all infertile men to undergo a thorough evaluation?) the first line investigations should include Physical Examination, History and Semen Analysis. Abnormalities in these lead to further investigations. YCMD, Y chromosome microdeletion; CFTR, CF transmembrane conductance regulator.

Studies were regarded as eligible if they presented primary data on predictive values [or odds ratios (OR)] concerning multiple or single analysis parameters. Four studies provided data on multiple assessments of parameters (Zinaman *et al.*, 2000; Guzick *et al.*, 2001; Jedrzejczak *et al.*, 2008; van der Steeg *et al.*, 2011, total $n = 5022$) and 10 studies focussed on single ejaculate parameters as predictors of fertility. Studies identifying a single semen parameter with significant predictive power for fertility are relatively common but the discriminatory power is very often low e.g. expressed as receiver operating characteristic curve/area under the curve (ROC–AUC) close to 0.500 or OR including 1.00 in the 95% CI. In contrast, studies that have investigated multiple parameters, which are more reflective of testicular production/function and maturation, for example Guzick *et al.* (2001) and Jedrzejczak *et al.* (2008), present OR or predictive values, that are comparable to diagnostic laboratory tools in other areas of modern clinical medicine (Boyd, 2010). As such the conclusion of this analysis was that examination of multiple parameters was more predictive.

In summary, we strongly recommend based on a high quality of evidence that the assessment of a combination of several ejaculate parameters is a better predictor of fertility success than a single parameter (Table I).

The second fundamental question is commonly posed but rarely answered: Is an evaluation of a single ejaculate versus two ejaculates sufficient for referral to infertility investigation and treatment? This was considered important globally, due to the low level of male engagement to address reproductive health issues in many settings. As above, the analysis was limited to studies published after 1 January 2000 primarily due to the presumed advances in quality in laboratory andrology. In this analysis, studies were only eligible if they provided appropriate information to ascertain the reliability of the data obtained from semen analysis, and they presented primary data concerning the usefulness of repeat analyses [e.g. intraclass correlation coefficient (ICC), where an ICC close to 1.00 indicates high reliability between a pair of assessments].

Only five studies (Francavilla *et al.*, 2007; Stokes-Riner *et al.*, 2007; Mishail *et al.*, 2009; Leushuis *et al.*, 2010; Christman *et al.*, 2013 total $n = 6482$) provided information that could be used to support or reject a recommendation concerning analysis of a single versus two ejaculates (see Supplementary Data for a detailed analysis of the studies). Analysis that included measures such as the ICC demonstrates the reliability of a single ejaculate for referral to infertility investigation and treatment leading to the conclusion that examination of a single ejaculate is sufficient. This conclusion is consistent with ASRM and EAU recommendations. For example, the ASRM (2015a) state 'that at a minimum, the initial screening…should include analysis of at least one semen sample' and the EAU state that 'if the results of semen analysis are normal according to WHO criteria, one test is sufficient' (Jungwirth *et al.*, 2015). The question is whether there is a sub-group of men being investigated for infertility that require a repeat semen analysis. If so, which group would this be? It is most likely to be men with ejaculate results in between very good and very poor, i.e. those

in the 'intermediate' range (Guzick *et al.*, 2001). Guzick *et al.* (2001) presented a model where patients were divided into three categories: poor, intermediate and good ejaculate results. Creating three groups for men undergoing infertility investigation may seem somewhat hypothetical. However, there is a considerable overlap in ejaculate analysis results from fertile and infertile men and the intermediate range corresponds largely to this mixed group. For men with results in the 'intermediate' group a repeat analysis could provide further information—confirming earlier results or pointing to a less or more severe problem. It is, therefore, logical to use a 'borderline zone' between good and very poor results (Guzick *et al.* 2001; Björndahl, 2011), and perhaps restrict repeat analysis to this mixed group.

However, a recommendation that analysis of a single ejaculate is sufficient to determine the most appropriate investigation and treatment pathway is a controversial strategy. In some cases, this appears contrary to conventional clinical practice where a plethora of well-documented variables are known to affect semen analysis thus potentially reducing the clinical value of a single ejaculate. Additionally, analyses of only one ejaculate is contrary to previous standard WHO recommendations. However, a primary reason for the variability in semen parameters is the failure of some laboratories to adhere to standard WHO recommendations (Mallidis *et al.*, 2012) and failure to control for key parameters e.g. abstinence, that increases the variability of the test. Importantly, the majority of these can be mitigated by adopting and adhering to appropriate practices and WHO procedures (WHO, 2010). Notwithstanding this, adoption of a strategy of analysis of a single ejaculate should be accompanied by a detailed cost-benefit analysis to examine if and at what stage additional semen assessment, particularly in the borderline zone, is appropriate. It is also important to emphasize that this recommendation applies only to referral for infertility investigation and treatment, and is not relevant, for example, if the aim of a study it to establish a 'true' value of sperm production or sperm output rate, for example, where a single ejaculate is not sufficient (Amann and Chapman, 2009).

In summary, we strongly recommend that based on a high quality of evidence that analysis of a single ejaculate is sufficient to determine the most appropriate investigation and treatment pathway although semen analysis could be repeated if one or more abnormalities are found (Table I).

There are a number of significant areas for future. First, for example, there is a need for large multi-centre studies to examine the predictive values in semen analysis to identify men likely to contribute to spontaneous pregnancy, ART pregnancy, fertilization failure, pregnancy loss/miscarriage, time to pregnancy (TTP) and live birth. Second, a fundamental problem with developing new therapies or diagnostic tests for male infertility is the limited understanding of the formation, maturation and physiological workings of the normal and dysfunctional spermatozoon. There is an urgent requirement to understand these cellular, molecular biochemical and genetic mechanism(s) in order to formulate appropriate diagnostic assays and rational therapy for the male.

What key male lifestyle factors impact on fertility?

This is, and is likely to remain, a topical issue. The focus of the analysis was on obesity, smoking and heat exposure.

Does obesity influence semen parameters?

Obesity is a global health problem. It impacts not only cardiovascular diseases but also on many other related health disorders. Obesity may adversely affect male reproduction by endocrinologic, thermal, genetic and sexual mechanisms (Reis & Dias, 2012). As such, obesity must be considered as a potential causal factor in male infertility. However, two key meta-analyses published in this area show conflicting data (MacDonald *et al.*, 2010; Sermondade *et al.*, 2013) with the latter concluding: 'overweight and obesity were associated with an increased prevalence of azoospermia and oligozoospermia'. Additionally, there are a number of cross-sectional and longitudinal studies (MacDonald *et al.*, 2013; Eisenberg *et al.*, 2014 and Andersen *et al.*, 2015) that reported some negative associations between semen parameters and obesity. A cross-sectional study of New Zealand males (2013) showed that morphology was the only parameter that correlated with BMI (MacDonald *et al.*, 2013). A longitudinal study of American males reported an increased OR for decreased ejaculate volume and total sperm count associated with obesity (Eisenberg *et al.*, 2014). Furthermore, the OR for lower sperm concentration and total sperm count increased with waist circumference. A cross-sectional study of Norwegian men found a significant decline in all standard semen quality markers with increasing BMI. BMI was also negatively associated with hormones of reproduction (Andersen *et al.*, 2015). A cross-sectional study of 4400 men attending infertility clinics in the USA reported a significant negative relationship between obesity and semen parameters (Bieniek *et al.*, 2016). Moreover, the incidence of azoospermia and oligozoospermia was more prevalent in obese men. Data from the CHAPS-UK study found no evidence for an effect of BMI on either motile concentration (Povey *et al.*, 2012) or sperm morphology (Pacey *et al.*, 2014). The ASRM concluded in their 'Obesity and reproduction: a committee opinion paper committee opinion' paper in 2015 that 'obesity in men may be associated with impaired reproductive function' (ASRM, 2015b).

Based on the number of papers able to be included in each meta-analysis paper (n = 31 and 25 for MacDonald *et al.*, 2010 and Sermondade *et al.*, 2013, respectively) and the contradictory results, along with cross-sectional and longitudinal study outcomes, a reasonable conclusion is that additional well-controlled, population-based trials are necessary before stronger conclusions regarding the potential impact of obesity on semen parameters can be made. The methods used to assess obesity should also be standardized. Obesity studies should include measurement of reproductive hormones, as the only meta-analysis paper (MacDonald *et al.*, 2010) that included hormonal parameters concluded 'There was strong negative relationship for testosterone, SHBG and free testosterone with increased BMI.' In a recent cross-sectional study, (Andersen *et al.*, 2015) the correlation between the three hormones and obesity was affirmed. Studies on the impact of weight-loss nutritional interventions on reproductive health are missing. Bariatric surgical intervention reports exist, however the outcomes are mixed and access is often restricted based on socio-economic status.

In summary, the evidence supports a detrimental effect of obesity on many aspects of health, and evidence is conflicting about a potential effect on reproductive function. Therefore, we strongly recommend, based on a moderate quality of evidence, that males presenting for fertility evaluation should be counseled about weight-loss strategies when

the BMI and waist circumference data demonstrate obesity and especially morbid obesity (Table I).

Does exposure to heat adversely affect semen parameters and/or male fertility?

Perhaps surprisingly, the quality of the evidence linking heat to human male infertility is relatively poor. Data from animals, primarily by experimental manipulation of the testis, strongly suggest an adverse effect of heat on spermatogenesis and subsequent fertility (Durairajanayagam *et al.*, 2015). Additionally, it is well established that cryptorchidism is associated with abnormal spermatogenesis attributable, at least in part, to heat exposure of the testes to core body temperature (Hutson *et al.*, 2013).

A key question is: When the testes are in the scrotum, does heat exposure adversely affect semen parameters and male fertility? Many studies link certain activities to increases in scrotal temperature (for example use of saunas, hot baths), but do not follow this up with information about the effect on semen parameters (Durairajanayagam *et al.*, 2015). Of the studies that do investigate the effect of temperature on semen parameters, fewer still provide indicators of fertility outcomes such as live birth rate or TTP. Only Thonneau *et al.* (1997) assessed TTP, and this study alone, with a small sample size, was insufficient to draw robust conclusions about the effect of heat exposure on fertility. The reported reduction in semen parameters caused by heat is often small and it is unclear what effect, if any, this would have on biological fertility. For example, Hjollund *et al.* (2000) reported a lower average sperm concentration in men with higher scrotal temperatures, but this was still in the normal range (above 15×10^6/ml, WHO, 2010). Additionally, some studies did not measure scrotal temperature, attributing a difference in semen parameters to an activity without providing evidence that there was any rise in testicular temperature (e.g. a study of taxi drivers Figà-Talamanca *et al.*, 1996).

No RCTs were found and almost all of the data were collected retrospectively. Previous studies often suffer from small sample size (e.g. Garolla *et al.*, 2013) and confounding factors such as lifestyle factors (e.g. Figà-Talamanca *et al.*, 1996). Very few of the studies used control groups which further complicates interpretation. No systematic reviews or meta-analyses have been performed, which is probably because of the significant variation in study design. The available studies use different ages of participants, different outcomes, subjective definitions of heat exposure, and even different criteria for semen analysis (Zorgniotti & Seaflon, 1988 and Cherry *et al.*, 2014).

Some studies investigated the effect of occupational heat exposure on male fertility, for example welders. The evidence that this activity affects male fertility is low, and extreme heat exposures such as this do not represent the normal heat exposures of the general population (caused by wearing different types of underwear, sedentary position etc.). Povey *et al.* (2012) and Pacey *et al.* (2014) concluded that there was no significant effect of lifestyle factors, including heat exposure, on semen parameters, and others also failed to find a significant effect, for example, Støy *et al.* (2004) and Eisenberg *et al.* (2015), although Priskorn *et al.*, (2016) did report a negative association between watching television for 5 h/day and sperm concentration but there was no measurement of heat exposure.

Suffice it to say that further work is required to elucidate the effects of heat exposure on male fertility. Ideally, scrotal temperature, semen parameters and a measure of fertility outcomes, such as live births, would have to be measured in prospective cohort studies. Studies must use comparable measures of semen analysis, methods for measuring scrotal temperature and definitions of infertility in order for comparisons to be made. This would allow a systematic review or meta-analysis of the evidence to be carried out.

In summary, we strongly recommend, based on a very low quality of evidence, that there is insufficient evidence to conclude that exposure to heat, be it occupational or as a result of clothing or body position, affect semen quality and/or male fertility (Table I).

Does cigarette (tobacco) smoking adversely affect semen parameters and/or male fertility?

Most of the published literature on cigarette (tobacco) smoking and male fertility only looks at the effect of smoking on semen parameters. The quality of the recent evidence linking cigarette smoking to decreased semen quality is moderate, as a systematic review and meta-analysis of 46 cross-sectional studies (Li *et al.*, 2011) found that smoking was associated with reductions in all of the semen parameters. A further meta-analysis of the literature since 2010 analysing 20 studies with 5865 participants (Sharma *et al.*, 2016) also concluded a significant negative effect of cigarette smoking on all semen parameters. One prospective cohort study was identified which examined the effect of smoking status on semen quality (Yang *et al.*, 2015). This study reported a significant reduction in total sperm count ($P = 0.012$) and concentration ($P = 0.023$) after multivariate analysis. Several cross-sectional and case-control studies found differing effects of smoking on semen quality. For example, Jeng *et al.* (2014) reported a decreased proportion of sperm with normal morphology in those who smoked >10 cigarettes/day ($P = 0.04$), whilst Povey *et al.* (2012) found no significant effect of smoking on motile sperm concentration and Pacey *et al.* (2014) found no significant on sperm morphology. Several studies reported an association between smoking and changes in blood hormone levels. For example, Jeng *et al.* (2014), Al-Matubsi *et al.* (2011) and Lotti *et al.* (2015) all reported increased serum testosterone in smokers compared to non-smokers.

The definitions of smoking status vary between studies, with some studies failing to define which participants they classed as 'smokers' (Caserta *et al.*, 2013) and some studies using cotinine levels to evaluate smoking status rather than pack-years or number of cigarettes smoked per day. Lotti *et al.* (2015) considered those that had smoked for <1 year to be 'never smokers'. The studies also varied on how they dealt with ex-smokers—in some studies only those who currently smoked were 'smokers' and in others, anyone who had exceeded 1-pack-year at any time in their life were 'moderate smokers' (Anifandis *et al.*, 2014). In some cases, smoking status was divided into light, moderate and heavy, and again, these definitions were inconsistent. These inconsistencies made it challenging for Li *et al.* (2011) to establish a dose-dependent trend of smoking and reduced semen quality. Much of the current evidence comes from men presenting to infertility clinics, and may not represent the effect of smoking on semen quality and/or fertility in the general population. Also, smoking status was generally self-reported. This could introduce bias to the evidence based.

There are few studies that examine the effect of smoking on indicators of fecundity, such as TTP. One retrospective cohort study,

Mutsaerts *et al.*, (2012) found that paternal smoking had no effect on TTP. ASRM (2012a) concluded that there was insufficient evidence of the association between smoking and male infertility, despite the fact that smoking has been shown in many studies to affect semen quality, however, a review of this recommendation is due to be published in the near future.

Systematic review and meta-analysis are required to investigate the effect of smoking on blood hormone levels, and of measures of fecundity, such as TTP. However, this evaluation would require a greater number of well-designed, prospective cohort studies with consistent definitions of smoking status. The participants could be stratified into levels of smoking, which could be assessed by biochemical methods to reduce self-reporting bias. The outcomes should include measurement of semen parameters as well as measures of fecundity and fertility, such as TTP and live birth rate.

In summary, we strongly recommend based on a moderate quality of evidence that there is some evidence to suggest a negative effect of cigarette (tobacco) smoking on semen quality/male fertility but not all studies report this. However, as smoking has an adverse effect on general health and wellbeing it is recommended that men trying for a pregnancy should abstain from smoking (Table I).

As stated previously the effect of lifestyle (and environmental factors) affecting male infertility is likely to remain a rapidly developing and topical issue. There are a large number of significant areas for future research. Primarily large-scale prospective multi-centre trials encompassing a variety of geographical locations are required to examine the effects of lifestyle (e.g. obesity, heat exposure, smoking, recreational and medical drug use, high-intensity sports) and environmental factors (e.g. occupational exposures, endocrine disrupting chemicals (EDCs)/toxins) on spermatogenesis, semen analysis, male fertility and fertility outcomes, including the health of subsequent offspring.

Do supplementary oral antioxidants and herbal therapies significantly influence fertility outcomes for infertile men?

There is a significant body of data to support the concept that oxidative stress plays a key role in sperm dysfunction and male infertility (Aitken *et al.*, 2014). Consequently, antioxidant treatment of the infertile man may improve semen quality and/or fertility. However, the key question is: Are there data to support this? To address this question two approaches were used: use of a recent Cochrane review (updated; Showell *et al.*, 2014); and primary analysis including analysis of the literature on herbal therapies.

The Cochrane review (Showell *et al.*, 2014) examined the use of antioxidants. Forty-eight published studies were included in the systematic review with 37 studies included in the meta-analysis. In summary, only 7/48 trials reported on clinical pregnancy rate, only four of which went on to report live births. The authors recommended that until live birth and clinical pregnancy rate are robustly reported by all infertility trials, it is not possible to draw clear conclusions on the use of antioxidants for infertile men. Additionally, they concluded that the low-quality evidence from only four small RCTs suggested that antioxidant supplementation in infertile males may improve live birth rates. Data were lacking on other adverse effects. Importantly they suggested that 'further large well-designed randomized placebo-controlled trials are needed to clarify these results'. Our primary analysis of the literature came to a similar conclusion as the Cochrane review (Showell *et al.*, 2014) as there were very few additional studies examined (e.g. Raigani *et al.*, 2014).

Far fewer studies examined the use of herbal therapies (Y virilin, Saffron, Addyzoa). Two-thirds of the studies showed some improvement in semen parameters and one study reported a positive effect on sperm membrane integrity (Omu *et al.* 1998). Two studies showed improvements in sperm DNA integrity after herbal therapy (Omu *et al.* 2008; Raigani *et al.* 2014). After the use of herbal therapies only one of three (33.3%) studies reported a positive influence on pregnancy rates. Adverse effects were reported in 16.6% of the studies and most of them were mild to moderate. However, it is worth noting the study of Safarinejad *et al.* (2011) within which a large number of patients had adverse haematological reactions during treatment with the compound Linn Crocus sativus (Saffron).

An inevitable conclusion was that the methodological quality of most studies in the literature on antioxidants and herbal therapies to treat male infertility is poor. Additionally, the heterogeneity of the selected studies makes meta-analysis challenging. A further complication is that techniques to measure oxidative stress, antioxidant capacity and/or DNA damage are not standardized between all the trials. Moreover, there is often a lack of clear pre-selection of a subgroup for testing e.g. confirmed high-reactive oxygen species/DNA damage, reduced antioxidant capacity. Taking these factors into account, oral antioxidant therapy may improve seminal oxidative status in infertile men either by decreasing oxidative stress or by increasing the total antioxidant capacity but the evidence is of poor quality. In some cases, positive relationships are manifested in improvements in semen parameters, most often sperm motility. This may explain the higher pregnancy rates after antioxidant therapy compared to placebo but further detailed studies are required. Studies evaluating the supplementation with herbs constitute only a small part of the available literature. For these studies, the heterogeneity of the trials do not allow a robust conclusion to be drawn. Suffice it to say, there are no high-quality data to support the use of a single antioxidant or a specific combination of antioxidants. Further, it is not possible to recommend an effective treatment regimen.

In summary, we strongly recommend based on low-quality evidence that there are insufficient data to recommend the use of supplemental antioxidant therapies for the treatment of men with abnormal semen parameters and/or male infertility. Additionally, we strongly recommend based on very low quality of evidence that there are insufficient data to recommend the use of herbal therapies for the treatment of men with abnormal semen parameters and/or male infertility (Table I).

There are significant areas for future research; there is an absolute and urgent requirement for large, well-designed placebo-controlled randomized trials with primary outcomes of TTP and live births (including health of these births) reported in well-characterized groups to examine, for example, the effects of dietary supplementation, vitamins and herbal remedies.

What are the evidence-based criteria for genetic screening of infertile men?

The determination of whether an infertile male benefits from having a genetic evaluation depends on the aetiology of the reproductive

compromise and the severity. A detailed history and comprehensive PE, coupled with adjunctive tests, such as a semen analysis, hormone assays and on occasion testis biopsy, help clarify to which diagnostic category the patient belongs and, as a helpful consequence, assists in determining which genetic studies may be fruitful. For example, if a male has a reproductive history consistent with a known cause of resultant spermatogenic failure, such as chemotherapy, bilateral mumps, orchitis with resultant atrophy or current use of anabolic steroids, and is presently severely oligozoospermic or azoospermic, it can be assumed that these are the proximate reasons for the reduced/absent spermatogenesis and no genetic evaluation needs to be undertaken. The focus of the recommendations was on Karyotype, Y micro deletions and cystic fibrosis (CF) mutation analysis.

Y-chromosomal microdeletions and karyotype in men with spermatogenic dysfunction

No Cochrane reviews were identified. The primary evidence was from studies by Rozen *et al.* (2012), and Krausz *et al.* (2014) and practice statements from ASRM (2012b), AUA (2011) and EAU (Jungwirth *et al.*, 2015). Based upon the evidence, we recommended that in men who have a history, PE and hormonal assays consistent with severe oligozoospermia or non-obstructive azoospermia (NOA), both a karyotype and Y-chromosomal microdeletion assay should be offered.

Karyotype: Men with a sperm count <5 million/ml show a much higher rate of autosomal abnormalities than fertile populations (around 4%) while the highest frequency is found in NOA men (mostly Klinefelter syndrome). Klinefelter syndrome [47,XXY including variants (48,XXXY), and XX males (SRY+ and SRY−)] is the most common of the sex chromosomal aneuploidies. Translocations may be found in a relatively small percentage of men with severe oligozoospermia and azoospermia (Yatsenko *et al.*, 2010). The benefits of knowing if there is a chromosomal abnormality are in the planning for therapy and in the future follow up of the patient. As such, karyotype analysis should be performed prior to either use of ejaculated sperm in conjunction with ICSI, or prior to operative testis sperm extraction (TESE). The *a priori* knowledge of a chromosomal translocation, depending upon its exact nature, may significantly alter the thought process and therapeutic strategy of an upcoming ICSI cycle by employing PGS to allow the transfer of only balanced or normal embryos while discarding those that are chromosomally unbalanced (e.g. Dul *et al.*, 2012).

In summary, we strongly recommend, based on a high quality of evidence, that karyotype testing should be performed on all males with severe oligozoospermia (<5 × 10^6/ml) or NOA prior to any therapeutic procedure (Table I).

Y-chromosomal microdeletion assay: The molecular geography of the Y chromosome is such that microdeletions (not recognizable by cytogenetic methods) may occur that partially or completely eliminate the azoospermia factor (*AZF)a* or the *AZFb/c* region from the genome, and, consequently, any important 'spermatogenic necessary' genes that reside in those intervals. Frequency data compiled by Rozen *et al.* (2012) show an overall incidence of Y microdeletions in the *AZFc* region in 1/27 men, which varied depending on the Y haplotype. The importance of Y microdeletion testing in the severely oligozoospermic or azoospermic male prior to any therapy (ICSI using ejaculated sperm or TESE) is for prognosis and consideration of PGD. For example, data show that when a complete *AZFa*, *AZFb* or *AZFb/c* microdeletion is present (~1–2% incidence of each in the NOA man) no spermatozoa will be found on TESE. When there is no possibility that sperm will be present, it is unhelpful and hurtful for the male to be operated upon and, in the latter circumstance, for the female partner to have an ovarian stimulation unnecessarily. Men with *AZFc* microdeletions can produce spermatozoa that are capable of fertilization, embryo development and term pregnancy (Oates *et al.*, 2002). An *AZFc* microdeletion results in a quantitative reduction in spermatogenesis with maintenance of spermatozoa quality and function. All males born will directly inherit the *AZFc* microdeletion.

In summary, we strongly recommend, based on a high quality of evidence, that Y chromosome microdeletion (YCMD) testing should be performed on all males with severe oligozoospermia (<5 × 10^6/ml) or NOA prior to any therapeutic procedure (Table I).

CF-mutation analysis in men with Congenital Bilateral Absence of the Vas Deferens or clinical CF

No Cochrane reviews were identified. The primary evidence was from studies by Yu *et al.* (2012), Xu *et al.* (2014) and Lommatzsch and Aris (2009), and practice statements from ASRM (2012b), AUA (2011) and EAU (Jungwirth *et al.*, 2015).

Men with clinical CF (pulmonary and pancreatic dysfunction) will also have absence of the vasa and seminal vesicles bilaterally and will, consequently, have a low volume, low pH, and an azoospermic ejaculate. The incidence in males of northern European heritage is 1:2000. An equal frequency of men with low volume, acidic pH, azoospermia will have congenital bilateral absence of the vas deferens (CBAVD) with little respiratory or pancreatic disease, the vast majority of whom will possess mutations/pathogenic abnormalities in both maternal and paternal CF alleles. Whether one presents with respiratory tree (including sinuses) and/or pancreatic disease, simply absence of the vas deferens or somewhere clinically between these phenotypic extremes depends upon exactly which mutations/abnormalities in the alleles are inherited. There are >1985 recognized mutations in the CF transmembrane conductance regulator (*CFTR*) gene (Cystic Fibrosis Mutation Database: The Hospital for Sick Children, Genetics and Genomics Biology. Toronto: 1989 [Accessed: August 2014]. Available at: http://www.genet.sickkids.on.ca/cftr/app.) The *CFTR* gene has 27 exons spanning 250 kb of chromosome 7 (7q31) and encodes an mRNA of 6.5 kb and the final protein contains 1480 amino acids. Certain mutations, such as c.1521_1523delCTT (legacy name: F508del), severely impair either quantity or functional quality of the CFTR protein determined by that allele. Other abnormalities, such as the 5 T polymorphism in intron 8 (5 T), only mildly impair quantity or functional quality of the CFTR protein determined by that allele. It is the combination of the two that correlates to the severity of disease expression. If a person is homozygous for c.1521_1523delCTT, for example, s/he will have problematic respiratory and pancreatic disease manifested and diagnosed in childhood. However, if a male has inherited the '5 T' allele and c.1521_1523delCTT on the opposite allele and is therefore a compound heterozygote, pulmonary and pancreatic function may be clinically normal and CBAVD is the only recognizable phenotypic consequence. Bilateral vasal absence, then, is the most sensitive indicator of a biallelic CF gene abnormality as there is differential expressivity and sensitivity to CFTR in different epithelial tissues. In addition, it has been postulated that severity of phenotype may be modified by

polymorphisms in unrelated genes such as TGFB1 (transforming growth factor) and EDNRA (endothelin receptor type A) (Havasi *et al.*, 2010). In a recent meta-analysis by Yu *et al.* (2012) of CBAVD patients, 78% had at least one mutation identified, 46% had two mutations identified and 28% had only one mutation identified. The most common heterozygous mutation pairing was F508del/5T (17% of CBAVD cases) and F508del/R117H (c.350G > A; 4% of CBAVD cases). The polythymidine tract in intron 8 has three alleles consisting of 5, 7 and 9 thymidines that are found in 5, 85, and 10%, respectively, of the general population. In the presence of 5T, there is reduced splicing of Exon 9 and, as a consequence, reduced expression of full-length CFTR. Because 5T acts as a 'mutation' when trans (on the opposite allele) to a defined *CFTR* mutation, e.g. F508del, the poly-T tract in intron 8 must be defined in cases of CBAVD. Poly-T tract analysis is often only a reflex assay when R117H is detected (Chen and Prada, 2014). However, many of the studies in the meta-analysis of Yu *et al.* (2012) were conducted in the early years after discovery of the association of CBAVD and CF mutations, when only a small cohort of mutations was known and searched for (Anguiano *et al.*, 1992). The more comprehensive the assay, the more patients will have their second abnormality identified. Although a *CFTR* mutation genetic basis underlying most cases of CBAVD was a statistical certainty, this meta-analysis provides well-defined summary values which, in all likelihood, will be modified upwards in future years as even more complete *CFTR* assessment is accomplished for men with CBAVD.

The distribution of *CFTR* mutations and polymorphisms differs depending upon the ethnic/geographical origin of the patient/population being studied. As reviewed by Lommatzsch and Aris (2009), F508del is the most common mutation leading to CF worldwide but varies in its frequency based upon ethnicity/geographical location: 70–80% in CF patients from northern Europe, 50% in CF patients from southern Europe, 48% in African Americans, 46% in US Hispanics, 30% in Ashkenazi Jews, 18% of Tunisian CF patients and rarely in Native Americans. Furthermore, in the Ashkenazi Jewish population c.3846G > A (legacy name W1282X) is the most common mutation found (48% frequency). The meta-analysis by Xu *et al.* (2014) which specifically looked at F508del, 5T, and M470V, supports the above findings, concluding that there are significant associations between F508del and CBAVD ($P < 0.001$, OR = 22.20, 95% CI = 7.49–65.79), 5T and CBAVD ($P < 0.001$, OR = 8.35, 95% CI = 6.68–10.43).

In situations of CF or CBAVD, it is always necessary to screen the female partner for CF gene abnormalities so as to have a proper assessment of the risks of any offspring inheriting one of the two paternal mutations and the maternal mutation and presenting with clinical CF or, at the least, CBAVD if male. In addition, the benefit of testing the male with CBAVD helps provide information for his siblings who have a 75% chance of harbouring at least one (or possibly both) of the mutations inherited by the patient from his parents. Finally, patients may have mild CF symptoms such as 'sinusitis' or 'bronchitis', not previously recognized to be CF-mutation related and which, with a full understanding of their genetic basis, may be therapeutically managed in a different fashion. Not all CBAVD appear to be caused by/associated with *CFTR* mutations and abnormalities, and these cases may be secondary to a distinct genetic aetiology that affects mesonephric duct development. The phenotypic end-product may be CBAVD and unilateral renal agenesis, as described by McCallum *et al.* (2001). Therefore, in cases of CBAVD where no *CFTR* mutations are identified, renal ultrasonography is indicated.

In summary, we strongly recommend based on the high quality of evidence that appropriate *CFTR* mutation analysis should be offered to all males with CBAVD or CF (Table I).

There are significant areas for future research in the genetic screening of the infertile male. For example, what are the long-term health outcomes of children born from infertile men, can cost-effective tools for genetic screening in men (karyotype, Y micro deletions and CF-mutation analysis) in low-income settings be developed, and what is the genetic basis of unilateral absence of the vas associated with unilateral renal agenesis?

How does a history of neoplasia and related treatments in the male impact (his and his partners) reproductive health and fertility options?

In a number of aspects this was a very challenging question to address. Although there were several reviews in the area, for example Tournaye *et al.* (2014) and Samplaski and Nangia (2015), and key recommendations from national societies, for example American Society of Clinical Oncology (ASCO) (Loren *et al.*, 2013), the therapeutic agents and treatment regimens are continually evolving. Additionally, there were limited data on key aspects of the question, such as advice on the contraception window post-treatment and health of the offspring of both juvenile and adults cancer survivors.

However, on a general level, there is consistency in the recommendations of the major medical societies in Europe and USA: the European Society for Medical Onclology (Peccatori *et al.*, 2013) and ASCO (Loren *et al.*, 2013). For example, storage of semen samples is the primary option to potentially preserve fertility of men (and boys producing sperm in the ejaculate) who are undergoing chemo/radiotherapy regimes (Loren *et al.*, 2013; Peccatori *et al.*, 2013). As such, the overwhelming evidence suggests that all patients should be provided with information about the impact of their cancer treatment on spermatogenesis and the option of sperm banking. Whether regimens carry a high or lower likelihood of long-term fertilty impairment, given the variability in individual response to treatment and the potential for relapse, the evidence would recommend that sperm cryopreservation should always be considered and services be available and affordable.

Counselling should also include the fact that there is little chance of recovery from azoospermia after 10 years following radiotherapy (Sandeman, 1966), total body irradiation (Rovó *et al.*, 2006) or chemotherapy (Meistrich *et al.*, 1992, Heikens *et al.*, 1996), however, contraception should continue to be considered if paternity is not desired. Actively attempting pregnancy during cancer treatment must be avoided, however, if an accidental pregnancy would occur during cancer treatment this should not automatically be considered an indication for elective termination. The pregnant couple should be offered counselling and appropriate foetal diagnostic interventions, for example, minimally an evaluation of the foetus through ultrasound (De Santis *et al.*, 2008).

Although the fertility of male cancer survivors is reduced, registry data have identified subgroups dependent on cancer type, age at

onset, treatment modality and dose, whose fertility is not different from the general population (Tournaye et al., 2014). Pregnancy outcomes, such as pre-term delivery, low-birth rate and miscarriages, seem to be comparable to the general population but there are conflicting data regarding the risk of malformations (Ståhl et al., 2011). Generally, a co-ordinated approach between the healthcare professionals involved in cancer treatment and the reproductive medicine specialists is highly recommended although regrettably not always achieved.

In summary, we strongly recommend that based on moderate quality of evidence that every male cancer patient should be provided with information about the impact of his cancer treatment on spermatogenesis and the option of sperm banking. Additionally, we strongly recommend based on low quality of evidence that: patients should be advised to use contraception if they do not wish to procreate even after prolonged periods of azoospermia following radiotherapy, as recovery is possible; male cancer patients should be informed that pregnancy outcomes in partners are good but a slightly higher risk of congenital anomalies in their offspring cannot be excluded (Table I).

There are significant areas for future research. For example, there are insufficient data to advise men regarding the contraception window post-treatment and a lack of systematic data on the risk of birth defects following accidental conception during treatment. It also has to be determined if there are any effects in second generations of cancer survivors following cancer treatment; there are no data regarding the risk to partners or offspring from chemotherapeutic agents in semen.

What is the impact of varicocele on male fertility and does correction of varicocele improve semen parameters and/or fertility?

For this question, and after discussion with this working group of experts and with the leads of the WHO GDG, a recommendation was made to use the Practice Committee of the ASRM; Society for Male Reproduction and Urology (2014). Report on varicocele and infertility: a committee opinion (evidence level IV). As the methodology used to construct this report was not equivalent to the WHO GRADE assessment, the conclusions cannot be suggested as a recommendation but as an opinion based on a review of the literature. As such these are suggested as Good Practice Points, namely:

- Treatment of a clinically palpable varicocele may be offered to the male partner of an infertile couple when there is evidence of abnormal semen parameters and minimal/no identified female factor, including consideration of age and ovarian reserve.
- IVF with or without ICSI may be considered the primary treatment option when such treatment is required to treat a female factor, regardless of the presence of varicocele and abnormal semen parameters.
- The treating physician's experience and expertise, including evaluation of both partners, together with the options available, should determine the approach to varicocele treatment.

An analysis by Shridharani et al., (2016) presents a detailed assessment of the EAU, ASRM and AUA recommendations on varicocele. The differences in their recommendations and the complexities of conducting a long-term prospective trial that would definitively answer this question clearly indicate that significant further research is necessary to guide clinical management.

Challenges and future research opportunities

It was perhaps an inevitable conclusion that, in conducting these analyses, gaps in the literature would be identified. It would be surprising if this was not the case. However, what was surprising was the substantial nature of the gaps where effectively little or sometimes no research had been performed. Of additional note was the sometimes low quality of the available evidence. Such a combination makes formulating informed evidence-based decisions for the diagnosis of the male difficult (see Table I for summary). However, conversely, a number of issues were identified with clear and significant opportunities for the way forward.

Key themes in developing these recommendations

Areas for research focus

We present key areas for research focus that demand investigation. Overall one high priority area for research was to gain a better understanding of the production, formation and workings of a human spermatozoon. There is an urgent requirement to understand these cellular, molecular biochemical and genetic mechanism(s) in order to formulate appropriate diagnostic assays, develop rational therapy for the male, and understand how external factors, such as the environment, negatively or positively influence these processes. Not surprisingly, this is not unique to our discipline. For example, a new strategy to understanding neurodegeneration with a primary focus on the formation and function of the cell is now strongly advocated as absolutely essential to accelerate progress for understanding neurodegenerative disorders (Kosik et al., 2016). Although research in understanding the workings of the human spermatozoon has progressed significantly over the last 10 years, there is still the need to catch up and then keep pace with the knowledge base in other cellular systems. It is unlikely that a series of robust diagnostic tools for sperm function can be developed without further detailed understanding of the working of the normal and dysfunctional cell. Additionally, without this knowledge, the complementary development of a drug(s) that a man can take or have added to his spermatozoa *in vitro* to improve sperm function will continue to remain elusive. Premature introduction of putative but unproven diagnostic and/or therapeutic tools into clinical practice in ART can hinder rather than advance progress in the field for the long-term (Harper et al., 2012, 2017; Spencer et al., 2016).

An additional high priority theme area for research was to examine the long-term health outcomes of the children born from men with compromised fertility (including those who may also have been treated with ART) whatever the nature of the compromising event(s) (e.g. genetics, environmental, iatrogenic and/or occupational). Moreover, this analysis needs to assess the effects in various geographical locations. In addition to the research themes which accompany each question, we identified other areas that require addressing. For example, what is the impact of age on male fertility? What are

the underlying causes related to the male for IVF–ICSI treatment failure? Which gene (or epigenetic) defects in men can predict ART outcomes? What are the most effective educational initiatives towards improving understanding of male infertility? What are the attitudes of men and women (in various geographical locations) towards the investigation of male fertility? Certainly, in presenting this analysis, a plethora of research questions has been generated.

Requirement to obtain robust data

A consistent theme was the focus on the quality of evidence available to support any recommendations. The criteria used can be presented in two, not mutually exclusive, formats: Traditionally, the quality of evidence is represented by using a range of different levels. However, it is clear that a considerable degree of the research related to the diagnosis of male infertility does not easily fit into these categories. The evidence is sometimes observational and not easily amenable to meta-analysis. With respect to the scientific and clinical evaluation of diagnostic methods i.e. methods that measure characteristics of the individuals, RCT are not usually appropriate. Primarily there is no inclusion of a separate group to be used as a control i.e. a group not obtaining the investigated treatment. In the evaluation of a diagnostic method, the control of the diagnostic method can be obtained with another independent method, or from the final clinical outcome. From this point of view, the highest level of evidence cannot easily be obtained through a RCT.

Where data/evidence were available the quality of the evidence was often judged to be sub-optimal. One issue was the robustness of studies and thus general applicability. The robustness of the evidence base in reproductive medicine has been discussed (Evers, 2013, Barratt, 2016a, 2016b; Glujovsky *et al.*, 2016) and is of course not just a theme in reproductive medicine (Baker, 2016). Robust methods must be developed and subsequently utilized. One example where we have perhaps made relatively little progress is the technical challenges of semen analysis. However, with a greater appreciation of the difficulties in this arena (Carrell and De Jonge, 2016) now is perhaps the time for the WHO to produce a 6th version of the semen assessment manual. The evidence based for the current (5th version) manual is at least 10 years out of date and a lot has changed. Nevertheless, there are areas of good practice: European Academy of Andrology guidelines and recommendations for quality control of the Y-chromosomal microdeletion assay (Krausz *et al.*, 2014) are commonly used. Robustness also applies to the verification of key recommendations from, for example, professional societies (see Ventimiglia *et al.*, 2016). In the future we need to heed the lessons of the past and make sure the evidence base, including the use of robust tools, is significantly improved.

The theme of international studies

Male infertility is a global and significant health problem. A consistent theme is the requirement for national and international efforts with large-scale, multi-centre studies encompassing different geographical locations. Considerable regional variations in key indices of male reproductive health have been reported (Skakkebaek *et al.*, 2016) but these are often on a relatively local scale. It is critical to understand potential variations in sentinel markers of male reproductive health, in other countries/regions as well as in low and middle resource settings globally, in order to inform on further policy and practice.

Context of resource setting

A key theme is the question of global implementation of diagnostic tools and therapeutic procedures, and especially in low-resource settings. Basic procedures do require resources and some recommendations although very simple procedures (e.g. sperm banking prior to potentially sterilizing cancer therapy) may be feasible in most settings but unfortunately they may not be in some areas without specialized storage facilities.

Overall summary: the road to a healthy future for male infertility

There are significant advances in male reproductive health from developments on *in vitro* spermatogenesis to dissecting the workings of the mature spermatozoon. However, progress in this arena is comparatively slow. The robustness of the data is sometimes wanting, and thus the ability to provide the strongest evidence-based guidelines has proven to be a challenge. We provided recommendations based upon the evidence available. However, when looking to the future there is a need to understand why it has happened that the data are limited and not forthcoming, in order to inform the direction of future initiatives and endeavours that could result in more studies that yield robust evidence from men residing in high-, middle- and low-income country settings.

What is the scale of the problem of male infertility and its consequences?

It is fundamental to know the prevalence of a disease in order to provide resources, estimate impact, make effective health economic arguments, present rational research questions and manage patients. However, there is a paucity of clinical data on the scale of the reproductive health problems and infertility in men on a global level, across all health economies. The best estimates on infertility from demographic health studies use the heterosexual woman, as she self-reports a problem with becoming pregnant, which further assumes a diagnosis of female infertility (due to minimal if any reporting of clinical diagnosis of the female), or possibly, an assumption of an infertile relationship; and, coupled with an even larger paucity of male diagnosis and management of infertility in low- and middle-income countries, any male infertility prevalence values based upon demographic health reports or from small private clinics are, at best, greatly extrapolated and highly inaccurate (Mascarenhas *et al.*, 2012). As a consequence, there is limited information on fundamental key markers such as the socio-economic impact that infertility and other diseases or disorders may have on the individual and society as a whole. Emphasizing the importance of the engagement of the male partner in the assessment of the infertile couple and the education of the public about male infertility should improve the care of the couple and expand our knowledge of the scale of the problem.

Moreover, there is an urgent need to determine the potential consequences of male infertility. This is a wide area of investigation which

should extend beyond the confines of the couple and their immediate relationships to investigate, for example, the relationship of infertility with other diseases and what impact this has on the disease. There is increasing evidence (albeit currently primarily in animals) of the potential transmission to the next generation of damaging insults to the male germ line by epigenetic mechanisms (Siklenka *et al.*, 2015). This may become especially relevant with the effect of significant potential changes in the external environments on the fidelity of the germ line, and also the likely future potential use of more immature (Tanaka *et al.*, 2015), and even *in vitro* generated, gametes.

Funding the science to match the scale of the problem

The lack of data in key areas reflects the overall paucity of high quality long-term national and international funding streams to support reproductive medicine, and male infertility in particular. Whilst this has been discussed previously it remains a fundamental block to further progress and a critical issue (Evers, 2013; Barratt, 2016a, 2016b). The WHO has not published evidence-based guidelines in this area, and the previous infertility/fertility manual for management of the infertile male based upon then current practice, was published in 1992. This effectively means ~25 years without an update on best practice and never any official guidelines for adoption and adaptation at country level. This is an unacceptable situation as the lack of universal international guidelines does not help advance the field of male infertility or male reproductive health in general. However, it is anticipated that these new evidence-based guidelines for the male will act as a platform for studies helping to raise the profile of male reproductive health.

Fundamentally, it is necessary to make more robust arguments to national and international agencies to help drive the research agenda and subsequently place reproductive medicine, with demonstrated impact on couples and individuals, at the vanguard of the funding landscape. The coupling of EDCs and male reproductive health may provide an example. In this field strong scientific, socio-economic healthcare and political arguments have consistently been marshalled to support research into what has been termed an 'epidemic' of male reproductive health problems (Hauser *et al.*, 2015; Skakkebaek *et al.*, 2016; Trasande *et al.*, 2016). The burden of the disease (via EDC) has been estimated and an impact assessment made (e.g. Olsson, 2014; Hauser *et al.*, 2015). For example, it is estimated that the EDC may contribute substantially to male reproductive disorders and disease equivalent to a staggering €15 billion cost in the European Union (Hauser *et al.*, 2015) although these figures are subject to considerable debate (e.g. Olsson, 2014; Woodruff, 2015). This type of analysis needs to be developed and continually fine-tuned for male reproductive health as a whole if additional support for the basic science and clinical base of male infertility are to be realized. We also need to realize that this support needs to encompass educating future students (some of whom will be consumers and others leaders in the field) by stressing the importance of studying reproductive health in the school/further education system (Finney and Brannigan, 2017). Developing and marshalling arguments to decision makers are a complex and continuous challenge. Male reproductive health needs firstly a series of hard-hitting documents providing an evidence based for the importance and challenges in our discipline. However, this is only the start of the process as delivery on these recommendations requires a myriad of discussions with international agencies, politicians and key decision makers. Evidence alone does not determine action—as exampled by the challenges in implementing regional, let alone global, policy to deal with the increasing CO_2 in the atmosphere (Malakoff, 2017). We need to marshal a whole series of skills to effect policy, and scientific evidence on its own is insufficient for major policy changes (see Cairney, 2012, 2017). Stating the obvious—evidence-based guidelines require high-quality evidence, which will only be achieved with appropriate funding that will result in obtaining that data. Without robust data from all segments of society, the status of male reproductive health will remain invisible and the reproductive health needs of men will remain neglected into the foreseeable future.

Supplementary data

Supplementary data are available at *Human Reproduction Update* online.

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The authors were members of the Evidence Synthesis Group (see above) and were responsible for the synthesis and presentation of the literature. All authors contributed to the synthesis of the literature, writing and finalizing of the manuscript.

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Conflict of interest

CLRB is Editor-In-Chief of *Molecular Human Reproduction*.

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Paper 24:

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OPINION

'Man Up': the importance and strategy for placing male reproductive health centre stage in the political and research agenda

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ABSTRACT: Approximately 1 in 20 young men today have sperm counts low enough to impair fertility, whereas this may not have been the case historically. The cause(s) of such a decline in male reproductive health is unknown, despite it being a global health issue. Concomitantly, little progress has been made in answering fundamental questions in andrology or in developing new diagnostic tools or alternative management strategies to ICSI in infertile men. We advocate formulation of a detailed roadmap for male reproductive health to facilitate development of a research agenda that highlights the present unmet needs and key unanswered questions, and seeks to deliver effective funding and investment to address them. This vision we term 'a Male Reproductive Health Ecosystem'.

Key words: andrology / contraception / IVF ICSI / male infertility / sperm count / spermatogenesis

Introduction

Whilst formulating new World Health Organization (WHO) guidelines for the diagnosis of male infertility a striking observation was the paucity of high quality data on which to base recommendations (Barratt *et al.*, 2017). Even simple questions did not have sufficient data to formulate 'low' let alone 'strong' recommendations. Similar deficiencies are evident regarding other longstanding fundamental questions in andrology. For example, spermatogenesis is absolutely dependent on high levels of testosterone, yet the mechanistic pathways via which testosterone achieves this remains a black box. This is despite our achievements in successfully manipulating androgen action specifically in every cell type in the testis via transgenesis (O'Hara and Smith, 2015). This example is symptomatic of much broader 'andrological ignorance', illustrated by the almost complete lack of effective treatments for male infertility or for male reversible contraception other than the condom—a stark contrast with the female, for whom there are multiple effective treatments for both infertility and contraception. This has been thrown into sharp relief by recent confirmation that sperm counts have been falling steadily for >40 years yet we do not know why (Levine *et al.*, 2017). Therefore, across the andrology spectrum, from basics to the clinic, there is a fundamental lack of knowledge that obstructs research, diagnosis and patient management. So this is where we are now, but how did we get here and how can we move forward in a structured way to improve our understanding and management of male reproductive health issues?

The illusion of progress

Male infertility is part of a dynamic and rapidly growing health industry. ART is a worldwide, highly innovative, billion dollar enterprise. Combined with the fact that reproductive medicine is newsworthy and rapidly captures the attention of the general public, the perception from the outside is that all is well in the world of male reproduction. This is an illusion. Numerous basic clinical and scientific questions in andrology remain unanswered—some for over 50 years. A sentinel

example is the lack of any real progress in developing robust methods for semen analysis, despite it being the cornerstone of infertility investigations. If a simple diagnosis cannot be correctly identified then how can we progress? These limitations have been well rehearsed elsewhere but do not diminish our collective universal failure in this area (Carrell and De Jonge, 2016). Moreover, diagnostic tools/treatments are introduced too soon, usually without proof of efficacy. A recent assessment demonstrated that the overwhelming majority of 'add ons' in ART (including andrology examples) had no robust evidence to support their use is damning in this respect (Spencer *et al.*, 2016). To a large extent, this situation is simply a consequence of our continuing ignorance about male infertility, as it creates a vacuum that encourages the premature introduction of new putative diagnostics assays and/or treatments because there is nothing else to offer patients. Couples seeking ART because the male partner has poor semen quality are likely to grasp at any new initiative, irrespective of cost, and are in no position to judge the benefits or efficacy. The paucity of effective non-ART treatments for male infertility, at least since 1992, simply adds to the pressure.

Explanations for 'andrological ignorance'—the ICSI paradox

Contrary to the negative perspective above, advances in male reproductive health have been delivered historically. We (the community of reproductive biology/medicine specialists) have a good history of basic research leading to clinical benefit, thus demonstrating that genuine advances in male reproductive health can be delivered. One example is the development of robust protocols for the cryopreservation of human sperm (Bunge and Sherman, 1953), which has had a profound impact on management of subfertile couples. However, the most transformative example was the truly remarkable development of ICSI for management of male infertility. Following the first birth of an ICSI-conceived child in 1992, ICSI use has mushroomed worldwide and is increasingly used even when no male problem exists (EIM, 2016; Boulet *et al.*, 2015). However, here's the paradox. ICSI is not a treatment, as the man's fertility status remains unchanged (the gametes are manipulated); it is a treatment of the female partner (encompassing ovarian stimulation, egg recovery and embryo transfer). Thus, the woman carries the treatment burden for male infertility, a fairly unique scenario in medical practice. Ironically, ICSI's success has effectively diverted attention from identifying what causes male infertility and focussed research onto the female, to optimize the provision of eggs and a receptive endometrium, on which ICSI's success depends. Thus, since its introduction 25 years ago, ICSI has effectively served to roadblock further scientific advancement in andrology—a widely argued viewpoint (Skakkebaek, 2017).

The situation described above has arisen over a long time period. Simply put, we are where we are so why change now?

Five timely reasons for immediate action

The first evidence on the legacy of ICSI has emerged—sperm counts in young men conceived by ICSI are, as a group, ~50% of those in young men conceived naturally (Belva *et al.*, 2016), and as sperm counts may be a barometer of overall health and longevity (Glazer *et al.*, 2017; Hanson *et al.*, 2018), this could be a double whammy for these men. Are we being complacent about passing on other health problems for the next generation to deal with?

Significant evidence suggests that the health of future generations may be influenced epigenetically by the quality of their father's sperm, which may have been altered by his diet and/or lifestyle (Lane *et al.*, 2014; Siklenka *et al.*, 2015); maybe such effects are the explanation for the fall in sperm counts (Levine *et al.*, 2017).

Fertile mouse sperm can now be generated *in vitro* (Saitou and Miyauchi, 2016) and the technology (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated protein-9 nuclease: CrispR-Cas9) for editing DNA in gametes has arrived (Vassena *et al.*, 2016). The pressures to use both approaches in human male infertility are so substantial, that it will undoubtedly happen. As with ICSI, are we going to sit back complacently whilst it happens? Use of these techniques may have no downside for future generations, but we owe it to our children and future generations to base this on evidence rather than blind presumption, as done largely with ICSI.

In many countries, couples are delaying their first attempt at conception until the female partner is in her 30s when her fertility is declining progressively (ACOG, 2014). For example in UK, the average age at first pregnancy in 2016 was 28.8 years and 54% of all pregnancies were to mothers aged > 30 years (Office for National Statistics, 2016). Therefore, sperm quality in the male partner should be optimal to maximize chances of a pregnancy, yet sperm counts are falling (Levine *et al.* 2017) such that >15% of young men today have suboptimal semen quality, with Denmark as an example of the worst case (Skakkebaek *et al.*, 2016). Evidence of impact is that births in Denmark due to assisted reproduction (>6%; EIM, 2016) are some of the highest in the world.

However, the most concerning issue of all is the burgeoning world population. Population growth creates significant pressure on limited world resources and productivity and, without dramatic policy change(s), is becoming increasingly unsustainable (Gerland *et al.*, 2014; Tilman *et al.*, 2017). The United Nations (UN) Population Division's 95% centile prediction of ~13.2 billion people by 2100 is frankly staggering and frightening in its potential impact (UN, 2017). It is remarkable then that contraceptive choices are still very limited and that the current global contraceptive strategy is suboptimal as evidenced by the continual high rates of elective terminations (Department of Health, 2016). Moreover, current strategies focus almost entirely on women. For example Family Planning 2020 (www.familyplanning2020.org), a global partnership whose aims are to increase contraception use by 120 million users by 2020, only includes women as users! No effective, reversible and widely available form of contraception has been developed for the male since the condom. Thus the burden once again falls to the female and is a price unfairly paid because of an inadequate understanding of the male reproductive process. Effective voluntary family planning is fundamental to delivery on the UN 17 Sustainable Development Goals which will transform our world (Starbird *et al.*, 2016; FP 2020; Fig. 1). To achieve this, it is critical that the reproductive input of males be considered of equal importance as females (Hardee *et al.*, 2016).

As outlined above, the present sidelining of andrology has arisen over many decades due to a combination of reasons. So how do we redeem the situation? We suggest the development and execution of a

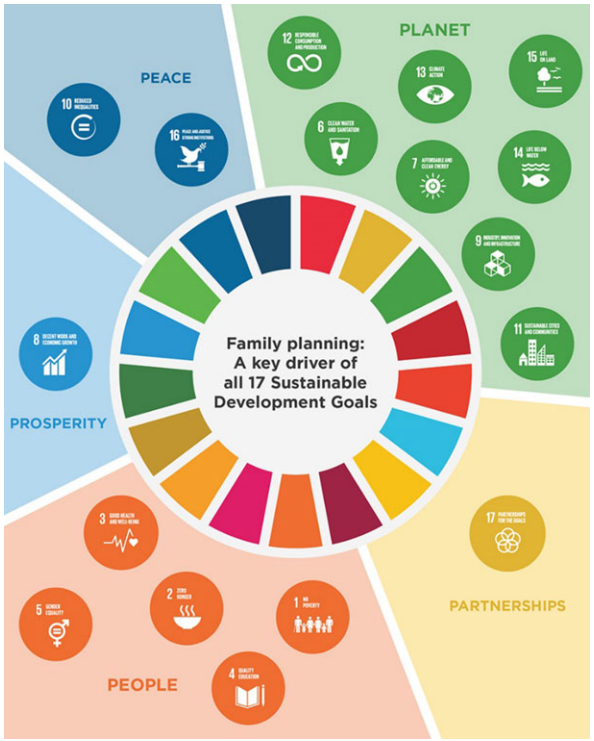

Figure 1 Family planning as key to success of the 17 Sustainable Development Goals. Family planning is centre stage to achieving the United Nations Sustainable Development Goals. Detailed information is provided in Starbird *et al.* (2016). See also FP 2020. Momentum at the Midpoint. 2015–2016 Progress Report. www.familyplanning2020.org. Diagram adapted from FP 2020 Progress Report.

detailed roadmap—'Male Reproductive Health Ecosystem' (Fig. 2). We propose that it should encompass the areas outlined below.

Identify research gaps

A fundamental part of a strategic roadmap is the identification of research gaps. The WHO has recently published a summary of the new evidence-based infertility guidelines (Barratt *et al.*, 2017), which could act as a foundation for developing strategies to improve research, as well as the diagnosis and treatment of male reproductive health disorders, in particular infertility. Nevertheless, it needs updating and expanding. Moreover, several critical aspects are missing from the WHO analysis. Primary among these is a detailed assessment of the economic and societal burden of male reproductive health. Many other diseases have compelling evidence of economic effects, for example infectious diseases (Bloom *et al.*, 2017), but this is all but absent for male reproductive health. A recent study (Hauser *et al.*, 2015) attempted to address this, although its focus was to suggest that this burden was attributable to endocrine disrupting chemicals, for which the evidence is rather limited. But the important point the article makes is that the cost to the European Union (EU) of male reproductive health disorders is substantial ~15 billion Euros per year. Such figures will likely surprise politicians and placing this information more into the public spotlight is one way to raise awareness and the political

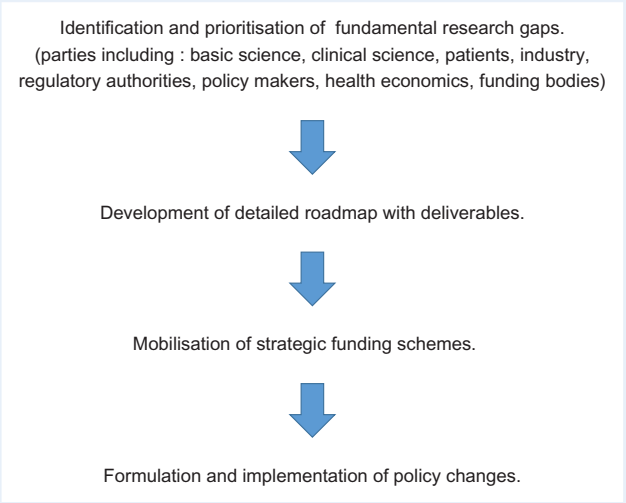

Figure 2 Male reproductive health ecosystem. The gaps in research are basic, translational and clinical. The proposal is to include key representatives from a spectrum of disciplines early on, for example policy experts, as in the final analysis some changes in policy strategy will be required. As for the World Health Organization (WHO) (Barratt *et al.*, 2017) this could be actioned by Expert Synthesis Groups led by a key expert. As we propose a strategic plan, overall we must be cognisant of initiatives in other areas such as growth of big data (Frégnac, 2017; Insel, 2017), whole cell maps (Horwitz and Johnson, 2017) and their biology (Kosik *et al.*, 2016). Funding for this initial approach would be required and may come from, for example, The Wellcome Trust. It is initially difficult to imagine identifying the gaps outwith the auspices of national professional societies. The default and easy route would be to get societies to do this. However, often these are talking shops and action can get stifled by political wrangling. Moreover, some work at glacial speed. An international consortium will require an international, co-ordinated effort across the discipline if it is to be effective. The proposal would work, throughout, by informing and interacting with key societies such as ESHRE, The American Society for Reproductive Medicine, the Society for the Study of Reproduction, the International Society of Andrology, and international bodies such as WHO, but not be dominated by them. Any effective strategy needs to be cognisant with what is working so far. For example, there is a renewed interest in funding work on male contraception (Bill and Melinda Gates Foundation, National Institutes of Health). Several countries have very effective networking for research delivery in infertility, e.g. The Netherlands. How did they achieve these? What can other societies/disciplines/models teach us? For example, the role of specific charities in collaboration with national funding agencies. Parkinson's.org.uk is a good example of a charity who have a £20 million UK research commitment (Parkinson's.org.uk) for a health issue that is less prevalent than male infertility. Effectiveness of national programmes of research should be investigated and benchmarked. Whilst any solutions involve significant new funding for male reproductive research (only ~3.6% of the UK Medical Research Council Populations and Systems panel budget was provided for male infertility research from 2014 to 2017), a piecemeal approach is not the answer (Spradling, 2016; Bloom *et al.*, 2017). The roadmap is presented in a simplistic linear fashion but there would be much dynamic movement between stages.

profile for andrology. A fundamental priority is therefore to obtain a detailed assessment of the economic and societal burden of male reproductive health disorders. This also needs to take account of the

impact of infertility at a time when family size across most of the EU is below replacement level (UN, 2015), as this has huge future implications for national economics. The increasing contribution of the male to couple infertility, for reasons outlined above, provides another opportunity for moving andrology back onto the research priority stage.

Effecting change

Once the gaps in knowledge are identified and articulated, a coherent plan for closing them is required. However, identifying the gaps in knowledge and formulating a plan are only the first steps for an effective long-term strategy. The key is for this to trigger changes in funding and, where necessary, policy. This is where it gets difficult. Evidence alone does not determine action and we need to marshal a whole series of skills to influence policy (Cairney, 2012, 2017). As Chris Tyler, who studied how evidence is used in the UK parliament, elegantly concluded 'for research to truly inform policy, it is not enough to hope that the stars will align. The stars need to be wrestled into position' (Tyler, 2017). One approach would be 'a New Male Reproductive Health Ecosystem' akin to that proposed for cancer research (Horning, 2017). This can and should involve co-ordination of a host of different parties including health care providers, patients and regulatory industries working as one interconnected community. Concomitantly, our aspirations for research financial support need to be raised logarithmically. For example, male reproductive health should be incorporated into the Global Fund to fight disease, which has been successful worldwide (Sachs and Schmidt-Traub, 2017), and aligned with worldwide challenges facing non-reproductive health of the male. Sperm counts may be a barometer for overall male health (Glazer *et al.*, 2017; Hanson *et al.*, 2018), so evidence that sperm counts have fallen 59% in ~40 years (Levine *et al.*, 2017) is a wake-up call. Baker and Shand (2017) in 'Men's Health: Time for a new approach to policy and practice' show how men are ignored or given minimal priority in policy and practice to effect the UN's Sustainable Development Goal 3 on health and wellbeing (GDG 3). Current policies are not equally applied to men and women, in part because men are reluctant to talk about their health problems, whether reproductive or not (Courtenay, 2000; Himmelstein and Sanchez, 2016).

Part of the execution strategy must involve raising the visibility of male reproductive health and engaging public support to press for change. Women could play a key partnership role in this. They have suffered invasive treatment, on behalf of male infertility management, with silent dignity in pursuit of a baby, but in a world in which we claim to be addressing inequalities between men and women, this is a standout example of the infringement of basic human rights and dignity. Maybe women undergoing treatment during ICSI can begin to apply pressure at the point of delivery of (their) treatment, asking 'why can't you treat him rather than me?' Initiatives such as March for Science (www.marchforscience.com) are a starting platform to explain the value of male reproductive health research, building on what has been learnt from initiatives such as Andrology Australia (www.andrologyaustralia.org) who have taken the lead in 'normalizing' men's health disorders, such that they can be discussed without stigmas (Hammarberg *et al.*, 2017). Urgent action is needed and under one banner—'Man up' (with its deliberate euphemism) is an example. There needs to be a concerted campaign to educate the public about the enormous gulf in understanding and practice regarding male versus female reproductive health, making clear this is not because all the problems are on the female side or because the female carries the biggest 'reproductive burden'.

Undoubtedly the development and delivery of a new male reproductive health ecosystem is a daunting task. However, we need to take strength from key advantages to develop a comprehensive strategy. For example, the combination of a dynamic health industry and large public interest provides an excellent starting point from which to go forward. Moreover, in some countries, a highly innovative regulatory framework is aligned with a strong translational arm (ART industry), which should facilitate and oversee the effective execution of novel research in humans (www.hfea.gov.uk; National Centres for Translational Research in Reproduction and Infertility www.nichd.nih.gov/research/supported/NCTRI; Reproductive Medicine Network http://c2s2.yale.edu/rmn/).

Future perspectives

Medicine currently faces the exciting challenge of incorporating new technology (e.g. stem cell therapy, gene editing), with its huge potential benefits, into patient care. To do this effectively, with minimal side-effects, understanding in a given area needs to be sufficiently advanced to enable technological advances to 'slot in'. We are far from this position with male reproductive health, yet the new technology is almost ready to use. Unless we 'Man up' our research now, the present gulf in knowledge and effective therapy for infertility between male and female will grow, and it will become easier to use *in vitro* generated male germ cells than naturally produced sperm to achieve couple fertility, making infertile men truly redundant. More worrying, however, is that such applications will be founded as much on guesswork as on understanding. The future wellbeing of children resulting from assisted reproduction should not be left to chance, and we should have a clear understanding of the risks versus benefits before embracing these new developments.

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Conflict of interest

R.M.S. is a Deputy Editor of Human Reproduction. C.L.R.B. is Editor-in-Chief of Molecular Human Reproduction and chair of the World Health

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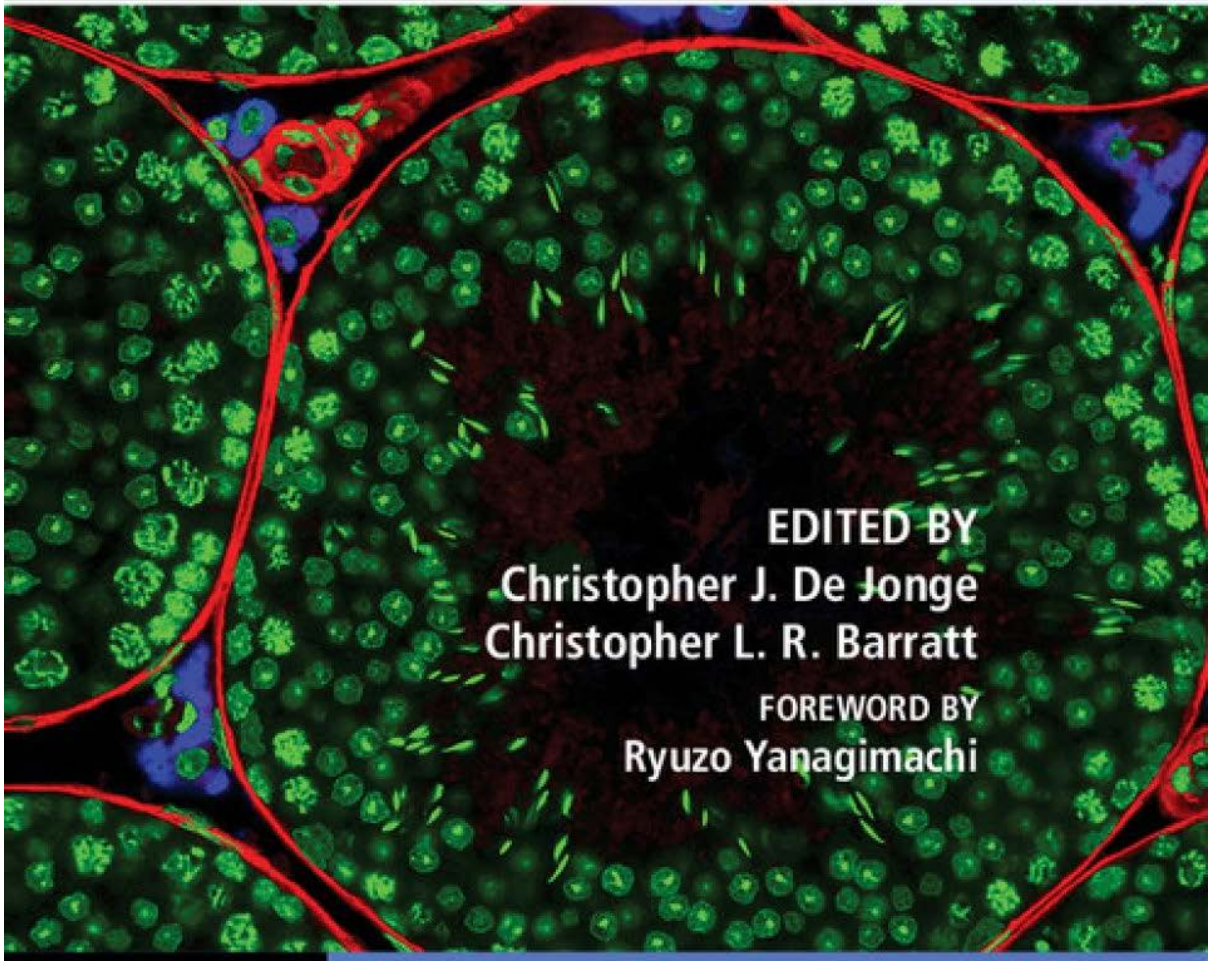

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