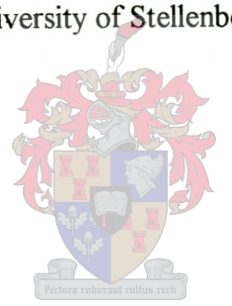
THE INTERACTION BETWEEN HUMAN SPERMATOZOA AND ITS HOMOLOGOUS ZONA PELLUCIDA: SCIENTIFIC ADVCANCES AND CLINICAL SIGNIFICANCE

Sergio C Oehninger

Dissertation presented in fulfillment of the requirements for the degree Doctor Philosophy

at the Faculty of Health Sciences,

University of Stellenbosch



Promoter

PROF DR FRANKEN

DECLARATION

I the undersigned, hereby declare that the work in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree

SIGNATURE.

DATE

ABSTRACT

Infertility is a very common problem worldwide. Recent data have shown that disorders of the male represent the most common single defined cause of infertility. This proposal examines the clinical significance and fundamental physiological aspects of human gamete interaction. These studies are focused on the assessment of the cellular-molecular mechanisms involved in human sperm binding to its homologous zona pellucida resulting in the physiologic induction of the acrosome reaction. We have developed and validated in vitro bioassays that assess specific steps of the fertilization process that are critical for early embryo development. The results of our translational research have already had a significant impact on the overall evaluation of male infertility and on the clinical management of the infertile man in the assisted reproduction arena. Furthermore, the unveiling of the basic mechanisms involved in human gamete interaction will ultimately allow for both (i) the development of new male reproductive diagnostic capabilities and (ii) the design of improved and safer therapies aiding conception in childless couples suffering from male infertility.

OPSOMMING

Menslike onvrugbaarheid is 'n algemene wêreldwye probleem en onlangse data toon aan dat die manlike factor die grootste enkel bydraende factor tot hierdie toestand is. Die werk loods 'n intensiewe ondersoek na die kliniese betekenis en basiese fisiologiese aspekte wat 'n rol tydens spermsel en eisel interaksie speel. Hoofstuk 3 fokus op die sellulêre en molekulêre meganismes wat betrokke is tydens spermsel en eisel binding wat gevolglik lei tot akrosoomreaksie van die spermsel. Die werk verteenwoordig die resultate van 10 jaar se navorsing tussen die kandidaat en die promoter. Dit gee oorsprong aan 'n reeks bio-toetse wat die bevrugtingsproses koriografiese ontleed en verskaf dus 'n stap-vir-stap uiteenseting van menslike bevrugting en gevolglike embrio ontwikkeling. Die resultate in Hoostuk 4 bring vernuwing in die begrippe van die manlike faktor en die rol in die kinderlose huwelik. Die resulate soos in Hoofstuk 3 en 4 uiteengesit, vorm nie net die basis vir die moontlike ontwikkeling van nuwe diagnostiese benaderings tot die hantering van die man nie, maar speel oojk 'n rol die daarstelling van verbeterde terapeutiese hantering van die kinderlose egpaar. Hoofstuk 5 gee kortliks riglyne en aanbevelings tot opsigte van die gebruik van die spermsel-zona pellucida bindingstoets en akrosomreaksie. Die kandidaat bevel aan dat die genoemde twee bio-toetse deel van die laboratorium ondersoeke van die man gebruik moet word.

Acknowledgements

Reproductive science is built on knowledge that results from valuable contributions of indefatigable clinicians and reproductive biologists. My work for the last 15 years has allowed me to interact and establish collaborations with scientists from not only the Jones Institute but also from many parts of the world. All of these scientists, their knowledge, common discussions and exchanges, have influenced my career and helped my academic accomplishments.

I am grateful to past and present professors and mentors of the Clinical Division of Reproductive Endocrinology and Infertility of the Jones Institute: Anibal Acosta, Zev Rosenwaks, Suheil Muasher, William Gibbons, Georgeanna Jones and Howard Jones, Jr. I am equally thankful to my mentor of the Scientific Division of the Jones Institute, Gary Hodgen. They have all represented the solid foundation of an ever-growing Institute founded on science and dedicated to life.

This work could not have been possible without the valuable assistance of the Andrology group that we organized throughout the years: Mahmood Morshedi, Co-Director of the Andrology Laboratory and Steve Taylor from the Jones Institute; Alessandro Schuffner (from Brazil), Gerardo Barroso (from Mexico), Hakan Duran, Kemal Ozgur and Namik Duru (from Turkey), Ahmed Nassar (from Egypt), Diana Vaamonde (from Spain) and Hadley Bastiaan (from South Africa), superb and hard-working collaborators.

Molecular biology aspects of this thesis were made possible thanks to the collaboration, insight and dedication of Ke Wen Dong, Simon Chi, Zhi Lin and Ming Hsu, all from the Jones Institute.

Special recognition should be given to Klaus and Elvira Hinsch (from Giessen, Germany) for their unselfish and highest quality assistance. An example of how science leads to long-term productive relationships and friendship.

I would like to acknowledge Mary Mahony, from the Jones Institute, Peter Blackmore, from Eastern Virginia Medical School, Gabor Huszar, from Yale University, Carlos Sueldo, from

the University of California at Fresno, and Willem Ombelet, form Genk, Belgium, for successful collaborations throughout the years.

Needless to say, my colleagues from the Reproductive Biology Unit at Tygerberg Hospital, Cape Town, South Africa, have provided indispensable help in the design and execution of many of the studies present herein. Top scientists on their own merit, Daniel Franken and Thinus Kruger have been collaborators and close friends for over a decade. My gratitude goes to them and their families.

This thesis presents the studies that I have performed with all the above-mentioned collaborators and includes research in the clinical, reproductive biology and molecular biology areas of our specialty. Some of this research has already been published in peer-reviewed journals and the latest manuscripts have recently been sent for publication. I consider all contributions to science of significance; they are always the basis for future developments, in this case with the ultimate goal of having an impact on the diagnosis and treatment of male infertility. Scientific advances depend upon ideas, creativity, people and resources. If anything, I hope that this effort projects to the present and younger generations that throughout the journey of scientific life, dedication, perseverance and teamwork can eventually lead to fruition.

Norfolk, July 2002

Dedication

I dedicate this thesis, its results and extensions to Dr. Washington H. Vignolo Puglia, Professor of Physiology, at the University of the Republic, Montevideo, Uruguay. Although physically not with us any more, his unbreakable spirit and convictions have guided me during this sojourn. Mentor and best friend since the early days of medical school, he taught me how to learn and how to teach, and how to think and advance science as a clinician.

Table of contents

Chapter 1.

Significance, objectives, speficic aims and development of bioassays for sperm function testing

1a. Significance

Assisted reproductive technologies: What new goals are immediately reachable?

In the last two decades we have lived an explosion of knowledge in basic reproductive biology and in the clinical success of assisted reproductive technologies (ART). A review of such achievements underscores the continuous need for: (i) re-examination and formulation of new questions related to physiologic and biochemical mechanisms involved in gametogenesis, fertilization and implantation, including the challenge of pre-existing dogmas; (ii) application of strict monitoring criteria for advancement of higher-quality laboratory practices and development of new hormonal preparations and techniques; (iii) improved reporting on clinical successes/failures; and (iv) appropriate socio-legal and ethical discussions that may allow novel ideas be transformed into realities in an expedited and safe manner.

Numerous areas are presently being scrutinized by active basic research endeavors. Notwithstanding such efforts, and at a clinical level, the more significant relevant questions appear to be: (1) can cycles of treatment in ART be further simplified in terms of monitoring and procedures? (2) Can diagnostic tools and predictors of success be improved in order to advance clinical management of both male and female infertility and to offer more accurate prognostic counseling to the infertile couple? (3) Can fertilization, but more importantly, implantation and pregnancy rates still be enhanced? And finally, (4) can the major complications of multiple pregnancy and ovarian hyperstimulation syndrome be eliminated?

Infertility clinics and In Vitro Fertilization (IVF) centers throughout the world struggle to achieve those aims. Better ways of diagnosing the ovarian reserve, but more fundamentally, a more accurate prediction of oocyte and embryo quality are needed. A better definition of male infertility, sperm parameter thresholds predictive of fertility potential and improved andrological diagnostic methods is warranted. The assessment of the developmental capacity and implantation potential of cultured embryos is still very imperfect. Our ability to select the best embryos requires the search for so far elusive biomarkers. And even the culture and transfer of blastocysts appears to be successful only in selected populations. All such areas of clinical research need to be encouraged as they may result in enhancement of diagnostic capabilities, implantation rates, and a decrease in multiple pregnancies.

A more profound understanding of the impact of ovulatory disorders, tubal factors (i.e., presence of hydrosalpinges) and endometriosis is being gained. The contribution of the male gamete to embryogenesis, including the impact of chromosomal/genetic factors and presence of DNA damage is being recognized. At the fertilization level, although ICSI (Intracytoplasmic Sperm Injection) is undoubtedly the procedure of choice in cases of moderate to severe male infertility, the technique does not appear to be universally indicated in all couples applying for ART. At least not until all safety issues are elucidated. The "black-box" of implantation and its failures remains unresolved and major efforts should be dedicated to unravel its secrets using classical and more novel approaches.

A few programs throughout the world have published outstanding implantation rates (approximately 40-50% chance of implantation per embryo transferred) and overall pregnancy rates (50-60% per transfer cycle) following blastocyst culture and transfer to the uterine cavity. Can those rates be achieved by all centers and even improved by optimized culture conditions, transfer techniques and luteal support? The answer is most likely affirmative. In our own program at the Jones Institute we have documented a total reproductive potential (combining the impact of "fresh" plus the cryopreserved-thawed embryo transfer cycles that followed a single stimulation of a given couple) of 55-65% in good ovarian responders of young age. Such results highlight the potential of the IVF technique.

We also have to keep in mind that in most occasions ART are "palliative" as opposed to "curative" of the underlying pathology. The truth of the matter is that in many female and male infertility cases, the reason of the underlying disease is unknown or idiopathic. Consequently, there is a fundamental need to carry out research directed to understand the pathophysiology as well as to be able to prevent those conditions resulting in infertility (i.e., sexually transmitted diseases, reproductive bio-hazards and others). These steps should lead to the development of simpler, safer and universally applicable therapies.

Progress has been made in the ethical and socio-legal aspects of IVF and other ART. Serious discussions at different levels have resulted in development of new guidance policies and/or legislation to regulate the procedures. The scientific community has made good progress in presenting its case and educating both government agencies and the public. Early worries about potential for abuse have been contained as the procedures have become mainstreamed. However,

constant monitoring of newly developed techniques and their application are needed in order to guarantee that they are used appropriately and not in an exclusive fashion favoring certain social groups.

There is no doubt that, thanks to the comprehension of infertility specialists, the advances in basic science that have translated into clinical interventions, and the inquisitiveness of the public in general, more and more couples arrive at the IVF scenario earlier and in increasing numbers. The cost-efficiency of the more advanced ART appears to be consolidated and most of the techniques are proven to be safe and with increasing success. The possibility to diagnose genetic disease in the embryo is here, and genetic banking, gene therapy and the growth of embryonic stem cells to be used in multiple areas of medicine are in the immediate horizon. Consequently, our efforts should be geared to introduce more scientific and technological advances while resting on a solid philosophical and ethical foundation and dedicated to serving society as a whole.

Male infertility: a major culprit

The successful implementation of ICSI has provided a unique means to allow couples suffering from severe male infertility to achieve their reproductive goals. However, despite the great therapeutic advantages of the technique, ICSI provides solution to clinicians often in the absence of an etiologic or physiopathologic diagnosis. The significance of this reality is highlighted by the very high frequency of male infertility (approximately 40 to 50% of infertile couples have male infertility) and by the limited knowledge as to its probable causes (50% of cases are still considered idiopathic).

It is well established that male infertility is typically associated with defective sperm parameters (frequently but not limited to, abnormal concentration, motility and/or morphology). Nevertheless, the information gained by the results of the "basic" semen analysis usually does not provide high prediction for the outcome of reproduction or an inkling on the underlying putative pathogenic mechanisms.

Recent work derived from the IVF arena has clearly demonstrated that an abnormal sperm-zona pellucida interaction is frequently observed in infertile men. Such finding can be observed in the presence of normal or abnormal basic sperm parameters. An impaired sperm-zona pellucida interaction results in fertilization failure or low rates of fertilization, thereby decreasing chances of

pregnancy even in IVF. Consequently, there is a real need to assess sperm functional competence in the "extended" evaluation of the infertility work-up. Although the cellular and molecular mechanisms involved in gamete interaction are beginning to be unraveled in several animal species, little is known about the basic mechanisms involved in human sperm-zona pellucida interaction. The studies proposed herein will therefore translate basic and clinical information into improved diagnostic techniques and more directed therapies to help alleviate male infertility.

1b. Overall objective, enunciation of specific aims and hypotheses

The **overall objective** of these studies was to investigate basic and clinical aspects of human sperm-zona pellucida interaction in order to gain knowledge on the biology of human fertilization and improve management of infertility. Notwithstanding the major impact derived from previous animal studies that have addressed and advanced the knowledge on gamete biology and fertilization, our experiments focused on the human spermatozoa and the human zona pellucida. It is our expectation that the information provided by these studies can be immediately translated into clinical applications.

Because our work focused on the use of human gametes (albeit not involving fertilization and embryo development) all studies presented herein were approved by the Institutional Review Boards at Eastern Virginia Medical School and Stellenbosch University. Furthermore, all laboratory procedures and personnel involved adhered strictly to the guidelines for human andrology laboratories dictated by the American Society of Reproductive Medicine and other regulatory agencies.

The **specific aims** of these studies were:

(1) To develop and validate an *in vitro* bioassay (the hemizona assay or HZA) for the assessment of tight sperm binding to the homologous zona pellucida. *Hypothesis:* Capacitated spermatozoa bind in a specific, tight and irreversible manner to the homologous, biologically intact zona pellucida, and undergo a physiologically induced acrosome reaction (exocytosis triggered by components of the zona pellucida). This hypothesis was tested by incubation of spermatozoa and the zona pellucida from microbisected oocytes followed by determination of the kinetics, sperm concentration-, sperm morphology-, and time-dependency of binding. We also examined the impact of oocyte storage conditions and maturational stage, and sperm acrosomal status on tight binding (Completed and Published studies).

(2) To develop and validate an *in vitro* bioassay (a micro volume assay) for the evaluation of the physiologically induced acrosome reaction of sperm in suspension by human solubilized zona pellucida. *Hypothesis:* Capacitated spermatozoa respond to the agonistic biological stimulus (i.e., zona pellucida) with maximal induction of the acrosome reaction, an exocytotic event resulting in the release of acrosomal enzymatic contents and exposure of the inner

acrosomal membrane. This hypothesis was tested by stimulation of the acrosome reaction after incubation of sperm with various concentrations of human, solubilized zona pellucida in capillary tubes (micro assay) and assessment of induction rates using a specific lectin-indirect immunofluorescence method (Completed and Published studies).

(3) To examine the capacitation-dependency of the spontaneous and agonist-induced acrosome reaction. *Hypothesis:* Agonist-dependent acrosomal exocytosis is maximally induced following defined time- and medium-dependent sperm incubation conditions and is associated with protein phosphorylation at the level of tyrosine residues and membrane translocation of phsophatidylserine, a biomarker of plasma membrane instability. This hypothesis was tested by simultaneous monitoring of phosphatidylserine translocation and acrosome reaction in sperm populations under various *in vitro* incubation conditions using annexin V binding and FITC-PSA immunofluorescence. Immunoblotting was used to examine tyrosine phosphorylation (Completed and Unpublished studies).

(4) To examine the intracellular cascades involved in the zona pellucida-induced acrosome reaction. *Hypothesis:* Solubilized zona pellucida triggers acrosomal exocytosis via a transmembrane signaling cascade involving heterotrimeric G proteins (pertussis toxin-sensitive); an alternative pathway involves modulation of intracellular calcium levels. To test this hypothesis capacitated spermatozoa were treated with pertussis toxin and/or EGTA (and respective negative and positive controls) prior to induction of acrosome reaction with solubilized zona pellucida. The priming effect of progesterone and synergistic activity of human follicular fluid were also examined (Completed and Unpublished studies).

(5) To establish the predictive value of the HZA for fertilization outcome in IVF. *Hypothesis:* Spermatozoa from infertile men have a statistically significant lower capacity to effect tight binding to the homologous zona pellucida (and consequently a lower ability to undergo a physiologically-induced acrosome reaction) when compared to fertile controls resulting in lower fertilization rates in IVF therapy. HZA results and IVF rates were assessed in men with normal and abnormal semen parameters in a prospective fashion and results assessed using logistic regression and cluster analyses (Completed and Published studies).

(6) To integrate the HZA and induced-acrosome reaction assays into a novel, sequential, diagnostic scheme in andrology and to compare results of methodologies currently

available to assess sperm functional capacities by a meta-analytical approach (evidence-based medicine). *Hypothesis:* Tight binding of sperm to the zona pellucida and the zona-induced acrosome reaction are critically impaired in infertile men and results of these assays can be used to identify patients at risk for fertilization failure with robust statistical power (high sensitivity and specificity). The predictive ability of currently used sperm function assays was compared using a meta-analysis; predictive values, specificity and sensitivity for fertilization outcome were determined by ROC curves and likelihood ratios (Completed and Published studies).

(7) To present a state-of-the-art strategy for the clinical management of male infertility. How should we manage andrology diagnosis and treatment in the new millennium? *Hypothesis:* Infertile men should be subjected to a standardized and updated sequential diagnostic scheme. The diagnosis should lead to the recommendation of an individualized and optimized therapeutic approach providing maximal chances of conception (Completed and Published studies).

1c. Development of bioassays for sperm funtion testing

***Specific aim 1*: To develop and validate an *in vitro* bioassay (the hemizona assay or HZA) for the assessment of tight sperm binding to the homologous zona pellucida.**

Hemizona assay and its impact on the identification and treatment of human sperm dysfunctions

[1]S. Oehninger, [2] D. Franken, [3]N. Alexander and G.D. [1]Hodgen

Andrologia 1992; 24:307-321

[1]The Jones Institute for Reproductive Medicine, Dept. Ob/Gyn, Eastern Virginia Medical School, Norfolk, Virginia; [2]Reproductive Biology Unit, Tygerberg Hospital, Dept. Ob/Gyn, University of Stellenbosch, Cape Town, RSA; and [3]National Institutes of Child Health and Human Development, Bethesda, MD

Zona binding test

S. Oehninger

ESHRE Andrology Special Interest Group

Excerpts on Human Reproduction 1996; (3) 11-13

The Jones Institute for Reproductive Medicine, Dept. Ob/Gyn, Eastern Virginia Medical School, Norfolk, Virginia

Abstract

The HZA, a functional test for human gamete interaction, has become a useful and valuable experimental tool for physiological and cellular analysis of the early events leading to fertilization. The analysis of the conventional semen parameters with the emphasis on sperm morphology (as judged by strict criteria) and motion characteristics (evaluated by computer assisted analysis) constitutes the first obligatory step for a critical evaluation of male factor patients. Patients in whom fertilization disorders are suspected should be evaluated through bioassays of sperm function of established accuracy. The HZA, a bioassay of sperm-zona binding capacity is here proven to be highly predictive of IVF outcome. Ultimately, our increasing knowledge of sperm biology and dysfunction will provide a basis for a better diagnosis (membrane receptor defects and metabolic/biochemical abnormalities?) as well as better therapeutic interventions in patients with sperm disorders. It seems likely that the HZA man be eventually replaced by standardized test kit in which recombinant human DNA-derived zona receptors mimic the natural function of the hemizonae currently used. This ZP3 regent may also be a useful antigen for contraceptive development. The HZA therefore constitutes a useful adjuvant in the armentarium for the diagnosis and therapy of male-factor patients.

Introduction

The hemizona assay (HZA) has been introduced as a new diagnostic test for the binding of human spermatozoa to human zona pellucida to predict fertilization potential (Burkman *et al.*, 1998). In the HZA, the two matched zona hemispheres created by microbisection of the human oocyte provide three main advantages: 1) the two halves (hemizonae) are functionally equal surfaces allowing controlled comparison of binding and reproducible measurements of sperm binding from a single egg, 2) the limited number of available human oocytes is amplified because an internally controlled test can be performed on a single oocyte, and 3) because the oocyte is split microsurgically, even fresh oocytes cannot lead to inadvertent fertilization and pre-embryo formation (Burkman *et al.*, Hodgen *et al.*, 1988).

Sperm-zona pellucida binding tests evaluate the first, crucial step of sperm-oocyte interaction that leads to fertilization, that is, tight binding of spermatozoa to the zona pellucida (Yangimachi, 1981). Overstreet and Hembree (1976) were the first to propose an assay for the evaluation of zona pellucida penetration by human spermatozoa using human oocytes recovered from the ovaries of cadavers. Initially developed as a zona penetration assay, sperm binding to the zona was not defined as an endpoint for the assay. The two most common zona binding tests currently used are the hemizona assay (HZA) (Burkman *et al.*, 1988) and a zona pellucida-binding test (Liu *et al.*, 1988, 1989). Both bioassays have the advantage of providing a functional homologous test for sperm binding to the zona comparing populations of fertile and infertile spermatozoa in the same assay. The internal control offered by the HZA represents an advantage by decreasing the number of oocytes needed during the assay and diminishing the intra-assay variation (Burkman *et al.*, 1988; Hodgen *et al.*, 1988; Oehninger and Alexander, 1991; Oehninger and Hodgen, 1991; Oehninger *et al.*, 1992d).

Before a spermatozoon can penetrate the zona pellucida there is a specific need for firm attachment between the gametes. When spermatozoa and the zona are mixed in vitro, some of the spermatozoa adhere rapidly and reversibly to the zona by a process called sperm 'attachment'. Subsequently, an irreversible adhesion occurs between the two gametes. This phase is called 'tight binding'. Tight binding is attributed to the presence of complimentary binding sites or receptors on the surface of the gametes and typically these receptors manifest a high degree of species specificity (Yanagimachi, 1981; Ahuja, 1985). Because of the binding of gametes is a critical step in fertilization and pre-embryo development; the HZA has many potential uses in reproductive

medicine. Initially developed as a test to investigate male infertility and predict fertilization potential, this bioassay has been used to assess sperm function after contraceptive treatment (Burkman, 1988; Hodgen, 1989; Oehninger and Alexander, 1991; Oehninger and Hodgen, 1991; Oehninger *et al.*, 1991d). As has often been the case, new findings in reproductive medicine can usually be projected toward infertility or contraceptive technology depending on the focus of our interest (Hodgen, 1989).

The objective of this chapter is to summarize studies that were performed in order to develop and validate a model (an in vitro bioassay, the hemizona assay or HZA) for the assessment of tight sperm binding to the homologous zona pellucida. Because the chapter is the results of numerous studies I took the liberty to modify its format. Methodology, results and discussion have been combined and in addition the information is presented in three sections including: 1) use of the HZA to predict human spermatozoa fertilization potential, 2) use of the HZA in contraceptive technology, and 3) use of the HZA to examine the biological basis of the human fertilization process.

Methodology, results and discussion

Use of the HZA as a predictor of human spermatozoa fertilization potential

Male-factor infertility remains one of the most challenging barriers to successful (viable) pregnancy despite the application of assisted reproductive technologies. While some cases of male related infertility can be suspected based on standard evaluations before attempting in vitro fertilization (IVF) or related techniques, some are unanticipated and alarming for couples involved. A better way to identify and classify cases of sperm dysfunction would aid in prognostication and allow for a plan before failed or poor fertilization is confirmed.

Conventionally measured features of semen using the World Health Organization (WHO) criteria (World Health Organization, 1987), including sperm concentration, motility, and morphology, all effect rates of fertilization in vitro and the outcome of assisted reproductive techniques, as demonstrated in both IVF and gamete intrafallopian transfer (GIFT) (Mahadevan and Trounson 1984; Acosta *et al.*, 1989; Rodriguez-Rigau *et al.*, 1989). The evaluation of sperm morphology by a more stringent technique (strict criteria) has enhanced objectivity and decreased intra- and inter-assay variations while examining this sperm parameter (Kruger *et al.*, 1986, 1988).

Using strict criteria, sperm morphology has been shown to significantly enhance the prediction of IVF outcome (Acosta *et al.,* 1989; Kruger *et al.*, 1986,1988,1990). Although positively correlated with fertilization rates in IVF, the assessment of sperm motion characteristics by computer assisted semen analysis still cannot be reliably used to predict fertilization outcome (Oehninger and Alexander, 1991; Oehninger and Hodgen 1991; Oehninger, 1992). Therefore, attention has focused in bioassay of sperm-oocyte interactions (Overstreet and Hembree, 1976; Overstreet *et al.,* 1980; Burkman *et al.,* 1988; Liu *et al.,* 1988, 1989). Three categories of tests compose this battery of bioassays: 1) sperm-zona pellucida binding tests, 2) sperm-zona pellucida penetration tests, and 3) sperm-oocyte fusion tests.

A highly specific type of binding is necessary for fertilization to ensure, and some disorders of male infertility may be due to a failure of such sperm function. Therefore, the HZA provides a unique homologous (human) bioassay to assess sperm function at the fertilization level (Fig 1). Different sources of human oocytes can be used in the assay, oocytes recovered from surgically removed ovaries or post-mortem ovarian tissue, and surplus oocytes from the IVF program. Since fresh oocytes are not always available for the test, different alternatives have been implemented for storage. Others have described the storage of human oocytes in dimethylsulfoxide (DMSO) at ultra low temperatures (Overstreet and Hembre, 1976). Additionally, Yanagimachi and colleagues showed that highly concentrated salt solutions provided effective storage of hamster and human oocytes such that the sperm-binding characteristics of the zona pellucida were preserved (Yanagimachi *et al.,* 1979). In developing the HZA, we have examined the binding ability of fresh, DMSO and salt-stored (under controlled pH conditions) human oocytes and have concluded that the sperm binding ability of the zona remain interact under all these conditions (Yoshimatsu *et al.,* 1988; Franken *et al.,* 1989a; Kruger *et al.*, 1991). Subsequently, we have assessed the kinetics of sperm binding to the zona showing maximum binding at 4-5 h of gamete coincubation with similar binding curves both for fertile and infertile semen samples (Burkman *et al.,* 1988; Franken *et al.,* 1989a) (Fig.2).

Detailed description of oocytes collection, handling, and micromanipulation as well as semen processing and sperm suspension preparations for the HZA have been described elsewhere (Burkman *et al.,* 1988, 1990). The assay has been standardized to a 4 h gamete coincubation, exposing each hemizona to a sperm droplet (100 µl of a dilution of 500,000 motile sperm ml^{-1} prepared after swim-up). Ham's F-10 medium supplemented with foetal cord serum is usually the medium utilized for sperm preparation and gamete coincubation. After coincubation, the hemizona

are subjected to pipetting through a glass pipet through a glass pipet in order to dislodge loosely attached sperm. The number of tightly bound spermatozoa on the outer surface of the zona is finally counted using phase contrast microscopy (x 200). Results are expressed as the number of sperm tightly bound to the hemizona for control and patient and also as hemizona index (HZI) the number of sperm tightly bound for the control sample (x 100) (Burkman *et al.,* 1988). The assay has been validated by a clear-cut definition of the factors effecting data interpretation, i.e., kinetics of binding, egg variability and maturation status, intra-assay variation and influence of sperm concentration morphology, motility and acrosome reaction status (Coddington *et al.,* 1991; Oehninger *et al.,* 1991e; Franken *et al.,* 1989a, 1990b, 1991b, 1991c) (Fig. 3).

Over 90 days evaluation, spermatozoa from fertile men do not exhibit a time-dependent change in zona binding potential, thus, reassuring their utilization as controls in the bioassay (Franken *et al.,* 1991b). Within each pool of donor's utilized in the assay, a cut-off value or minimal threshold of binding has to be established in order to validate each assay for the purpose of identification of a poor control semen specimen and/or a poor zona. In our control population, this cut-off value is approximate 20 sperm tightly bound to the control hemizona (fertile donor) (Franken *et al.,* 1991b). Therefore, each laboratory should statistically assess its own control data to establish a reasonable lower limit assay acceptance.

If the control hemizona (matching hemizona exposed to fertile semen) has a good binding capability, that is, tightly binds at least 20 spermatozoa after the 4 h incubation period (information derived from a statistical evaluation of a pool fertile donors), then a single oocyte will give reliable information about the fertilizing ability of test spermatozoa (sperm from infertile patients) under IVF conditions. Because of the definition of the assay's limitations and its small intra-assay variation (less than 10%) the power of discrimination of the HZA has been maximized. Conversely, for other sperm-zona binding tests, several oocytes have to be used because of the high inter-egg variation and in fact an intra-assay coefficient of variation of 30% has been reported (Liu *et al.,* 1988, 1989).

The inter-egg variability is high not only for oocytes at different stages of maturation (immature vs. mature eggs) but also within a certain population of eggs at the same maturation stage. However this factor is internally controlled in the assay by the utilization of matching hemizona that allows a comparison of binding of a fertile vs. an infertile semen sample in the same assay under the same oocyte quality conditions. Incubating matching hemizona from eggs at the

same maturational stage with homologous spermatozoa from the same fertile ejaculate, we have been able to determine the intra-egg (intra-assay viability) both for human and monkey (cynomolgus) oocytes (Oehninger, *et al.,* 1989b, 1991e, 1992a). Overall, the mean value of the difference between the two matching halves shows an intra-egg variability of approximately 10% for all categories of egg nuclear maturation (Table 1). Additionally, we have shown that full meiotic competence of human and monkey oocytes are associated with an increased binding potential of zona pellucida. It would appear that zona maturation is associated with the nuclear development progression of the oocyte, perhaps in parallel with cytoplasmic and membrane maturation, leading to a fully fertilizable status.

The specificity of the interaction between human spermatozoa and the human zona pellucida under HZA conditions is strengthened by the fact that the sperm tightly bound to the zona are acrosome reacted (see below). Results of interspecies experimented performed with human, cynomolgus monkey and hamster gametes have demonstrated a high species specificity of human sperm/zona pellucida functions under HZA conditions providing further support for the use of this bioassay in infertility and contraception testing (Table 2).

In prospective blinded studies, we have investigated the relationship between sperm binding to the hemizona and IVF outcome (Franken *et al.,* 1989b; Oehninger *et al.,* 1989a, 1991c). Results have shown that the HZA can successfully distinguish the population of male-factor patients at risk for failed or poor fertilization. Using either a cut-off value of fertilization rate of 65% (mean minus 2 standard deviations of the overall fertilization rate in the Norfolk Program for non-male-factor patients), or distinguishing between failed vs. successful fertilization (0% vs. 1-100%) the hemizona assay results expressed as HZI provide a valuable means to separate these categories of patients (Oehninger and Hodgen, 1991; Oehninger, 1992; Oehninger, *et al.,* 1992c) (Table 3).

Powerful statistical results allow us to use the HZA in the prediction of fertilization rate (Oehninger, 1992; Oehninger *et al.,* 1992c) (Fig 4). Therefore, the HZA can distinguish a population of male-factor patients that will encounter low fertilization rates in IVF, and when combined with the information provided by other sperm parameters (morphology and motion characteristics) gives reliable and useful information in the clinical arena. Of the classical sperm parameters, sperm morphology is the best predictor of the ability of spermatozoa to bind to the zona pellucida (Fig 4). Patients with severe teratozoospermia ('poor prognosis" pattern or less than 4%

normal sperm scores as judged by strict criteria) have an impaired capacity to bind the zona under HZA conditions (membrane/receptor deficiencies?) (Oehninger, 1992). We agreed with Liu et al., that the ability of sperm to achieve tight binding to the zona pellucida might reflect multiple functions of human spermatozoa (Liu, *et al.,* 1988,1989).

In our studies, when the HZA was removed from regression analysis in order to identify the predictive value of other sperm parameters (sperm concentration, morphology and motion characteristics), the percent of progressive motility was the second best predictor of fertilization outcome (Oehninger, 1992). We speculated that the relationship between sperm morphology and IVF results depends upon an effect on zona binding. On the other hand, motility seems to affect the prediction of fertilization rate outside the prediction of the HZA. It would appear that although important in achieving binding, motility may be more important for cumulus penetration and zona pellucida penetration, factors not directly evaluated in the HZA. Logistic regression analysis provided a robust HZI range predictive of the oocytes potential to be fertilized. This HZI cut-off value is approximately 35%. Overall, for failed vs. successful and poor vs. good fertilization rate, the correct predictive ability (discriminative power) of the HZA was 80 and 85%, respectively (Table 4). The information gained may be extremely valuable for counseling patients in the IVF setting (i.e., considering a HZI below 35% the chances of poor fertilization are 90-100%, whereas for the HZI over 35%, the chances of good fertilization are 80-85%) (Oehninger, 1992; Oehninger *et al.,* 1992c).

Therefore, the assay has an excellent sensitivity and specificity with a low incidence of false positive results (Table 4, Fig 5). For an HZI of 35%, the positive predictive value of the HZA is 79% and its negative predictive value is 100% (considering good vs. poor fertilization rates). In the HZA, false positive results can be expected, since other functional steps follow the tight binding of sperm to the zona pellucida and are essential for fertilization and pre-embryo development. Thus, although binding is not crucial to achieve fertilization, it does not guarantee that fertilization will ensue. However, tight binding to the zona is an absolute prerequisite for fertilization to follow naturally.

Furthermore, in prospective studies we have examined the ability of the HZA to predict IVF results in excess oocytes donated by patients in the same IVF/GIFT treatment cycle. In addition, we assessed the power of the HZA to prognosticate the fertilization outcome in subsequent IVF cycles in the same patients. Results have shown that for same and subsequent

treatment cycles (repeating patients) an HZI value of 35% was again an excellent predictor (discriminator) do success/failure.

Under HZA conditions, the minimum concentration of motile sperm from fertile donors necessary to achieve valid results is approximately 250,000 motile spermatozoa ml^{-1}. The 'effective number of sperm' (morphological normal sperm with high motility) can be determined under theses HZA conditions and may be indication of the actual number of normal spermatozoa necessary to achieve binding and thereby anticipation successful fertilization (Franken *et al.*, 1990b). Others have proposed that the HZA may be particularly useful for predicting which patients may benefit from insemination within increased concentration (patients with severe oligozoospermiain in which micro-insemination methods are utilized to try to enhance fertilization rates (Hammit *et al.*, 1991).

Severe disorders of fertilization (failure or delayed fertilization) occur up to 8% of IVF cycles (Oehninger *et al.*, 1988, 1989c). We have analyzed couples with recurrent IVF failure in order it establish a pathophysiologic diagnoses for specific functional defects of sperm-oocyte interactions during fertilization process, and also to develop a serial, diagnostic scheme for managing these clinical problems (Oehninger *et al.*, 1991a). The results of predictive fertilization bioassay (HZA and hamster zona free egg/sperm penetration assay or SPA), IVF treatment, fertile donor cross-match tests with either sperm or oocytes, and oocyte micromanipulation techniques for assisted fertilization, were analyzed in sequence (Table 5). All patients had recurrent failed fertilization in multiple IVF attempts.

The sequential analysis depicted here allowed us to establish a specific diagnosis of sperm-oocyte defects. Clearly, one should also take into consideration technical problems during the IVF attempt, including iatrogenic failures due to sperm preparation techniques (sperm damage caused by production of reactive oxygen species after repeated pelletting of unselected sperm populations) (Aitken and Clarkson, 1987; Aitken, 1988; Aitken *et al.*, 1991; Mortimer, 1991). Results using this type of evaluation have indicated that specific defects could be demonstrated at the level of sperm-zona binding, zona penetration, and sperm-oocytes fusion. Therefore, they could possibly explain failure of fertilization at a particular point (Oehninger and Hodgen, 1991; Oehninger, *et al.*, 1991a, 1991e, 1992c).

Failure of fertilization due to a defective sperm-zona pellucida interaction may be relatively common problem, thereby underscoring the potential of the HZA as a diagnostic/predictive test.

However, one must realize that post-zona binding defects may occur, thereby stressing the necessity of evaluating patients with fertilization disorders utilizing other bioassays, which assess complimentary sperm functions. The order of progression of the predictive bioassays may be important because those sperm defects that prevent tight binding to the zona pellucida or its penetration will negate subsequent functional tests. Accordingly, the HZA should be applied first. Men whose specimens show adequate tight binding or penetration under HZA conditions should be examined next for acrosome reaction, oolema fusion, decondensation, and pronuclear formation in sequence. The results of this sequential analysis help clinicians in the management of these difficult cases and assist in the selection of optimal therapies (i.e., micro-insemination methods (van der Ven *et al.,* 1989), medical therapies if they finally become validated (Acosta *et al.,* 1991), or oocyte micromanipulation for assisted fertilization (Cohen *et al.,* 1988; Ng *et al.,* 1988; Palermo *et al.,* 1993) (Fig.6).

The presence of antisperm antibodies has been shown to negatively affect sperm function in several ways. Antibodies that agglutinate, immobilize, or opsonize sperm impair migration through the female reproductive tract and signifignificantly reduce the number of sperm at the fertilization site. Likewise, antibodies binding to the sperm surface may interfere with the normal process of sperm differentiation, capacitation, cumulus penetration, acrosome reaction, zona penetration, or sperm-oocyte membrane interactions (Alexander, 1989). In addition to the routinely used serum and semen tests for the detection of antisperm antibodies, the HZA may be a test that differentiates between those sperm populations that will have reduced fertility and those that will not (Speroff *et al.,* 1990; Alexander and Oehninger, 1991).

It has been previously shown that sperm specific isoantibodies and autoantibodies inhibit the binding of human sperm to the human zona pellucida and also that immobilizing antibodies have a blocking effect on sperm penetration of human zona pellucida (Broson *et al.,* 1989; Tsukui *et al.,* 1988). Sperm/zona binding assays provide another method of evaluation of the effect of different subtypes of antisperm antibodies on this crucial step of gamete interaction (Franken *et al.,* 1991a; Liu *et al.,* 1991; Mahony *et al.,* 1991a; Mahony and Alexander, 1992). Although the presence of antisperm antibodies seems to influence the transport capabilities of the gametes and the fertilization process in vivo, IVF and GIFT have been successful in treating patients immunologic infertility (Acosta *et al.,* 1989; Elder *et al.,* 1990; van der Merwe *et al.,* 1990).

We have utilized the HZA in order to try to determine the possible effect of peritoneal fluid from patients with endometriosis on sperm/oocyte interactions. Although small numbers of patients have been studied so far there appears to be a negative effect of peritoneal fluid obtained from patients with endometriosis on zona-sperm interaction (Coddington *et al.,* 1992) (Fig. 7). This might represent one multiple mechanisms of infertility in patients with endometriosis. It is not known whether detrimental changes are occurring at the level of the zona pellucida or the level of sperm resulting in decreased tight binding, but the effect seems to be greater as the stage of endometriosis increases (Coddington *et al.,* 1992). Not only can the HZA evaluate the effects of endometriosis on sperm/oocyte interaction, but it might be useful as a marker to monitor this effect in treated patients as well.

Use of the HZA in contraceptive technology

Being a functional bioassay that evaluates sperm-zona pellucida oocyte interaction, the requisite first step in fertilization and development, the HZA has a potential role in contraceptive technology. For example, contraceptive vaccines that depend on blocking antigens essential to sperm an oocyte functions involved in sperm binding require some means for prediction of clinical success. Such biological endpoints would help to monitor effective antibody titer attainment and maintance during and after the immunization regimen (Hodgen, 1989).

Similarly, the use of endocrine regimens to suppress spermatogenesis to obtain fertility control is fraught with concern that persistent oligozoospermia or episodic escape phenomenon might leave some subjects at risk for unwanted fertility. Even though there is evidence to suggest that this residual sperm may be qualitatively abnormal, their fertilizing potential may be high. Achievement and maintenance of azoospermia in men receiving GnRH agonists or other hormonal protocols for example, seems an exclusive and perhaps an impractical goal. Thus, a biological test that can reliably show the fertility potential of sperm may be scientifically and ethically informative during early clinical trials. Ongoing experiments are assessing the potential use of HZA in predicting contraceptive efficacy in these circumstances (antifertility assay) (Rodgers, *et al.,* 1990). Also, the HZA is an invaluable tool for the assessment of antifertilizing capacity of antihuman sperm monoclonal antibodies (Hodgen, 1989; Oehninger and Alexander 1991; Oehninger and Hodgen, 1991; Oehninger *et al.,* 1991d; Mahony and Alexander, 1991; Oehninger, 1992). Definition of the antigens involved in sperm-egg interaction (receptors) could provide important advances in the development of a contraceptive vaccine.

Since compelling evidence has demonstrated that sperm surface carbohydrates-binding proteins mediate gamete recognition by binding with the high affinity and specificity to complex-like glucoconjugates of the zona pellucida, we have investigated the role of complex saccharide moieties using the HZA system (Ahuja, 1985; Oehninger *et al.,* 1990). Fucoidin, a polymer of sulfated L-fucose had an immediate, dose-dependant and potent inhibitory effect of sperm binding to the zona pellucida, an effect that was present even after washing the sperm cells after the preincubation period (Fig. 8). This complex sugar has no effect on sperm viability or motion characteristics and does not affect spontaneous rate of acrosome reaction (Oehninger *et al.,* 1990, 1991b, 1992b). Furthermore, fucoidin inhibits the acrosome reaction exogenously stimulated with solubilized human zona pellucida and does not effect acrosome reaction triggered by a calcium ionophor (Mahony *et al.,* 1991b) (Fig 9). This information points to an inhibition of a receptor ligand-type interaction. Moreover, fucoidin has no effect on the influx of calcium triggered by progesterone (Fig 10). Therefore, the binding event inhibited fucoidin seems to be rather specific, although further experiments are required in order to validate this hypothesis. Potential contraceptive methods based on adding complex saccharides such as fucoidin (or others) to spermicidal formulations seem plausible if these carbohydrate moieties are effective and well tolerated in vivo. Therefore, the HZA can be a system in which both spermicidal, antifertilization and virucidal compounds can be tested for contraceptive purposes (inhibitors of sperm-egg interaction) (Oehninger, 1992).

Use of the HZA to examine the biological basis of human fertilization

The specificity of the interaction between human spermatozoa and the human zona pellucida under HZA conditions is strengthened by the fact that the sperm tightly bound to the zona are acrosome-reacted. This has been established by transmission electron microscopic evaluation of sperm bound to and penetrating through the hemizona and by the demonstration of hemizona induced acrosome reactions using the T-6 monoclonal antibody immunofluorscent techniques (Coddington *et al.,* 1990; Franken *et al.,* 1991d; Fulgham *et al.,* 1992). Results in interspecies experiments (see above) have also demonstrated a high species specifity of human gamete functions under HZA conditions, both in terms of tight binding as well as in the ability of the zona glycoprotein to trigger the acrosome reaction.

The HZA offers the arena in which prefertilization sperm events (capacitation and final maturation changes) can be tested under the appropriate conditions, i.e., zona pellucida. The temporal relationships between sperm-zona binding, hyperactivated motility, and the acrosome reaction have been recently illustrated in human gametes. Spermatozoa from fertile men showed a hyperactivation peak at about 2h, followed by a slow decline through 5 h, coincident at 3 h 30min (Burkman, 1984, 1990). Under the conditions of this particular study (stimulated/synchronized system) there was a shift in acrosomal status, which occurred between 2 and 3.5 h. As previously reported, the kinetics of change in the extent of hyperactivation and in acrosomal loss although measured in different sperm population are consistent with and association between these two events (Robertson *et al.,* 1988). Furthermore, the kinetics of the acrosome reaction of human spermatozoa may correlate with sperm fertilizing capacity under IVF conditions (Takahashi *et al.,* 1992).

Although the sequence of biochemical/physiologies events leading to capitation is not fully understood, an efflux of cholesterol from the sperm plasma membrane resulting in a modification of membrane fluidity may be an important factor. In the mouse model, the binding of ZP3 to a sperm receptor initiates gamete interaction. This receptor-ligand interaction then triggers intracellular transduction signals resulting in exocytosis, i.e. the acrosome reaction (Greve and Wasserman, 1985; Bleil and Wasserman, 1986; Jones, 1990; Wasserman 1990; Sailing, 1989, 1991). Based on the present evidence in the human model under HZA conditions, it seems that capacitation-zona binding-acrosome reaction may occur in the same sequence. However, it has been shown that acrosome intact and acrosome reacted human sperm can initiate binding to the zona (Morales *et al.,* 1989). Even so, the question remains about the physiological significance of those two different sperm populations. Acrosome reactions may occur asynchronously both in vivo as well as under in vitro conditions. Nevertheless, the physiologically relevant acrosome reaction in human sperm may well be the one that occurs at the level of zona pellucida, triggered by this structure after initial recognition and tight binding has occurred.

Time course studies using the HZA system support the concepts that 1) the human zona pellucida is an important trigger for the acrosome reaction, and 2) that sperm are an asynchronous population in regard to acrosomal status; those spermatozoa which achieve tight binding to the hemizona progress rapidly through the acrosome reaction (ZP3 induced?), while those sperm cells not tightly bound have a very low frequency of spontaneous acrosome reaction. Bound sperm would then be ready to penetrate the zona by binding to successive ZP2 molecules in their

transverse through the matrix, while acrosin and possibly other proteases cleave the ZP2/ZP3 filaments in their path.

Studies have shown that progesterone can induce the acrosome reaction in capacitated human sperm (Suarez *et al.*, 1986; Thomas and Meizel, 1988). It has also been demonstrated that progesterone can induce a rapid influx of calcium ions in either capacitated or non-capacitated human sperm (Thomas and Meizel, 1988; Blackmore *et al.*, 1990). The induction of the acrosome reaction is believed to follow this influx of calcium ions. This rapid effect of progesterone to stimulate calcium influx indicates a non-genomic response and suggests that cell surface receptors exist for progesterone (Blackmore, *et al.*, 1990,1991). It has recently been shown that a distinct non-genomic cell surface receptor for progesterone exists in human sperm (Blackmore *et al.*, 1991). High levels of progesterone are present in human follicular fluid. Therefore, we have investigated the potential effects of progesterone on sperm functions associated with the fertilization process. After a 1 h preincubation with sperm, progesterone at the dose of 1 $\mu g/ml^{-1}$ significantly enhanced binding of sperm to the zona pellucida in fertile men. Additionally, progesterone at the same dose enhanced the penetration of zona-free hamster eggs (SPA) both with an enhancement of the capacity to penetrate zona-free eggs after 1 h and 24 –h sperm preincubation (Sueldo *et al.*, 1992) (Table 6). Furthermore, although progesterone did not enhance acrosome reactions in short incubations times, there was a positive effect after 24 h of coincubation. We have also observed a significant enhancement of hyperactivation motility after exposure to progesterone (Mbizvo *et al.*, 1990). Therefore, progesterone could represent a means to enhance sperm function at the fertilization level at the time of IVF. Current studies are testing this hypothesis.

We have reported negative correlations between the percentage of morphologically abnormal spermatozoa and the results of assisted reproduction techniques (Kruger *et al.*, 1988; Acosta *et al.*, 1989). Patients with teratozoospermia demonstrated poor sperm binding to the sperm binding to the zona pellucida and low IVF rates (Cohen *et al.*, 1988, Franken *et al.*, 1990b). Additionally, we have reported a significant linear relationship between the number of sperm tightly bound to the hemizona and the percentage of spermatozoa with normal morphology in the semen evaluated according to strict criteria (Oehninger, 1992). These findings suggested that a natural selection occurs against morphologically abnormal spermatozoa at the site of zona pellucida. That is, under in vivo conditions natural barriers are present minimizing migration and transport of abnormal spermatozoa through the female genital tract and cumulus cells, thus diminishing the chances of these abnormal spermatozoa to play a role in fertilization. These natural barriers of the reproductive

tract are not present under IVF or HZA conditions except for the selection in motility and morphology following swim-up separation.

Therefore, we investigated the morphology of those spermatozoa tightly bound to the zona pellucida using HZA as a bioassay model and we compared the morphological normality of zona-bound spermatozoa to the observed in the original semen samples and swim-up fractions (Menkveld *et al.,* 1991). A significantly higher number of normal forms was found among the zona-bound sperm, both for normospermic as well as teratozoospermia patients (Menkveld *et al.,* 1991) (Table 7). These results indicate that a selection process against abnormal spermatozoa occurs at the site of the zona pellucida. Spermatozoa classified as normal or slightly abnormal have the potential for selectively achieving binding to the zona in favor of abnormal (bizarre) spermatozoa; particularly those sperm cells with acrosome abnormalities and other sever head defects are either actively excluded or simply cannot bind to the zona or do it with a low efficiency due to inherent defects. This newly identified human zona property of sperm selectivity points to another potential use of HZA (i.e., selection of sperm to be used in assisted fertilization). Examples of this may include microinjection of a selected sperm directly into the ooplasm or transfer of multiple spermatozoa into the perivitelline space to achieve assisted fertilization. For such purposes, using micromanipulators, morphologically normal sperm can be identified more efficiently and removed from the hemizona after which they can be used for surgical fertilization (Menkveld *et al.,* 1991). Additionally, this would ensure that the physiological acrosome reaction had occurred.

In support of this exciting development, Huszar and co-workers have utilized creatine kinase-immunocytochemistry to evaluate human hemizona – sperm complexes to examine whether the distribution of creatine kinase-stained cells bound to the hemizona would follow the incidence of these sperm cells in semen samples, or if there is preferential binding by the normal sperm (Huszar *et al.,* 1994). Huszar *et al.,* have previously shown a relationship between sperm creatine kinase concentrations and fertilizing potential in men (Huszar *et al.,* 1990; Huszar and Vigue, 1990). The binding to the hemizona was selective for normal sperm as the incidence of intermediate and dark sperm in the semen samples was significantly higher than in those bound to the hemizona (normal = clear heads; intermediate and immature = light and dark staining. Huszar and co-workers have suggested that this high degree of selectivity is not related to the properties of the zona, but rather to the fact that immature and abnormal spermatozoa are defective in the site(s) of oocyte recognition and binding. Therefore, creatine kinase staining patterns in the hemizona

complexes support the use if strict criteria for identification of the male-factor population and the use of the HZA in assisted reproduction and in assisted fertilization.

References

1. Acosta AA, Oehninger, S, *et al.,* (1991). Possible role of pure human follicle-stimulating hormone in the treatment of severe male-factor infertility by assisted reproduction: preliminary report. *Fertil Steril*, **55**, 1150-1156.
2. Acosta AA, Oehninger, S *et al.,* (1989). Assisted reproduction in the diagnosis and treatment of the male factor. *Obstet Gynecol Surv*, **44**, 1-18.
3. Ahuja KK (1985). Carbohydrate determinants involved in mammalian fertilization. *Am J Anat*, **174**, 207-223.
4. Aitken, RJ, Irvine DS, *et al.,* (1991). Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol*, **164**. 542-551.
5. Aitken RJ, (1988). Assessment of sperm function for IVF. *Hum Reprod*, **3**, 89-95.
6. Aitken RJ and Clarkson JS (1987). Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil*, **81**, 459-469.
7. Alexander, NJ and Oehninger S (1991). Use of the hemizona assay to define sperm function. Comparative spermatology 20 years after. B. B. New York, Raven Press, 793-396.
8. Alexander, NJ (1989). Natural and induced immunological infertility. *Curr Opin Immunol*, **1**, 1125-1130.
9. Blackmore PF, Neulen J, *et al.,* (1991). Cell surface-binding sites for progesterone mediate calcium uptake in human sperm. *J Biol Chem*, **266**, 18655-18659.
10. Blackmore PF, Beebe SJ, *et al.,* (1990). Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem*, **265**, 1376-1380.
11. Bleil JD and Wassarman PM (1986). Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *J Cell Biol*, **102**, 1363-1371.
12. Bronson RA, Cooper GW, *et al.,* (1982). Sperm-specific isoantibodies and autoantibodies inhibit the binding of human sperm to the human zona pellucida. *Fertil Steril*, **38**, 724-729.
13. Burkman, L, Coddington C, *et al.,* (1990a). The hemizona assay: Assessment of fertilizing potential by means of human sperm binding to human zona pellucida. Handbook of the laboratory diagnosis and treatment of infertility. Keel BA and Webster BW. CRC Press, Boca Raton, FL, 213-228.

14. Burkman L. (1990b). Hyperactivated motility of human spermatozoa during in vitro capacitation and implications for fertility. Controls of sperm motility: biological and clinical aspects. G. C. Boca Raton, FL, CRC Press, 303-329.
15. Burkman LJ, Coddington CC, *et al.,* (1988). The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril*, **49**, 688-697.
16. Burkman LJ. (1984). Characterization of hyperactivated motility by human spermatozoa during capacitation: comparison of fertile and oligozoospermic sperm populations. *Arch Androl*, **13**, 153-165.
17. Coddington CC, Oehninger S, *et al.,* (1992). Peritoneal fluid from patients with endometriosis decreases sperm binding to the zona pellucida in the hemizona assay: a preliminary report. *Fertil Steril*, **57**, 783-786.
18. Coddington CC, Franken DR, *et al.,* (1991). Functional aspects of human sperm binding to the zona pellucida using the hemizona assay. *J Androl*, **12**,1-8.
19. Coddington CC, Fulgham DL, *et al.,* (1990). Sperm bound to zona pellucida in hemizona assay demonstrate acrosome reaction when stained with T-6 antibody. *Fertil Steril*, **54**, 504-508.
20. Cohen J, Malter H, *et al.,* (1988). Implantation of embryos after partial opening of oocyte zona pellucida to facilitate sperm penetration. *Lancet*, **2**, 162.
21. Elder KT, Wick, KL *et al.,* (1990). Seminal plasma anti-sperm antibodies and IVF: the effect of semen sample collection into 50% serum. *Hum Reprod*, **5**, 179-184.
22. Franken D, Kruger T, *et al.,* (1993) The ability of the hemizona assay to predict human fertilization in vitro in different and consecutive IVF/GIFT cycles. *Hum Reprod*, **8**, 1240-1244.
23. Franken, DR, Windt ML, *et al.,* (1991a). Assisted reproductive technologies may obviate apparent immunologic infertility. *Andrologia*, **23**, 291-295.
24. Franken, DR, Coddington, CC, *et al.,* (1991b). Defining the valid hemizona assay: accounting for binding variability within zonae pellucidae and within semen samples from fertile males. *Fertil Steril*, **56**, 1156-1161.
25. Franken, DR, Windt ML, *et al.,* (1991c). Comparison of sperm binding potential of uninseminated, inseminated- unfertilized, and fertilized-noncleaved human oocytes under hemizona assay conditions. *Mol Reprod Dev*, **30**, 56-61.

26. Franken, DR, Oosthuizen WT, *et al.*, (1991d). Electron microscopic evidence on the acrosomal status of bound sperm and their penetration into human hemizonae pellucida after storage in a buffered salt solution. *Andrologia*, **23**, 205-208.
27. Franken D, Burkman, L, *et al.*, (1990a). Human hemizona attachment assay. Human spermatozoa in assisted reproduction. Baltimore, Williams & Wilkins, 335-371.
28. Franken DR, Kruger, TF, *et al.*, (1990b). "Hemizona assay and teratozoospermia: increasing sperm insemination concentrations to enhance zona pellucida binding." *Fertil Steril*, **54**, 497-503.
29. Franken DR, Burkman, LJ, *et al.*, (1989a). Hemizona assay using salt-stored human oocytes: evaluation of zona pellucida capacity for binding human spermatozoa. *Gamete Res*, **22**, 15-26.
30. Franken DR, Oehninger, S, *et al.*, (1989b). The hemizona assay (HZA): a predictor of huan sperm fertilizing potential in *in vitro* fertilization (IVF) treatment. *J In vitro Fert Embryo Transf*, **6**, 44-50.
31. Fulgham, D, Coddington C, *et al.*, (1992). Human sperm acrosome reaction on zona pellucida: a time course study. *ARTA*, **3**, 25-34.
32. Greve JM and Wassarman PM (1985). Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. *J Mol Biol*, **181**, 253-264.
33. Hammitt DG, Walker DL, *et al.*, (1991). Treatment of severe male-factor infertility with high concentrations of motile sperm by microinsemination in embryo cryopreservation straws. *J In vitro Fert Embryo Transf*, **8**, 101-110.
34. Hodgen GD (1989). The hemizona assay: potential utility in predicting contraceptive efficacy and reversibility. *CONRAD Communication*: 5-7.
35. Hodgen GD, Burkman LJ, *et al.*, (1988). The hemizona assay (HZA): finding sperm that have the "right stuff". *J In vitro Fert Embryo Transf*, **5**, 311-313.
36. Huszar G, Vigue L, Oehninger S (1994). Creatine kinase immunocytochemistry of human sperm-hemizona complexes: Selective binding of sperm with mature kinase-staining pattern. *Fertil Steril*, **61**, 136-142.
37. Huszar G and Vigue L (1990a). Spermatogenesis-related change in the synthesis of the creatine kinase B-type and M-type isoforms in human spermatozoa. *Mol Reprod Dev*, **25**, 258-262.
38. Huszar GL, Vigue L, *et al.*, (1990b). Sperm creatine kinase activity in fertile and infertile oligospermic men. *J Androl*, **11**, 40-46.

39. Jones R. (1990). Identification and functions of mammalian sperm-egg recognition molecules during fertilization. *J Reprod Fertil Suppl*, **42**, 89-105.

40. Kruger TF, Oehninger S, *et al.,* (1991). Hemizona assay: use of fresh versus salt-stored human oocytes to evaluate sperm binding potential to the zona pellucida. *J In vitro Fert Embryo Transf*, **8**, 154-156.

41. Kruger T, Acosta A, *et al.,* (1990). Clinical importance of morphology. Human spermatozoa in assisted reproduction. Baltimore, Williams and Wilkins: 176-180.

42. Kruger TF,Acosta, AA, *et al.,* (1988). Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril*, **49**, 112-117.

43. Kruger T F, Menkveld R, *et al.,* (1986). Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril*, **46**, 1118-1123.

44. Liu DY, Clarke GN, *et al.,* (1991). Inhibition of human sperm-zona pellucida and sperm-oolemma binding by antisperm antibodies. *Fertil Steril*, **55**, 440-442.

45. Liu, DY, Clarke GN, *et al.,* (1989). A sperm-zona pellucida binding test and in vitro fertilization. *Fertil Steril*, **52**, 281-287.

46. Mahadevan, MM. and Trounson, AO (1984). The influence of seminal characteristics on the success rate of human in vitro fertilization. *Fertil Steril*, **42**, 400-405.

47. Mahony, M. and Alexander, NJ (1992). Sites of antisperm antibody action. *Hum Reprod*, **6**, 1426-1430.

48. Mahony, MC, Blackmore, PF, *et al.,* (1991a). Inhibition of human sperm-zona pellucida tight binding in the presence of antisperm antibody positive polyclonal patient sera. *J Reprod Immunol*, **19**, 287-301.

49. Mahony, MC, Fulgham DL, *et al.,* (1991b). Evaluation of human sperm-zona pellucida tight binding by presence of monoclonal antibodies to sperm antigens. *J Reprod Immunol*, **19**, 269-285.

50. Mbizvo MT, Burkman, LJ, *et al.,* (1990). Human follicular fluid stimulates hyperactivated motility in human sperm. *Fertil Steril*, **54**, 708-712.

51. Menkveld, R, Franken DR, *et al.,* (1991). Sperm selection capacity of the human zona pellucida. *Mol Reprod Dev*, **30**, 346-352.

52. Morales, P, Cross NL, *et al.,* (1989). Acrosome intact and acrosome-reacted human sperm can initiate binding to the zona pellucida. *Dev Biol*, **133**, 385-392.

53. Mortimer, D. (1991). Sperm preparation techniques and iatrogenic failures of in-vitro fertilization. *Hum Reprod*, **6**, 173-176.

54. Ng, SC, Bongso A, *et al.*, (1988). Pregnancy after transfer of sperm under zona. *Lancet*, **2**, 790.

55. Oehninger S, Morshedi M, *et al.*, (1993). Validation of the hemizona assay (HZA) in a monkey model. II. Kinetics of binding and influence of cryopreserved-thawed spermatozoa. *J Assist Reprod Genet*, **10**, 292-301.

56. Oehninger S, Clark GF, *et al.*, (1992a). Effect of fucoidin on human sperm-zona pellucida interactions. *J Androl*, **13**,519-525.

57. Oehninger S, Stecker JF, *et al.*, (1992b). Male infertility: the impact of assisted reproductive technologies. *Curr Opin Obstet Gynecol*, **4**, 185-196.

58. Oehninger, S, Acosta, AA, *et al.*, (1991a). Recurrent failure of in vitro fertilization: role of the hemizona assay in the sequential diagnosis of specific sperm-oocyte defects. *Am J Obstet Gynecol*, **164**, 1210-1215.

59. Oehninger, S, Alexander N, *et al.*, (1991b). Antifertilization assays. Gamete interaction: prospects for innumocontraception. N. Alexander, D. Griffin, J. Spieler and G. Waites. New York, Wiley Liss, 473-488.

60. Oehninger S. and Alexander NJ (1991c). Male infertility: the focus shifts to sperm manipulation. *Curr Opin Obstet Gynecol*, **3**, 182-190.

61. Oehninger, S, Clark GF, *et al.*, (1991d). Nature of the inhibitory effect of complex saccharide moieties on the tight binding of human spermatozoa to the human zona pellucida. *Fertil Steril*, **55**,165-169.

62. Oehninger S, Franken D, *et al.*, (1991e). Hemizona assay: sperm defect analysis, a diagnostic method for assessment of human sperm-oocyte interactions, and predictive value for fertilization outcome. *Ann N Y Acad Sci*, **626**, 111-124.

63. Oehninger S and Hodgen G (1991f). How to evaluate human spermatozoa for assisted reproduction. Assisted reproduction review. A. DeCherney. Baltimore, Williams and Wilkins, 15-27.

64. Oehninger, S, Veeck L, *et al.*, (1991g). Human preovulatory oocytes have a higher sperm-binding ability than immature oocytes under hemizona assay conditions: evidence supporting the concept of "zona maturation. *Fertil Steril*, **55**, 1165-1170.

65. Oehninger S, Acosta A, *et al.*, (1990). Antagonistic and agonistic properties of saccharide moieties in the hemizona assay. *Fertil Steril*, **53**, 143-149.

66. Oehninger S, Acosta A, *et al.*, (1989a). Delayed fertilization during in vitro fertilization and embryo transfer cycles: analysis of causes and impact on overall results. Fert*il Steril*, **52**, 991-997.

67. Oehninger S, Coddington CC, *et al.,* (1989b). Hemizona assay: assessment of sperm dysfunction and prediction of in vitro fertilization outcome. *Fertil Steril*, **51**, 665-670.

68. Oehninger S, Scott RT, *et al.,* (1989c). Validation of the hemizona assay in a monkey model: influence of oocyte maturational stages. *Fertil Steril*, **51**, 881-885.

69. Oehninger, S, Acosta, AA, *et al.,* (1988). Failure of fertilization in in vitro fertilization: the "occult" male factor. *J In vitro Fert Embryo Transf*, **5**, 181-187.

70. Overstreet, JW, Yanagimachi R, *et al.,* (1980). Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg: a study of fertile donors and infertile patients. *Fertil Steril*, **33**, 534-542.

71. Palermo G, Joris H, *et al.,* (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an occyte. *Lancet*, **340**, 17-18

72. Robertson L, Wolf DP, *et al.,* (1988). Temporal changes in motility parameters related to acrosomal status: identification and characterization of populations of hyperactivated human sperm. *Biol Reprod*, **39**, 797-805.

73. Rodriguez-Rigau LJ, Ayala C, *et al.,* (1989). Relationship between the results of sperm analysis and GIFT. *J Androl*, **10**, 139-144.

74. Saling, PM (1991). How the egg regulates sperm function during gamete interaction: facts and fantasies. *Biol Reprod*, **44**, 246-251.

75. Saling, PM (1989). Mammalian sperm interaction with extracellular matrices of the egg. *Oxf Rev Reprod Biol*, **11**, 339-388.

76. Speroff L, Friedman A, *et al.,* (1990). Who benefits from intrauterine insemination with washed sperm? *Contemp Obstet Gynecol*, **35**, 65-77.

77. Suarez S, Wold D, *et al.,* (1986). Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid. *Gamete Res*, **14**,107-121.

78. Sueldo, C. E., S. Oehninger, *et al.,* (1993). Effect of progesterone on human zona pellucida sperm binding and oocyte penetrating capacity. *Fertil Steril*, **60**, 137-140.

79. Takahashi K, WetzelsAM, *et al.,* (1992). The kinetics of the acrosome reaction of human spermatozoa and its correlation with in vitro fertilization. *Fertil Steril*, **57**, 889-894.

80. Thomas P and Meizel S (1988). An influx of extracellular calcium is required for initiation of the human sperm acrosome reaction induced by human follicular fluid. *Gamete Res*, **20**, 397-411.

81. Tsukui S, NodaY, *et al.,* (1988). Blocking effect of sperm immobilizing antibodies on sperm penetration of human zonae pellucidae. *J In vitro Fert Embryo Transf*, **5**, 123-128.

82. van der Merwe JP, Kruger TF, *et al.,* (1990). Treatment of male sperm autoimmunity by using the gamete intrafallopian transfer procedure with washed spermatozoa. *Fertil Steril*, **53**, 682-687.

83. van der Ven HH, Hoebbel K, *et al.,* (1989). Fertilization of human oocytes in capillary tubes with very small numbers of spermatozoa. *Hum Reprod*, **4**, 72-76.

84. Wassarman, P. M. (1990). Regulation of mammalian fertilization by zona pellucida glycoproteins. *J Reprod Fertil Suppl*, **42**, 79-87.

85. World Health Organization (1987). WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. Cambridge, UK, *The Press Syndicate of the University of Cambridge*, 7-17.

86. Yanagimachi, R. (1991). Mechanisms of fertilization in mammals. Fertilization and embryonic development in vitro. L. Mastroianni and J. Biddger. New York, *Plenum Press*, 81-187.

87. Yanagimachi, R., A. Lopata, *et al.,* (1979). Retention of biologic characteristics of zona pellucida in highly concentrated salt solution: the use of salt-stored eggs for assessing the fertilizing capacity of spermatozoa. *Fertil Steril*, **31**, 562-574.

88. Yanagimachi, R., H. Yanagimachi, *et al.,* (1976). The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod*, **15**, 471-476.

89. Yoshimatsu, N., R. Yanagimachi, *et al.,* (1988). Zonae pellucidae of salt-stored hamster and human eggs: their penetrability by homologous and heterologous spermatozoa. *Gamete Res*, **21**, 115-126.

Figure 1

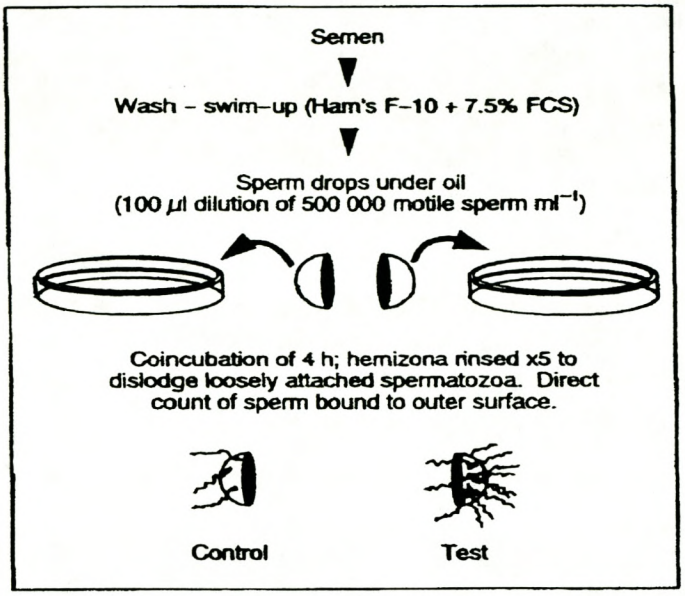


Figure 2

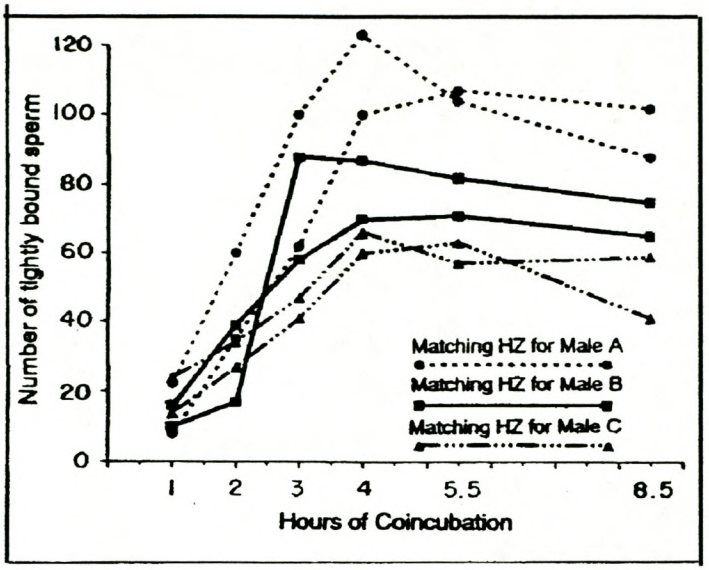


Figure 3

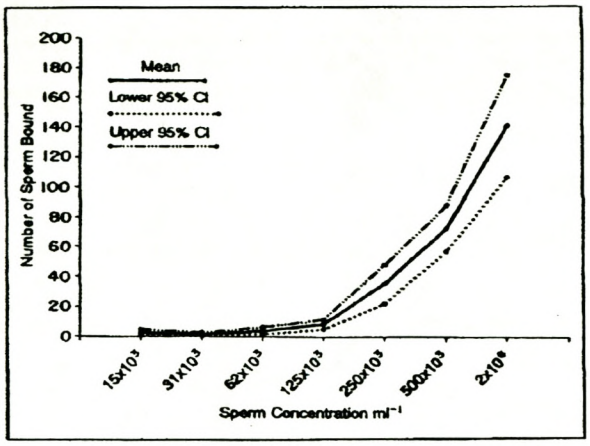


Figure 4

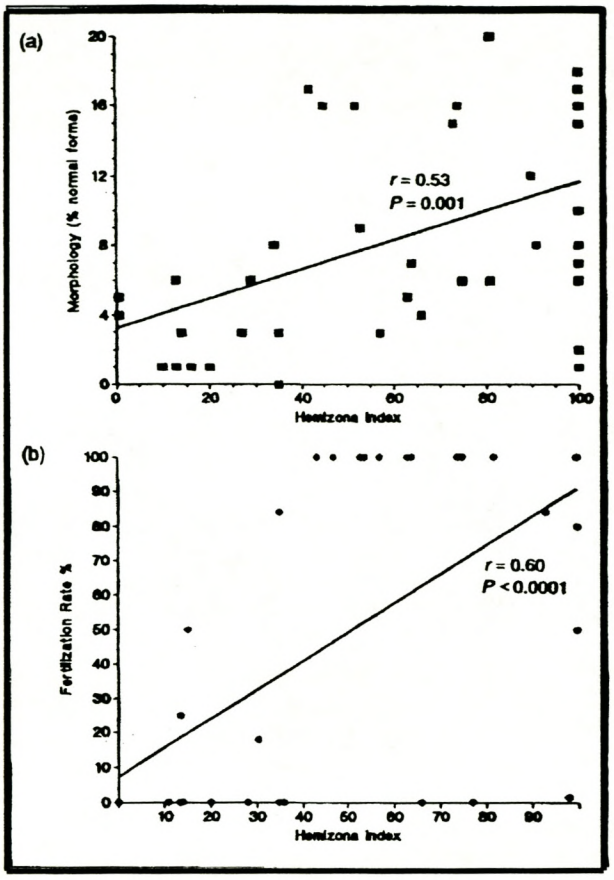


Figure 5

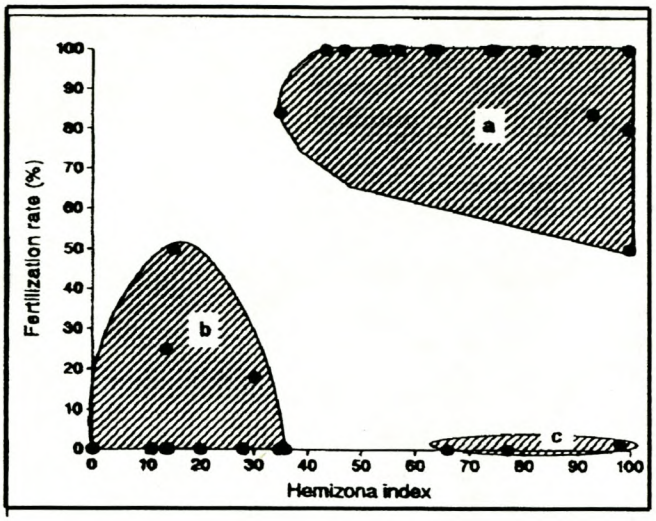


Figure 6

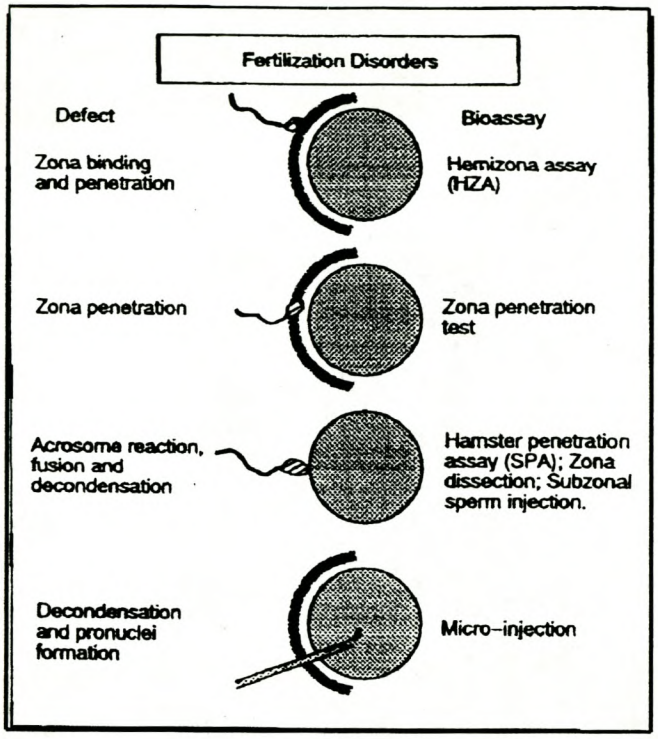


Figure 7

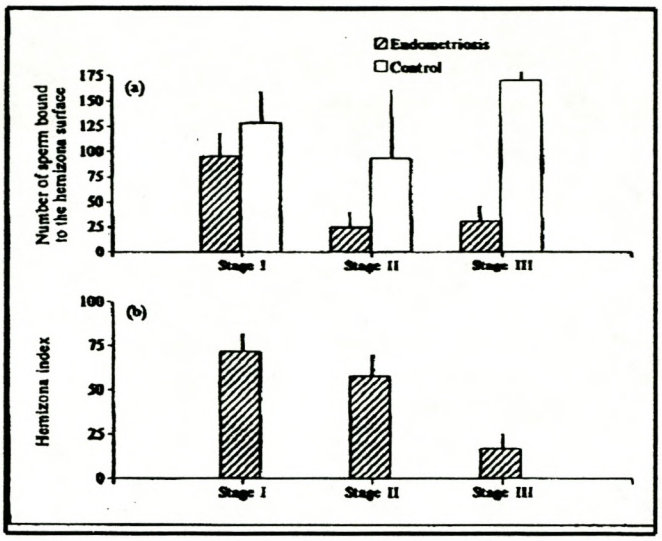


Figure 8

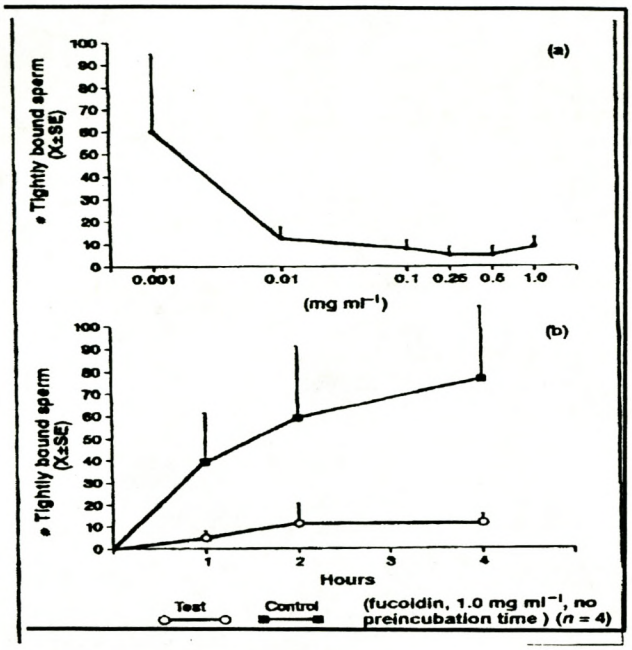


Figure 9

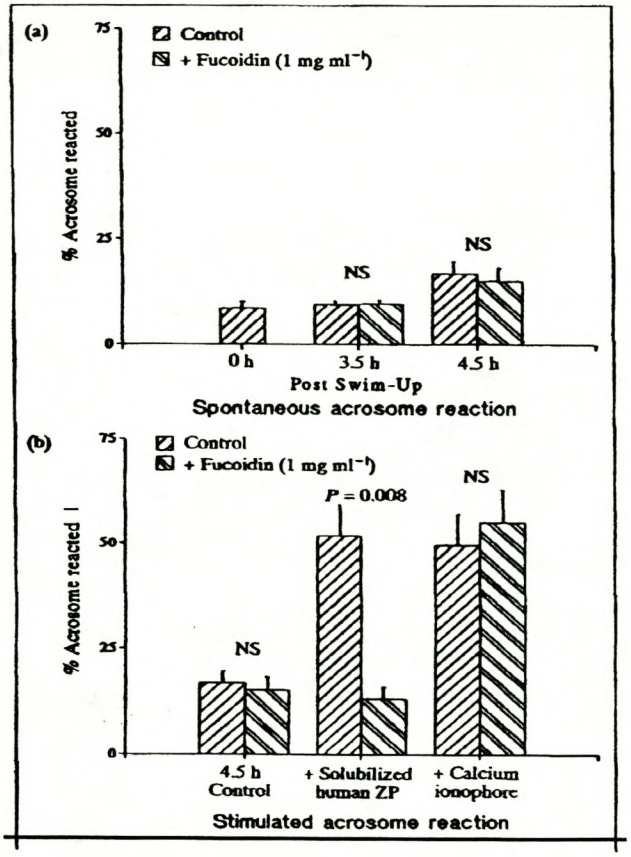
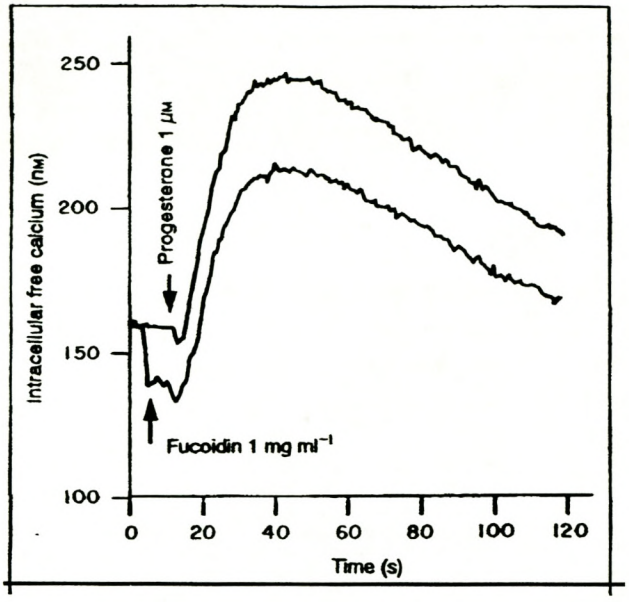


Figure 10



Legends to Figures

Figure 1. Hemizona assay: sperm-zona pellucida binding test.

Figure 2. The number of spermatozoa from fertile men tightly bound to the zona in the HZA (oocytes stored in DMSO or salt-stored for 15 days). Each curve represents the mean binding of two matching hemizonae: fertile male A ,▲△ ; fertile male B,■◇ ; fertile male ◯● Open symbols = salt-stored oocytes; closed symbols = DMSO-treated oocytes. (Adapted from: Franken *et al.* 1989a).

Figure 3. Pooled zona pellucida-binding data for two fertile men (control) using increasing sperm insemination concentration. (Adapted from: Franken *et al.* 1990b).

Figure 4. Relationship between (a) HZI and morphology (strict criteria) and (b) HZI and fertilization rate under IVF conditions (Adapted from: Oehninger, 1992).

Figure 5. Cluster analysis of HZI and fertilization rate considering a cut-off HZI of 35% (a) high HZI, successful fertilization; (b) low HZI, poor fertilization; (c) false positive results.

Figure 6. Fertilization disorders: establishing a pathophysiologic diagnosis through the sequential application of predictive sperm/oocyte bioassays, IVF and assisted fertilization techniques (Adapted from: Oehninger *et al.*, 1991a).

Figure 7. (a) Average number of spermatozoa bound to the hemizona surface when exposed to peritoneal fluid (PF) from endometriosis patients. (b) Hemizona index (number of sperm bound to the hemizona from test/number of sperm bound to the hemizona from control x 100) for each stage of endometriosis (Adapted from: Coddington *et al.*, 1992)

Figure 8. Effect of fucoidin on human sperm-zona interaction. (A) Dose-response curve (1 h pre-incubation of fucoidin with sperm prior to HZA with and without fucoidin (Adapted from: Oehninger *et al.*, 1990).

Figure 9. Effect of fucoidin on spontaneous (A) and (B) stimulated acrosome reaction (Adapted from: Mahony *et al.*, 1991b).

Figure 10. Evaluation of calcium influx into sperm: effect of progesterone and fudoidin (Adapted from: Oehninger *et al.*, 1992b).

Table I

HZA results according to nuclear maturation stage of human and cynomolgus monkey oocytes (Adapted from: Oehninger *et al.*, 1989b, 1991e).

	Prophase I	Metaphase I	Metaphase II
Human Oocytes			
Hemizonae	26	16	10
Tightly bound sperm			
($\bar{X} \pm SE$)	22 ± 4	24 ± 7	50 ± 11^a
(range)	(2–73)	(2–94)	(15–94)
Monkey Oocytes			
Hemizonae	16	12	14
Tightly bound sperm			
($\bar{X} \pm SE$)	21 ± 3	44 ± 5^b	69 ± 6^a
(range)	(5–52)	(17–64)	(25–115)

HZA results according to nuclear maturation stage of human and cynomolgous monkey oocytes

[a] $P<0.01$ compared to prophase I oocytes

[b] $P<0.01$ compared to prophase I and metaphase II oocytes

Table II

Interspecies experiments

(a) HZA results

	Human sperm	Monkey sperm
Human zona	93.2±15.8* ($n=10$)	3.9±1.3* ($n=10$)
Monkey zona	2.8±1.6‡ ($n=5$)	126.0±34.8‡ ($n=5$)
Hamster zona	6.5±2.5‡ ($n=5$)	6.2±1.0‡ ($n=4$)

*$P<0.001$ †$P=0.02$ ‡not significant

(b) Percentage of acrosome-reacted sperm (human and monkey) after swim-up separation in the parallel droplets in sperm exposed to but not bound to the hemizona (HZ), and in sperm tightly bound to the HZ under HZA conditions.

	Swim-up	Parallel droplet	Sperm exposed to HZ but not bound			Sperm tightly bound to the HZ		
			Human	Monkey	Hamster	Human	Monkey	Hamster
Human sperm	7.3±1.1	10.6±1.2	7.7±1.3	9.3±1.3	8.6±0.8	89.0±3.6*	9.8±6.9	10.5±4.8
Monkey sperm	7.7±1.7	12.1±2.1	8.4±2.1	10.4±2.2	5.0±2.1	22.0±6.8	90.5±4.8†	5.5±5.6

*$P<0.001$ †$P<0.001$

Table III

HZA results (absolute binding and HZI) according to fertilization rates in IVF (Adapted from Oehninger, 1992).

	Concentration ($\times 10^6$ ml^{-1})	Motility (%)	Morphology (%)	HZA		Hemizona index (HZI)	Fertilization rate (%)
				Control	Test		
Fertilization rate ≥65% 'Good fertility' (n=26)	94.5±13.1	71.6±2.8	11.2±1.0	82.6±10.8	84.5±10.9	81.4±4.4	98.0±1.2
Fertilization rate <65% 'Poor fertility' (n=18)	58.9±8.7	46.8±3.8	4.3±0.7	68.6±8.7	27.3±7.3	36.3±6.9	8.0±0.02
P value	0.04	<0.0001	<0.0001	0.5	<0.0001	<0.0001	<0.0001
Successful fertilization (1–100%) (n=30)	91.9±11.8	68.8±3.1	10.2±1.0	82.2±9.5	77.8±10.2	75.9±5.2	90.0±4.2
Failed fertilization (0%) (n=14)	54.3±11.0	45.8±4.9	4.6±1.0	65.4±12.6	25.4±9.5	35.1±9.3	0
P value	<0.04	<0.0001	0.002	0.3	<0.0001	0.0006	<0.0001

Table IV

Overall predictive value of the HZA considering a cut-off HZI of 35%.

	Failed vs. successful fertilization	Poor vs. good fertilization
True positive	27 (+PV = 82%)	26(+PV = 79%)
False positive	6	7
False negative	3	0
True negative	8(−PV = 73%)	11(−PV = 100%)

Table V

Sequential analysis for the diagnosis of specific sperm/oocyte defects. HZI: hemizona index. SPA: hamster egg/sperm penetration assay (Adapted from Oehninger, *et al.*, 1991a).

Couple	Hemizona index (%)	SPA	IVF	Ooocyte Micro manipulation	Cross-match test	
		Control vs. Test (%)	(Oocytes fertilized/ inseminated)		Donor Sperm	Donor Oocyte
#1	30	40–20	0/7	0/3 (PZD)	2/2	0/3
#2	27	31–21	0/13	2/6 (PZD)	–	–
#3	20	–	0/12	2/5 (SZI)	–	–
#4	75	28–27	0/11	2/7; (MI) 0/2 (PZD)	2/3	0/1
#5	65	100–0	0/18	6/10; (MI) 0/2 (PZD)	3/3	0/2
#6	0	32–0	0/4	–	–	-
#7	100	25–20	0/7	–	–	1/1
#8	12	–	0/20	0/3 (SZI)	7/15	–

Sequential diagnosis of sperm/oocyte defects in patients with recurrent failed IVF. (*PZD*: Partial zona dissection; MI: oocyte microinjection; *SZI*: subzonal sperm insertion)

Table VI

Effect of progesterone in HZA, SPA and acrosome reaction (P4: 1.0ug ml-1, 14 and 24 h sperm pre-incubation) (Adapted from Sueldo *et al.*, 1992).

	HZA (sperm tightly bound)		SPA (%)		Acrosome Reation (%)	
	1 h	24 h	1 h	24 h	1 h	24 h
Control	35	4	31	39	10	9
Progesterone	52*	4	60*	66*	18	30*

*$P<0.05$ compared to control

Table VII

Distribution of spermatozoa (%) in the original sample, swim-up fractions and on the hemizona-sperm complexes based upon morphology (strict criteria) (Adapted from Menkveld *et al.*, 1991).

	Semen			Swim-up			Hemizona		
	Normal	Slightly abnormal	Amorphous	Normal	Slightly abnormal	Amorphous	Normal	Slightly abnormal	Amorphous
Normospermic ($n=4$)	21	22	56	27	25	47	44[a]	25	29
Teratospermic ($n=11$)	3	9	87	5	13	81	15[a]	16	67

[a] $P<0.05$ compared to semen and swim-up fractions

***Specific aim 2*: To develop and validate an *in vitro* bioassay (a micro volume assay) for the evaluation of the physiologically induced acrosome reaction of sperm in suspension by human solubilized zona pellucida.**

Physiological induction of the acrosome reaction in human sperm: Validation of a micro-assay using minimal volumes of solubilized, homologous zona pellucida

[1]Daniel R Franken, [1]Hadley S Bastiaan and [2]Sergio Oehninger

Journal of Assisted Reproduction and Genetics 2000, 17: 156-161

[1]Department of Obstetrics & Gynaecology, University of Stellenbosch, Tygerberg, South Africa; and [2]Department of Obstetrics & Gynaecology,
Jones Institute for Reproductive Medicine,
Eastern Virginia Medical School,
Norfolk, VA 23507, USA

Abstract

The objective of these experiments was to develop a method that could accommodate micro-volumes of solubilized human (ZP) and sperm for assessing the induction of the acrosome reaction. For this purpose, we developed a micro-assay using 1µl of 2.5, 1.25, 0.6, 0.3 and 0.125 ZP/µl incubated with 1µL of a highly motile sperm suspension for 60 minutes. As a control and parallel to the micro-assay a standard acrosome reaction technique was performed. Results: No significant differences were observed between the percentage acrosome reacted sperm reported by the two assays under basal conditions (spontaneous) or after induction with a Ca^{2+}ionophore or solubilized ZP. At a ZP concentration of 0.6 ZP/µl, the percentages of acrosome reacted spermatozoa in both techniques were significantly higher compared to the spontaneous acrosome reaction results, namely 18% and 17%, compared to 10% and 10%, respectively. Approximately a 30% level of acrosomal exocytosis was induced with 2.5 ZP/µl in both methods. We concluded that this newly devised micro-technique is easy and rapid to perform, is repeatable and facilitates the use of minimal volumes of solubilized human ZP (even a single ZP) for assessment of the inducibility of the acrosome reaction of a homologous sperm population.

Introduction

The andrologic evaluation of the male partner relies on a thorough history and physical examination, followed by a urologic and endocrinologic work-up as indicated. Still, the semen analysis remains the cornerstone of diagnostic management. We and other, have been promoters of a sequential, multi-step diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics (Oehninger, *et al.,* 1991; Amman and Hammerstedt, 1993; Oehninger, *et al.,* 1997.) The proposed diagnostic scheme should include (i) assessment of the "basic" semen analysis and (ii) functional testing of spermatozoa (World Health Organization, 1992; Oehninger, 1995).

Different laboratories have highlighted the diagnostic power of a variety of tests that examine the functional competence of the male gamete. The World Health Organisation has incorporated some of them under the category of functional tests (WHO, 1992). At a recent Consensus Workshop in Advanced Andrology (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996.) it was concluded that because of their validation and unquestioned clinical value, the homologous sperm-zona pellucida (ZP) binding tests should be incorporated in the advanced stages of the work-up. However, it was also agreed that better standardisation of the currently used acrosome reaction techniques should be implemented prior to their introduction as a routine clinical tool. At the present time, there seems to be general agreement that more clinical information can be gained by the analysis of a stimulant-induced acrosomal exocytosis compared to the assessment of the spontaneous frequency of acrosome reactions (basal rate). The most widely utilised method is the challenge with a calcium ionophore agent where the acrosome reaction is identified with defined lectins in combination with indirect immunofluorescence (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996).

The acrosomal response of a given sperm sample has been illustrated to be a crucial event leading to fertilisation and many reports have aimed to correlate acrosome reaction response with in vitro fertilisation rates. Moreover, the precise timing of the acrosome reaction formed the rationale for the development of the ARIC-test (Cummins, *et al.,* 1991; Teasarik, 1996). The concept of acrosomal inducibility (Henkel, *et al.,* 1993; Henkel *et al.,* 1998) and the ARIC-test have gained more recognition, and there seems to be agreement that this method of evaluation is a more reliable predictor of sperm fertilising ability than those tests that simply measure the frequency of spontaneous acrosome reactions. The inducibility of the acrosome reaction, i.e. the difference

between spontaneous and percentage acrosome reacted sperm after induction, correlates significantly with in vitro fertilization outcome (Henkel, *et al.*, 1993).

The ZP in both the intact and solubilized state has been demonstrated to be a powerful and physiological inducer of the acrosome reaction (Cross, *et al.*, 1988; Mahony, *et al.*, 1991Bielfeld, *et al.*, 1994; Liu & Baker, 1994; Franke, *et al.*, 1996; Franken, *et al.*, 1997). During fertilisation, acrosome reaction failure can be caused by multiple factors; such as (i) inadequate sperm capacitation; (ii) an inability of the sperm membrane to undergo specific structural-functional changes after binding to the ZP; or (iii) an impaired capacity of the ZP of a specific oocyte to induce the acrosomal cascade.

In this investigation, we aimed to develop and validate a simple and rapid micro-assay for the accurate determination of the human sperm acrosome reaction mediated by minimal volumes of solubilized (or even a single ZP) homologous ZP.

Materials and methods

Preparation of sperm samples

Ejaculates from fertile men (donors) were used in these studies after approval by the local ethics committee. The sperm parameters of samples used were as follows (mean±standard deviation): concentration, 117.4±16 x10^6/ml; sperm motility, 60±5 %, and normal morphology (strict criteria), 17±2%. Motile sperm fractions (10x10^6 cells/ml, >90% motility) were retrieved using a double wash swim-up technique (15, 16). Before the onset of acrosome reaction studies, sperm were allowed to capacitate at 37°C in 5% CO_2 in air for 3 hours in synthetic human tubal fluid medium (HTF) supplemented with 3% BSA (17).

Preparation of solubilized ZP

Human oocytes were retrieved from post-mortem derived ovarian tissue following approval by the local ethics committee. Oocytes were stored in DMSO/sucrose at -196°C in liquid nitrogen (Hammit, *et al.*, 1993). Twelve hours prior to each experiment, oocytes were removed from storage and thawed at 37°C. Oocytes were placed in 0.25 M sucrose and 3% BSA in HTF medium for 20

minutes at room temperature, after which the oocytes were placed under mineral oil (Sigma Chem Co, St Louis, MO USA Cat no. M-3516) until used.

On the day of each experiment, 50 oocytes were vigorously pipetted with a small-bore glass pipette (inner diameter 80 microns) to separate the ZP from the ooplasm. The separated ZP were then placed in a plastic Eppendorf tube containing HTF medium supplemented with 3% BSA. The tubes were centrifuged for 15 minutes at 1,800x g, after which the HTF medium was carefully removed under microscopic vision (Olympus SZ40, Wirsam Scientific, Cape Town, South Africa), leaving only 50 ZP at the bottom of the tube. A total volume of 5µl 10mM HCl was added to the zonae pellucidae. Solubilization of the ZP was microscopically observed and controlled after which 5 µl of 10mM NaOH was added to the ZP to render a final zona volume of 10µl, containing 5ZP/µl. The final pH of the zona solution was 7.4.

Acrosome Reaction

Two sets of experiments, each using different volumes of solubilized ZP, were performed in a parallel fashion, namely, a micro-assay and a standard acrosome reaction assay. Following solubilization zona pellucidae were kept at 4°C for 7 days during which all experiments were performed. Ongoing studies in our laboratory currently evaluate the acrosome reaction inducibility of solubilized zona pellucidae recorded over an extended time period.

For the micro-assay, 1µl of ZP solution (concentration, 5ZP/µl stock solution), was aspirated into a Teflon pipette tip (Hamilton Pipette-tip, Cat 84254, Separations, Cape Town, South Africa), fitted to a micro-syringe (Hamilton 702, Separations, Cape Town, South Africa) with 1µl of sperm ($10x10^6$ sperm/ml, >90% motility), to render a final ZP concentration of 2.5 ZP/µl. Serial dilutions were made from the 5ZP/µl solutions (1:1, vol: vol) using HTF to equal final zona concentrations of 2.5, 1.25, 0.6, and 0.3ZP/µl. One microlitre of each dilution was separately added to 1µl of sperm to equal a final ZP concentration of 1.25, 0.6, 0.3 and 0.15 ZP/µl. Prior to aspiration into Teflon tips, all sperm/ZP suspensions were gently mixed in a well of a micro-titre plate (Microtest plate cat No. P43 Laboratory and Scientific, Cape Town South Africa). To prevent evaporation from the Teflon tips, aspirating HTF droplets into both sides of the Teflon tip sealed off sperm-ZP suspensions. Each sperm-ZP suspension was separated from the HTF droplets by air bubbles on both sides.

Due to the small volumes involved, progressive motility and percentage live cells (4), for both acrosome reaction techniques were manually performed on spotted slides (MAGV, Germany, XER 201B). Control and treated sperm samples were carefully removed from the teflon tips and placed on separate spots on the spotted slide and immediately evaluated for percentage live sperm under inverted phase contrast microscope (Nikon TMS-F, Research Instruments, Johannesburg, South Africa). During both techniques the percentage live acrosome reacted cells were recorded by aspirating/adding 1µl (1µg/ml) Hoescht-dye (supravital stain Hoechst 33258, B-2883; Sigma Chemical Co, St Louis, MO, USA) 5 minutes before termination of incubation of sperm and solubilized zona. During the evaluation of the acrosome status of each experiment, only live acrosome reacted spermatozoa were recorded.

In each study, negative and positive control experiments consisted of 1µl of sperm suspension plus 1µl HTF or 1µl 10µM Ca^{+2}-ionophore (Sigma, Chem. MO, USA, Cat C7522) incubated as the test conditions at 37°C, 5% CO_2, 95% humidity for 1 hour. For the standard acrosome reaction assay, larger volumes (i.e., 5µl of the sperm suspension plus 5µL of the above mentioned zona solutions (i.e. 2.5, 1.2, 0.6, 0.3, and 0.15 ZP/µl), were incubated in 0.4 ml Eppendorf tubes, under similar laboratory conditions for 1 hour. Prior to the onset of the study control experiments (i.e. exposure of sperm to HTF and Ca^{2+}-ionophore) were also included in the standard acrosome assay as described above.

Following the motility assessments, sperm droplets were allowed to air dry, after which the sperm were fixed in 70% ethanol for 20 minutes and evaluated for percentage live cells. Acrosomal status for both assays (and respective control conditions) was evaluated using fluorescein isothiocianate (FITC)-Pisum sativum agglutinin (PSA) staining (Sigma Chemicals, MO, USA, L0770.), with epi-fluorescence microscopy (Olympus BX40, Wirsam Scientific, Cape Town, South Africa) (Cross, *et al.,* 1998; Morales *et al.,* 1989; Morales & Cross, 1989; Mahony, *et al.,* 1991). Two hundred cells were counted in a blinded fashion in each well of the spotted slide and results were expressed as % acrosome-reacted sperm. The following staining patterns were evaluated as acrosome reacted spermatozoa; (i) patchy staining on acrosomal region, (ii) distinct staining in the equatorial region occurring as a equatorial bar and (iii) and no staining observed over entire sperm surface.

Intact ZP-induced acrosome reaction

Additional acrosome reaction studies were performed on intact ZP. Using the same sperm samples, parallel experiments to the micro- and standard acrosome assays were performed where the acrosomal status of ZP-bound sperm was examined after co-incubation of the male gametes with intact, previously salt-stored human oocytes. It has been demonstrated that oocytes stored under these conditions retain biophysical, biochemical and functional properties (Yanagimachi, *et al.*, 1979; Burkman, *et al.*, 1988; Franken *et al.*, 1991). At the time of the experiments, the oocytes were desalted, washed in culture medium and microbisected into matching hemizonae as previously described (Burkman, *et al.*, 1988). A total of 60 hemizonae (matching pairs from 30 oocytes) were individually incubated under oil in 50 µL droplets containing 25 x 10^6/ml motile sperm (post-swim-up) for 60 min at 37°C in 5% CO2 in air. The hemizonae were then removed from the suspension and after pipetting using a fine glass pipette to remove loosely attached sperm, the number of sperm tightly bound to each hemizonae were counted under phase-contrast microscopy (Burkman, *et al.*, 1988; Franken, *et al.*, 1991).

Thereafter, the sperm tightly bound to the zona were removed (stripped) by a shearing action, using a small-bore glass (60 microns inner diameter) pipette. Individual sperm were then placed on a spot glass slide, allowed to air dry, after which the acrosome reaction was determined as described above. The number of sperm tightly bound to each hemizona under these conditions was always >300 cells. Most of the zona-bound sperm could be stripped during the experiments for all hemizonae evaluated and at least 200 spermatozoa (per hemizona) could be assessed for acrosomal status.

Statistical Analysis

Comparisons of the percentage acrosome reaction for both methods (micro- and standard assays) under different experimental conditions (i.e., spontaneous, Ca^{2+}-ionophore and ZP-induced conditions) were performed with Fisher's exact paired t-test. The overall dose-dependency effect of varying solubilized ZP concentrations on acrosomal exocytosis was assessed by analysis of variance (ANOVA).

Results

The percentage acrosome reaction recorded for spontaneous (in HTF medium) and Ca^{2+}ionophore-induced did not differ between the micro- and standard acrosome reaction assays

(Table I). (Table II) shows acrosome reaction results for the various solubilized ZP concentrations used. Again, there were no differences in the induction of acrosomal exocytosis for the two methods. The percentage of live acrosome reacted sperm in both techniques were >80%. In addition, an obvious dose-dependent effect of solubilized ZP on the acrosome reaction was observed reaching an approximate 30% induction using a maximum dose of 2.5 ZP/µl for both the micro- and standard assays. Following a 1 hour incubation with intact hemizonae (intact ZP), on the other hand, the % acrosome reacted zona-bound sperm was significantly higher than the levels obtained with solubilized ZP or the Ca^{2+}-ionophore agent (84±9 % versus 32±2 and 28±3, respectively, $p< 0.001$).

Discussion

The need for a micro-volume assay to assess acrosome reaction has been identified previously (Morales, *et al.,* 1989). This is due to the fact that diagnostic andrology laboratories often lack sufficient biological material (i.e., human ZP) to perform a defined and specific test such as the examination of the physiological acrosome reaction. This is true for the natural ZP protein(s), but will also be relevant when recombinant human ZP proteins are to be tested for corroboration of their biological activity (Brewis, *et al.,* 1996; Chapman & Barratt, 1996).

The newly described assay is simple, can be performed quickly, and the results are reliable and repeatable. Therefore, because of the small volumes employed, it is an ideal technique for testing native and recombinant ZP (highly precious or scant material). The results of the present study indicated the use of a single ZP to be adequate for mediating the acrosome reaction of a sperm population in suspension. At a ZP concentration of 0.6 ZP/µL, the percentage acrosome reacted sperm, as determined by both the standard and micro- assays, was significantly higher than the spontaneous reaction, i.e. 18% (standard assay) and 17% (micro assay) compared to the spontaneous reaction namely, 10% (standard assay) and 10% (micro assay). The maximum levels of acrosomal exocytosis (28%) induced with the highest ZP concentration (2.5 ZP/µL final concentration) in the micro-assay were similar to the ones observed with the standard assay in our laboratory and in those of others (Franken, *et al.,* 1996; Mahony, *et al.,* 1991, Liu & Baker, 1994).

On the other hand, acrosome reaction induction by intact zonae pellucidae as detected during sperm-zona binding control assays was, as expected, higher than with the use of solubilized ZP. The incidence of zona-bound acrosome reacted spermatozoa found here is similar to the one we

reported using the hemizona assay model combined with transmission electron microscopy and the monoclonal T-6 antibody (Coddington, *et al.*, 1990; Franken, *et al.*, 1991). A more adequate configuration of the ZP proteins in the intact zona matrix and a higher number of zona protein molecules in the hemizona (as compared to lower numbers present in the very small volumes of solubilized ZP used here) are a possible explanation for this finding.

The routine introduction of a simple and reliable assay for the evaluation of the physiologically-induced acrosome reaction as a component of the previously proposed sequential diagnostic work up programme, will assist in the identification of specific sperm defects and may allow the development of more directed therapies. Andrology testing remains, in our opinion as well as those of others (Mortimer, 1994), an ever-growing component in the work up of the infertile couple. We enter the next millennium with many questions that remain to be answered by the hand of efficacious screening techniques and a new formidable therapy in ICSI (Oehninger, *et al.*, 1995). The analysis of the inducibility of the acrosome reaction, a critical step during fertilisation, aids the clinician in the management of male infertility. Finally, it is accepted that, once available, a biologically active recombinant human zona pellucida protein 3 (rhuZP3) will be the ultimate agonist or trigger substance for human sperm AR. Such a test will most certainly become the basis of the ideal acrosome reaction test (Tesarik, 1996). Once available in sufficient amounts, rhuZP3 moieties will have to go through comparative evaluation studies, using natural (solubilized) ZP as controls in subsequent (micro-volume) acrosome reaction assays.

References

1. Amman RP, Hammerstedt RH: (1993) *In vitro* evaluation of sperm quality: an opinion. *J Androl*, **14**, 397-406
2. Bielfeld PP, Faridi A, *et al.*, (1994) The zona pellucida induced acrosome reaction of human spermatozoa is mediated by protein kinases. *Fertil Steril*, **61**: 536-541.
3. Brewis IA, Clayton R, *et al.*, (1996) Recombinant human zona pellucida glycoprotein 3 induces calcium influx and acrosome reaction in human spermatozoa. *Human Reprod*, **2**, 583-589.
4. Burkman LJ, Coddington CC, *et al.*, (1988) The hemizona assay: development of a diagnostic test for the binding of human spermatozoa to the human zona pellucida to predict fertilization potential. *Fertil Steril*, **49**, 688-693.
5. Chapman NR, and Barrattt LR (1996) The role of carbohydrate in sperm-ZP3 adhesion. *Mol Hum Reprod*, 2, 767-774.
6. Coddington CC, Fulgham DC, *et al.*, (1990) Sperm bound to zona pellucida in hemizona assay demonstrate acrosome reaction when stained with T-6 antibody. *Fertil Steril*, **54**, 504-508
7. Consensus Workshop on Advanced Diagnostic Andrology Techniques. ESHRE Andrology Special Interest Group. (1996) *Hum Reprod*, **11**, 1463-1479.
8. Cross NL, Morales P, *et al.*, (1988). Induction of the acrosome reaction by the human zona pellucida. *Biol Reprod*, **38**: 235-244.
9. Cummins JM, Pember SM, *et al.*, (1991) A test of the human sperm acrosome reaction following ionophore challenge: relationship to fertility and other seminal parameters. *J Androl*, **12**, 98-103.
10. Franken DR, Bastiaan *et al.*, (1997). Zona pellucida mediated acrosome reaction and sperm morphology. *Andrologia*, **29**: 311-317.
11. Franken DR, Morales P, Habenicht UF. (1996) Inhibition of G protein in human sperm and its influence on acrosome reaction and zona pellucida binding. *Fertil Steril*, **66**, 1009-1011.
12. Franken DR, Oosthuizen WT, *et al.*, (1991) Electron microscopic evidence on the acrosomal status of bound sperm and their penetration into human zonae pellucidae after storage in a buffered salt solution. *Andrologia*, **23**, 205-208.
13. Hammit DG, Syrop CH, *et al.*, (1993) Conditions of oocyte storage and use of non-inseminated as compared with inseminated, nonfertilized oocytes for the hemizona assay. *Fertil Steril*, **60**, 131-136.

14. Henkel R, Franken D.R. Habenicht UF (1998) Zona pellucida as physiological trigger for the induction of the acrosome reaction. *Andrologia,* **30**, 275-280.

15. Henkel R, Müller *et al.,* (1993) Determination of the acrosome reaction in human spermatozoa is predictive of fertilization in vitro. *Hum Reprod*, **12**, 2128-2132.

16. Liu DY, Baker HWG. (1994) Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm-zona penetration and reduced fertilization in vitro. *Hum Reprod*, **9**, 1694-1700.

17. Mahony M, Oehninger *et al.,* (1991) Inhibition of zona pellucida-induction of the acrosome reaction in human spermatozoa by the complex polysaccharide, fucoidin. *Contraception*, **44**: 657-665.

18. Morales P, Cross NL, *et al.,* (1989a). Acrosome-intact and acrosome-reacted human sperm can initiate binding to the zona pellucida. *Dev Biol*, **133**, 385-392.

19. Morales PJ, Cross NL. (1989b) A new procedure for determining acrosomal status of very small numbers of human sperm. *J Histochem and Cytochem*, **37**, 1291-1292.

20. Mortimer D. (1994) The essential partnership between diagnostic andrology and modern assisted reproductive technologies. *Hum Reprod*, **9**, 1209-1213.

21. Oehninger SC, Franken DR, Kruger TF. (1997) Approaching the next millenium: how should we manage andrology diagnosis in the intracytoplasmic sperm injection era? *Fertil Steril*, **67**, 434-436.

22. Oehninger S.C. (1995) An update on the laboratory assessment of male fertility. *Human Reprod*, **10**, 38-45.

23. Oehninger S, Acosta A, Veeck, *et al.,* (1991) Recurrent failure of in vitro fertilization: role of the hemizona assay in the sequential diagnosis of specific sperm-oocyte defects. *Am J Obstet Gynecol*, **164**, 1210-1215.

24. Quinn P, Kerin JF, Warnes GM. (1985) Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril*, **44**, 493-498.

25. Tesarik J. Acrosome reaction testing. In: Consensus Workshop on Advanced Diagnostic Andrology Techniques. (1996) ESHRE Andrology Special Interest Group, *Hum Reprod*, **11**, 5-8.

26. World Health Organization. (1992) WHO Laboratory Manual for the Examination of Human semen and Sperm-Cervical Mucus Interaction, Cambridge University Press, Cambridge, 3rd Edition, pp.23.

27. Yanagimachi R, Lopata A, *et al.*, (1979) Retention of biologic characteristics of zona pellucida in highly concentrated salt-solution: the use of salt-stored eggs for assessing the fertilizing capacity of spermatozoa. *Fertil Steril*, **31**, 562-567.

Table I.

Acrosome reaction results recorded with a micro assay and a standard technique using calcium ionophore-induced and spontaneous reactions.

Standard assay*		Micro assay*		**Intact zona pellucida**
Spontan.-AR	Calcium ionophore-induced AR	Spontan.-AR	Calcium ionophore-induced AR	% AR of zona-bound sperm
10±2a	51±2b	10±3c	47±14d	84±9%

* mean % ±SD

a vs c: not significant, Fisher's exact paired t-test.

b vs d : not significant, Fisher's exact paired t-test.

AR: percentage acrosome reacted spermatozoa

Table II

Mean (SD) percentage acrosome reacted spermatozoa recorded for varying solubilized ZP concentrations.*

Zona pellucida conc. (ZP/µl)	**Standard Assay**	**Micro Assay**
	Percentage AR (Mean±SD)	Percentage AR Mean±SD
2.5	32*±2%	28±3%
1.25	26*±2%	23±2%
0.6	18*±3%	17±3%
0.3	14±2%	16±2%
0.15	14±3%	14±3%
Spontaneous	10*±2%	10±3%

Overall dose-dependency effect for both methods, $P<0.0001$ by analysis of variance.

- $p=<0.05$ for 2.5, 1.25 and 0.6 ZP/µl when compared to spontaneous acrosome reaction results.

Chapter 2.

Background

2a. Normal Fertilization

Fertilization involves a complex sequence of events leading to embryogenesis. Obligatory requirements for the successful completion of normal fertilization include a mature, metaphase II oocyte and spermatozoa that have completed the process of capacitation. The newly formed zygote undergoes early cleavage divisions depending upon the oocytes endogenous machinery and at the 4 to 8-cell stage initiates transcription of the embryonic genome (Braude, *et al.*, 1988). In vivo, these processes are synchronized with the preparation of the endometrial mucosa (window of implantation) thereby ensuring an adequate milieu receptive of the blastocyst. In vitro conditions can successfully mimic these physiological events. The clinical application of the knowledge gained in the disciplines of endocrinology, andrology and embryology has allowed many infertile couples achieve their reproductive goals through the therapeutic modality of in vitro fertilization (IVF) followed by embryo transfer. IVF and subsequently developed techniques such as embryo cryopreservation, intracytoplasmic sperm injection (ICSI) and pre-implantation genetic diagnosis have revolutionized the field of human reproductive medicine. These techniques will be, undoubtedly, the basis for future improved therapies that will help alleviate and even prevent human disease. Here, it is my aim to present current knowledge of the human fertilization process with emphasis on clinically relevant data.

The trip from oocyte to blastocyst

During the natural cycle, typically, a single preovulatory oocyte is released at the time of ovulation. Functionally competent (capacitated) spermatozoa, upon timely arrival to the fertilization site, will be able to successfully interact with the oocyte resulting in oocyte activation and pronuclear formation. This is followed by transport of the developing blastocyst to the implantation site. The ultimate goal in IVF, on the other hand, is to achieve the transfer of high quality embryos to the uterine cavity, thereby providing the infertile couple with maximal chances of conception. This goal is dependent upon the accomplishment of several inter-related sequential processes. These processes include: (a) the recruitment of multiple fertilizable oocytes; (b) the achievement of high normal (diploid) fertilization rates in vitro; (c) an adequate pre-implantation embryo growth under extra-corporeal culture conditions; and (d) the acquisition of an appropriate developmental potential of the transferred embryos resulting in timely implantation. A current priority of programs offering assisted reproductive technologies (ART) is the one to minimize the incidence of multiple pregnancies; in this regard, various culture conditions as well as selection policies are

being examined in order to transfer (and/or cryopreserve) those embryos demonstrating highest developmental competence.

Ovarian folliculogenesis, supported by a changing endocrine-paracrine milieu, requires dynamic interactions between the maturing oocyte, the nurturing granulosa cells and factors present in the follicular fluid. The onset of follicle growth involves physiologic, genetic and metabolic changes in the oocyte, as it prepares for the completion of meiosis, the prevention of polyspermy, and the initial stages of embryogenesis following fertilization. Both an adequate oocyte quality and quantity are needed for IVF. The number of recruited-growing follicles depends upon an inherent ovarian reserve, related to the woman's chronological age and basal serum FSH levels. Oocyte quality, on the other hand, is a direct consequence of metabolic changes, chromosomal–spindle apparatus intactness and an adequate genetic control of the mechanisms leading to growth-differentiation and to inhibition of programmed cell death (or apoptosis). Controversial data exist related to an increased incidence of apoptosis in poor quality oocytes such as observed in advancing age (Fujino, *et al.*, 1996; Perez, *et al.*, 1997; Van Blerkom, *et al.*, 1997).

Each embryo has a unique developmental potential and only a relatively small proportion of cleavage stage embryos is competent to implant following IVF and develop through gestation (Acosta *et al.*, 1988; Van Blerkom, 1997). There are developmentally lethal defects that occur in the female gamete prior to insemination. Mature oocytes are known to contain numerical chromosomal disorders (aneuploidism) and cytoplasmic structural defects (vacuolization and fragmentation) that predispose the fertilized oocyte to developmental failure (Van Blerkom, 1997; Van Blerkom *et al.*, 1997; Van Blerkom, 1998). These abnormalities may be the result of altered intra-follicular conditions during preovulatory oocyte maturation occurring either naturally or during controlled ovarian hyperstimulation. The extent to which aneuploidies detectable in mature human oocytes are a consequence of chromosomal defects that occur prior to the arrest of meiosis at the prophase I stage is unknown (Van Blerkom, 1997; Van Blerkom *et al.*, 1997; Van Blerkom, 1998). Clearly, poor culture conditions including use of suboptimal culture media, may generate or be additive to pre-existing defects.

Unfortunately, no single follicular factor(s) (secreted into the circulation or present in the follicular fluid) has been demonstrated to provide definitive prediction of the developmental competence of the oocyte or embryo. Recently, this issue was addressed by analyzing follicular fluid biochemistry (ATP, pO_2, pH, vascular growth factors), granulosa cell behavior in vitro (i.e.,

presence and polarization of oocyte regulatory proteins) and peri-follicular blood flow (using color pulsed Doppler ultrasonography) and correlating results with various oocyte/embryo developmental capacities under in vitro conditions. (Van Blerkom, 1997; Van Blerkom, *et al.*, 1997; Van Blerkom, 1998; Antczak and Van Blerkom, 1997). The demonstration of an association between follicular O_2 content, peri-follicular blood flow and embryo quality may provide a non-invasive and indirect indication of follicle health and possible of the developmental competence of the corresponding oocyte.

Following this line of investigation, we have also observed that follicles with a low peri-follicular blood flow resistance index (assessed by color Doppler the day after hCG administration) are associated with oocytes that after fertilization originated embryos of superior cleavage characteristics. More data are needed in order to define the correlation between peri-follicular blood flow and follicular homeostasis, and to determine whether changes in the follicular milieu (degree of hypoxia/acidosis or altered secretion of paracrine/autocrine factors) affect oocyte/embryo developmental competence.

Various clinical strategies are being used to try to improve oocyte quality in patients having repeated IVF cycles with poor ovarian response/oocyte morphology, fragmented embryos and/or multiple implantation failures. These strategies followed pre-clinical studies performed in non-human primates aiming at conferring maturity to prophase I oocytes by transferring cytoplasmic factor(s) from metaphase II oocytes (Flood *et al.*, 1990). Pregnancies have been achieved in preliminary clinical trials after cytoplasmic transfer in mature human oocytes (Cohen *et al.*, 1997; Cohen *et al.*, 1998). Therefore, this technique or modified therapeutic alternatives may be promising for patients with compromised embryos. Theoretically, cytoplasmic transfer may be able to rescue compromised oocytes by providing metabolic factors or regulatory products leading to stimulation of growth and/or suppression of apoptosis.

Embryo implantation potential in IVF can be correlated with morphological scores and cleaving status. However, the overall predictive value of these embryo features is relatively low. Embryos showing fragmentation or other obvious morphological abnormalities and retarded growth may have chromosomal abnormalities (Kligman, *et al.*, 1996). It has been suggested that the presence of multinucleated blastomeres at the 2-cell stage is indicative of failure to progress (Kligman, *et al.*, 1996; Sakkas, *et al.*, 1998). Therefore, the practice of embryo selection before

transfer can only be efficacious if inspections in the clinical laboratory are made at frequent intervals of observation (Van Blerkom, 1997).

For cryopreserved-thawed embryos, we found a significant relationship between average morphology and implantation rate (r= .41) with embryo grades 1-2 (highest morphology scores) showing odds ratio indicative of a 3-times higher chance of pregnancy. However, the positive predictive value for pregnancy was only 0.30. For fresh embryo transfers, we found that the average cleavage status on day 3 was a significant predictor of pregnancy (r= .26, $p<0.001$) better than average morphology score (r= .2, $p<0.001$). Pregnancy rates correlated positively with the number of good quality embryos transferred, but had a stronger and negative correlation with the number of poor quality embryos transferred. We also observed that the age-related decline in pregnancy was associated with impaired embryo cleavage rate and not poorer morphology scores. Results also revealed that IVF-derived embryos had better cleavage rates and morphology scores than ICSI-derived embryos; however, the implantation potential was similar for both groups. These results underscore the need for improved, non-invasive methods to assess pre-implantation embryo quality. It remains to be determined if measures of metabolic activity or other secreted proteins by cultured human blastocyst can be used as biomarkers of embryo developmental competence.

The ability to culture embryos to the blastocyst stage should help achieve the goals of selection of best quality embryos for transfer and reduction of multiple pregnancies. Therefore, the optimization and standardization of embryo culture media that support human embryo growth to the blastocyst stage should be considered an immediate priority for IVF programs. Recent data have been presented to support the move to blastocyst culture and transfer in human IVF (Sakkas *et al.*, 1998; Scholtes, *et al.*, 1996). We have performed a prospective randomized evaluation of two culture systems using cryopreserved-thawed human embryos and examined blastocyst development, trophoblast/inner cell mass ratios and cell death. Preliminary results demonstrated that blastocyst formation rates vary depending upon culture conditions and that the percentage of cell death is relatively low in embryos reaching blastocyst stage. Moreover, some culture media stimulated blastocyst formation of "deselected " embryos (embryos with poor morphological grades) thereby offering a means of separating human embryos with sub-optimal morphology into viable and non-viable categories. One of the major morphological anomalies observed in many human embryos is extensive cellular fragmentation. Embryo fragmentation seems to be associated with activation of programmed cell (Gardner, 1997). Advancements in clinical results should

follow acquisition of new knowledge in the areas of early embryo development and its control mechanisms.

Sperm capacitation

Spermatozoa are highly differentiated cells whose main function is to fertilize the oocyte leading to embryo development. They are the result of the complex process of spermatogenesis, which involves the phenomena of differentiation, multiplication (mitosis), determination of the haploid stage (meiosis) and a dramatic metamorphosis (spermatogenesis). Seminiferous tubule function and gametogenesis occur under strict endocrine and paracrine control. Spermatozoa are released into the epididymis (spermiation), where further maturational structural, biochemical and functional changes occur (capacitation). In order to successfully fertilize the oocyte, the spermatozoon must be able to perform these functions: migration (allowing transport to the fertilization site through adequate motion patterns), recognition and binding to the zona pellucida (an event dependent upon specific receptor-ligand interactions), penetration of the zona pellucida (secondary to the release of enzymes following the induction of the acrosome reaction by zona components), binding to the oolemma (also dependent upon interaction of complementary gamete molecules), oocyte activation, nuclear decondensation and participation in pronuclear formation leading to syngamy (Yanagimachi, 1994).

Only capacitated spermatozoa demonstrate the ability to respond to the adequate physiologic stimuli that result in the display of adequate motion characteristics, acrosome reaction responsiveness and competence to interact with the oocyte and its vestments (Figure 1). Several cellular changes that are manifested during capacitation include, among others, removal or modification of surface proteins, efflux of cholesterol from the membranes, changes in oxidative metabolism, achievement of hyperactivated pattern of motility and an increase in the phosphotyrosine content of several proteins. In addition to tyrosine phosphorylation of specific proteins, other modifications of cellular regulators occur such as a decrease in calmodulin binding to proteins and an increase in calcium uptake, intracellular pH and cAMP concentration (Yanagimachi, 1994; Visconti and Kopf, 1998).

The patterns of sperm hyperactivated motility are expressed in a reversible fashion under capacitating conditions (Jurisicova, *et al.*, 1996). Acrosome reaction, however, is an irreversible phenomenon, indicative of the completion of capacitation. Sperm tail protein phosphorylation

correlates positively and significantly with hyperactivated motility, indicating that phosphorylation of the tyrosine residues of sperm tail proteins could be one of the mechanisms through which hyperactivation occurs (Burkman, 1991). This stimulation is probably dependent on stimulation of a cAMP-dependent protein kinase (Yanagimachi, 1994).

Although hyperactivation is considered to be an integral part of capacitation, it has been suggested that the two processes may not be coupled (Nassar, 1998). There seems to be a temporal correlation between increased protein tyrosine phosphorylation and the development of hyperactivated motility in human spermatozoa. (Burkman, 1991; Suarez, *et al.*,1987; Leclerc, *et al.*,1996). These results do not preclude a possible role for tyrosine phosphorylation in additional events such as sperm binding to the zona pellucida or the acrosome reaction (Suarez *et al.*, 1987). Low levels of oxidative stress, as measured by the generation of reactive oxygen species (ROS) in spermatozoa, are able to support capacitation (Tomes *et al.*, 1998). An endogenous putative oxidase system located in the sperm plasma membrane plays an important biological role in supporting the tyrosine phosphorylation events associated with capacitation. Reports indicate that there is a causal association between basal ROS generation, tyrosine phosphorylation and sperm functions crucial for fertilization (Aitken, *et al.*, 2000) (Figure 2).

Sperm-zona pellucida interaction: binding and induction of the acrosome reaction

The early events that occur during fertilization may be viewed as a special form of highly complex cell-to-cell recognition. Cell-cell recognition mechanisms in many somatic cell systems involve carbohydrate side chains of membrane glycoproteins and several observations indicate that similar molecules may have a role in spermatozoa-oocyte binding in mammals. Compelling evidence has now demonstrated that carbohydrate-binding proteins on the sperm surface mediate gamete recognition by binding with high affinity and specificity to complex glycoconjugates of the zona pellucida (Fraser and Ahuja, 1988; Sailing, 1989, 1991 Yanagimachi, 1994; Miller, *et al.*, 1992; Oehninger, 2001).

In the mouse, the best characterized species so far; tight binding is achieved through interaction of the zona pellucida protein 3 (ZP3) and complementary sperm-binding protein(s) present in the plasma membrane. ZP3 triggers the acrosome reaction that is then followed by a secondary binding process involving the zona pellucida protein 2 (ZP2) and the inner acrosomal sperm membrane leading to zona penetration. Glycosylation appears mandatory for ZP3-ligand

function. It has been demonstrated that O-glycosylation, and particularly terminal galactose residues of O-linked oligosaccharides, are essential for maintaining mouse gamete interaction. Others have provided evidence that the amino sugar N-acetylglucosamine (NAG) is the key terminal monosaccharide involved in sperm-zona interaction in the mouse. In contrast, acrosome reaction-triggering activity of ZP3 seems to depend upon the integrity of the protein backbone (Wassarman, 1990a; Chapman and Barratt, 1996). Peptides synthesized based upon the published DNA sequence of ZP3 proteins are able to induce acrosomal exocytosis in some species (Wassarman, 1990a,b).

The molecular identity of the sperm surface receptor(s) for ZP3 is beginning to be unveiled. A number of candidate ZP3 receptor molecules have been proposed including potential carbohydate-binding proteins such as sp56, p95, β-1-4 galactosyltransferase and a D-mannosidase (Bleil and Wassarman, 1990; Leyton and Saling, 1989a,b; Miller, *et al.*, 1992; Cornwall, *et al.*, 1991).

A 95 kDa mouse (and human) protein with characteristics of a protein tyrosine kinase has also been proposed as a receptor for ZP3 (Leyton and Saling, 1989a,b; Leyton, *et al.*, 1992; Burks, *et al.*, 1995). Synthetic peptides corresponding to regions of the predicted extracellular domain of this receptor protein inhibited human sperm binding to the homologous zona pellucida (Burks, *et al.*, 1995). On the other hand, it has been proposed that sp56 is the mouse sperm protein responsible for recognition of ZP3 (Bleil and Wassarman, 1990; Bookbinder, *et al.*, 1995). According to this model, multiple interactions of ZP3 at the sperm surface would lead to aggregation (patching) of sp56, which in turn would generate the signal to trigger membrane fusion (Bleil and Wassarman, 1980, 1983, 1988, 1989, 1990). This possible patching mechanism is consistent with the following observations: (a) soluble ZP3, but not ZP3 glycopeptides induces the acrosome reaction (Florman, *et al.*, 1984) and (b) inability of ZP3 glycopeptides to induce the acrosome reaction can be overcome by dimerizing those glycopeptides with IgG directed against the ZP3 polypeptide chain (Leyton and Saling, 1989b).

In the mouse, ZP3-binding and ZP3-induced acrosomal exocytosis can be dissociated from each other, that is, seem to represent two independent processes (Kopf, 1990). There are differences in the concentration-dependency of ZP3 to express sperm binding activity and acrosome reaction-inducing activity. Specifically, the concentration response curve for ZP3-acrosome reaction inducing activity is shifted to the right of the concentration response curve for

ZP3-ligand activity. A model has been proposed predicting that ZP3 is composed of multiple "functional ligands", and that the interaction of these ligands with the sperm surface is responsible for both the sperm binding activity (through glycosylated epitopes) and the ability to induce a complete acrosome reaction (Kopf, 1990). Gamete recognition and adhesion probably depend upon a multivalent ligand interaction where the sperm protein receptor(s) binds to a number of different epitopes within the ZP3. These functional ligands do not necessarily have to be identical. The data concerning the involvement of either O- or N-linked glycosylation sites are also equivocal, particularly in the human. The lack of native human zona pellucida to perform direct carbohydrate analyses has made an unambiguous structural definition impossible so far. In the human, there is so far unequivocal data regarding the nature and specific function(s) of the zona binding protein(s) on the sperm surface.

Recently, full-length cDNA clones of ZP3 and ZP2 for different mammalian species (including mouse, hamster, human, rabbit, marmoset, pig, cat, cow and dog) have been isolated (Ringuette, *et al.*, 1986; Liang, *et al.*, 1990; Chamberlin and Dean, 1990; Kinloch, *et al.*, 1990; Liang and Dean, 1993; Lee, *et al.*, 1993; Thillai-Koothan, *et al.*, 1993; Schwoebel, *et al.*, 1991; Yurewicz, *et al.*, 1993; Harris, *et al.*, 1994). There is confusion in the literature as to the nomenclature of zona pellucida proteins. The names ZP1, ZP2 and ZP3 usually refer to molecular mass, although the terms ZPA, ZPB and ZPC have been coined to express functions and respective genes coding for these proteins. Genes encoding ZP2 and ZP3 are conserved among mammals and the DNA sequences of ZP3 cDNA coding regions show extensive homology between species studies do far.

Initially it was believed that cloning of the human ZP3 gene would circumvent this obstacle since a constant supply of recombinant material would be available. However, several of the laboratories dedicated to this task have been generally unable to purify a biologically active product so far (Chapman dn barratt, 1996). It seems clear that this is probably due to an inadequate and heterogeneous glycosylation of the protein by the different cell lines used. Very recently, we have been able to express and purify a human recombinant ZP3 that appears to demonstrate the full spectrum of biological activities (Dong *et al.*, 2001).

Signaling cascades involved in the acrosome reaction

Different views also exist in regard to the signaling cascades that follow the interaction between ZP3 and its receptor(s) leading to acrosomal exocytosis. It has been proposed that murine (and human) sperm-zona pellucida interaction leads to tyrosine phosphorylation of p95 (referred to as zona receptor kinase - ZRK); ZRK, therefore, contains intrinsic transmembrane signaling potential (Leyton and Saling, 1989a; Burks, *et al.*, 1995). Others have proposed that activation of GTP-binding proteins (G_i class) by ZP3 functions as a signal-transducing element distal to ZP3-mediated interactions. This results in coupling of receptor occupancy to changes in ionic conductance and/or a variety of intracellular second messenger cascade systems (Kopf, *et al.*, 1986; Kopf, 1990). Pertussis toxin can cross the plasma membrane and functionally inactivate G_i by ADP-ribosylating its α subunit (Casey and Gilman, 1988). This toxin does not affect the ability of mouse sperm to become capacitated but inhibits zona pellucida induced acrosome reaction in a dose-dependent manner (Kopf, 1990).

Increase of intracellular calcium concentration and a transient rise of internal pH also play a central role in acrosomal exocytosis. Recently, Arnoult, *et al.*, (1996, 1999) demonstrated in bovine and mouse spermatozoa that solubilized zona pellucida proteins depolarize sperm membrane potential by activating a pertussis toxin-insensitive pathway that has the characteristics of a poorly selective caution channel. However, zona pellucida proteins also activate a second pertussis toxin-sensitive pathway that produces transient elevations of sperm internal pH. Subsequently, voltage-sensitive calcium channels function as a coincidence detector by integrating two separate zona pellucida-induced signals.

In addition to the zona pellucida, another physiological inducer of the acrosome reaction, progesterone, has been demonstrated (Suarez, *et al.*, 1986; Thomas and Meizel, 1988; Osman, *et al.*, 1989; Blackmore, *et al.*, 1990). Progesterone stimulates calcium influx in sperm, a cellular event directly related to the acrosome reaction (Thomas and Meizel, 1989; Blackmore, *et al.*, 1990). The steroid's action is specific and presumably occurs through interaction with a surface receptor that is not modulated by pertussis toxin sensitive G proteins (Blackmore, *et al.*, 1990; Blackmore and Lattanzio, 1991; Tesarik, *et al.*, 1993a,b). Nevertheless, these results do not exclude the involvement of a pertussis toxin insensitive G protein, the presence of which has been demonstrated in bovine and human sperm (Hinsch, *et al.*, 1992, 1994a).

Moreover, it has recently been shown that when mouse spermatozoa were exposed first to progesterone and then to the zona pellucida, acrosomal exocytosis was enhanced to a greater

extent than when the agonists were presented together or in the inverse order (Roldan, *et al.*, 1994). This suggests that progesterone exerts a priming effect. The steroid similarly primed the generation of another intracellular messenger evoked by the zona pellucida, i.e., diacylglycerol (DAG) (Roldan and Harrison, 1992). Cross talk between pathways activated by ZP3 and progesterone may therefore be possible. These authors suggested that small amounts of progesterone may trigger intracellular calcium rises (Blackmore, *et al.*, 1990) that may be sufficient for DAG generation, but ZP3 may be needed for a further rise in calcium necessary for a complete acrosomal exocytosis (Roldan, *et al.*, 1994). Schematic diagrams of acrosomal exocytosis in mammals in general, and in humans in particular, are presented in (figures 3 and 4) (De Jonge, 1995; Doherty, *et al.*, 1995; Florman, *et al*, 1998; Wassarman, 1999).

Sperm-oocyte interaction: fusion, oocyte activation, pronuclear formation and contribution to early embryogenesis.

Spermatozoa that have undergone the acrosome reaction after interaction with and penetration through the zona pellucida are able to bind to the plasma membrane of the oocyte (oolemma). This also seems to be a specific recognition event involving putative molecules located in the equatorial segment of the sperm (sperm fusion proteins) and yet unidentified oocyte acceptors. Binding of the gametes leads to fusion of the membranes with incorporation of the entire spermatozoon into the ooplasm. The contact of the spermatozoon with the oocyte membrane triggers electrical membrane changes in the oocyte (membrane depolarization) and release of cortical granules, which represent fast and delayed protective mechanisms against polyspermy (Yanagimachi, 1994; 199).

There is still controversy as to the intimate mechanism(s) through which the spermatozoon activates the oocyte. Oocyte activation occurs in association with changes in the intracellular concentration of calcium ions, possibly modulated by a factor released by the spermatozoon once inside the oocyte. In the hamster, this protein has been identified as oscillin (Parrington, *et al.*, 1996). However, unequivocal identification of this factor in the human and other species has not yet been achieved. Sperm-oolemma binding and fusion is followed by activation of the oocyte's second messenger systems (calcium, PIP2), pH changes, protein synthesis, cyclin accumulation, DNA synthesis, nuclear envelope breakdown and the first cleavage division in some species. An increase in intracellular calcium is associated with microtubular rearrangement and pronuclear formation (Yanagimachi, 1994).

There is obviously extensive cross talk between the spermatozoon and the oocyte. In addition to the effects secondary to membranes fusion and the release of the oocyte activating factor(s) by the spermatozoon, the oocyte uses molecules that induce the sperm head decondensation (male pronucleus growth factor) and the substitution of protamines by histones (Yanagimachi, 1994; Thibault, *et al.*, 1975). Fertilization is achieved after the oocyte completes meiosis, female and male pronuclei are formed and syngamy (pronuclei union) is accomplished.

Abnormalities of fertilization: lessons from IVF and ICSI

Failure of fertilization during IVF treatment points out to one of three problems: (a) defective spermatozoa (intrinsic or due to poor preparation techniques), (b) poor quality oocytes (intrinsic or ovarian stimulation-derived), and/or (c) overall poor laboratory conditions (Oehninger *et al.*, 1988). We have been promoters of a sequential, multi-step diagnostic approach to assess those critical sperm functions involved in fertilization (Oehninger *et al.*, 1991; Oehninger *et al.*, 1997). Hopefully, based on such scheme we will soon be able to diagnose specific pathophysiological defects in cases of fertilization failure.

We and others have reported that sperm-zona pellucida is a crucial step and reflects multiple sperm functions (Oehninger, *et al.*, 1992; Liu and Baker, 1992). Many patients that are unable to fertilize oocytes under IVF conditions have a severe impairment of this functional step. A defective capacity to undergo the acrosome reaction is probably also a significant factor in some patients (ESHRE Andrology Special Interest Group, 1996). It has been recently shown that acrosomal exocytosis can be studied in vitro using small volumes of solubilized human zonae pellucidae and that G-proteins are involved as mediators. (Franken, *et al.*, 1996). This confirms previous studies that demonstrated the involvement of heterotrimeric G proteins in the induction of the acrosome reaction in other species (Kopf, 1990). It has also been demonstrated that funtional/biochemical/morpholgicl sperm immaturity (i.e., high content of creatine kinase) is present in many cases of male infertility resulting in fertilization deficiencies (Huszar and Vigue, 1994).

In recent studies, we investigated the structure of the human zona pellucida using antisera generated against synthetic ZP3 peptides (Hinsch, *et al.*, 1994a). Human metaphase II oocytes studied under a variety of conditions (i.e., fresh, refrigerated or salt-stored) normally reveal a strong immuno-reactivity to the specific anti-ZP3 antisera (Hinsch, *et al.*, 1994b). Of interest, in cases of

failed fertilization with either a poor ovarian response or morphologically defective oocytes (and in the presence of normal sperm parameters), we observed a marked alteration in the immuno-reactivity to the antisera (Oehninger, *et al.*, 1996). This finding demonstrates the presence of structural defects of the protein backbone of the zona pellucida and suggests that this defect may be a possible cause of fertilization failure.

Fertilization failure can be also due to sperm-oolemma fusion defects or an abnormal communication between the penetrating spermatozoon and the oocyte (i.e., lack or deficient sperm-oocyte activating factor, male pronucleus growth factor or other). Recent evidence from the ICSI setting clearly demonstrates that post-gamete fusion abnormalities may occur. Advances in fluorescent imaging by laser scanning confocal microscopy and other novel techniques permit a sophisticated high-resolution examination of gametes and embryos, including the fate of the sperm centrosome, the oocyte's microtubule organizing center, mitochondrial distribution and the initiation of embryo cleavage (Schatten, 1994). We remain enthusiastic about ongoing studies that may help in the elucidation of the contribution of the gametes (functional, biochemical-molecular and genetic) to early embryogenesis and in the identification of specific fertilization disorders.

References

1. Acosta A, Moon SY, Oehninger S, *et al.*, (1988) Implantation potential of each pre-embryo in multiple pregnancies obtained by in vitro fertilization seems to be different. *Fertil Steril,* **50**, 906-911.
2. Aitken RJ. (2000) Possible redox regulation of sperm motility activation. *J. Androl*, **21**, 491-496.
3. Aitken RJ, Gordon E, Harkiss D, *et al.*, (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Mol Reprod,* **59**, 1037-1046.
4. Aitken RJ, Paterson M, Fisher H, *et al.*, (1995) Redox regulation of tyrosine phosphorylatoin in human spermatozoa and its role in the control of human function. *J Cell Sci,* **108**, 2017-2025.
5. Antczak M and Van Blerkom J. (1997) Oocyte influences on early development: the regulatory proteins leptin and STAT are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Mol Hum Reprod*, **3**, 1067-1086.
6. Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, Florman HM (1999) Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membane hyperpolarizaion during capacitation. *Proc Nal Acad Sci USA,* **8**, 6757-6762
7. Arnoult C, Caardullo RA, Lemos JR, Florman HM (1996) Egg-activation of sprm T-type Ca^{2+} channels regulates acrosome reaction during mammalian fertilization. *Proc Natl Acad Sci USA,* **93**, 13004-13009
8. Blackmore PF, Neulen J, *et al.*, (1991) Cell surface for progesterone nediate calcium uptake in human sperm. *J Biol Chem,* **266**, 18655-18659.
9. Blackmore PF, Beebe SJ, Danforth DR, *et al.*, (1990) Progesterone and 17 a-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem,* **265**,1376-1380.
10. Bleil JD, Wassarman P. (1990) Identification of a ZP3-binding protein acrosome-intact mouse sperm by photoaffinity cross-linking. *Proc Natl Acad Sci USA,* **87**, 5563-5567.
11. Bleil JD, Wassarman PM. (1989) Identification of a mouse sperm receptor protein that recognizes ZP3. *J Cell Biol,* **109**, 125-129.

12. Bleil JD, Wassarman P. (1983) Sperm-egg interactions in the mouse, Sequence of events and induction of the acrosome reaction by zona pellucida glycoprotein. *Dev Biol,* **95**, 317-324.

13. Bleil JD, Wassarman PD. (1980) Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocytes zona pellucida. *Dev Biol,* **76**,185-202.

14. Braude P, Bolton V and Moore S. (1988) Human gene expression first occurs between the four and eight-cell stages of preimplantation development. *Nature* (London), **61**, 332-459

15. Burkman LJ. (1991) Discrimination between non-hyperactivated and classical hyperactivated motility patterns in human spermatozoa using computerized analysis. *Fertil Steril*, **55**, 363-371.

16. Burks DJ, Carballafa R, Moore HD, *et al.*, (1995) The interaction of a tyrosine kinase from human sperm with zona pellucida at fertilization. *Science,* **269**, 83-96.

17. Casey P, Gilman A (1988) A protein involvement in receptor-effectors coupling. *J Biol Chem,* **263**, 2577-2580.

18. Chamberlin ME. Dean J. (1990) Human homolog of the mouse receptor. *Proc Nat Acad Sci USA,* **87**, 6014-6018.

19. Chapman NR and Barratt CLR. (1996) The role of carbohydrate in sperm-ZP3 adhesion. *Mol Hum Reprod,* **108**, 767-774.

20. Cohen J, Scott R, Alikani M, *et al.*, (1998) Ooplasmic transfers in mature human oocytes. *Mol Hum Reprod,* **4**, 269-280.

21. Cohen J, Scott RT, Schimmel T, *et al.*, (1997) Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs. *Lancet,* **350**,189-7.

22. Cornwall GA. Tulsiani DR, Orgebin-Crist MC. (1991) Inhibition of the mouse sperm surface alohs-D-mannosidase inhibits sperm-egg binding in vitro. *Biol Reprod*, **44**, 913-921.

23. Dong KW, Chi TF, Juan YW, *et al.*, (2001) Characterization of biologic activities of a recombinant human zona pellucida protein 3 expressed in human ovarian teratocarcinoma (PA-1) cells. *Am J Obstet Gynecol,* **184**, 835-844

24. De Jong C. (1995) Hypothetical signaling pathways through which biological effectors stimulate acrosomal exocytosis in human spermatozoa. *J Androl*, **16**, cover.

25. Doherty CM, Tarchala SM, Radwanska E, *et al.*, (1995) Characterization of two-second messenger pathways and their interactions in eliciting the human sperm acrosome reaction. *J Androl*, **16**, 36-46

26. ESHRE Andrology Special Interest Group. (1996) Consensus Workshop in Advanced Diagnostic Andrology Techniques. *Hum Reprod,* **11**, 1463-1479.
27. Flood JT, Chillic CFl, Van Uem JF, *et al.*, 1990. Ooplasmic transfusion: prophase germinal vesicle oocytes made developmentally component by microinjection of metaphase II egg cytoplasm. *Fertil Steril,* **53**, 1049-1054.
28. Florman HM, Arnoult C, Kazam I, Chonqing L, O'Toole C (1998) A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. *Biol Reprod,* **59**, 12-16.
29. Florman HM, Bechtal K, Wassarman P. (1984) Enzymatic dissection of the fractions of the mouse egg's receptor for sperm. *Dev Biol*, **106**, 243-255.
30. Franken DR, Morales PJ and Habenicht UF. (1996) Inhibition of G protein in human sperm and its influence on acrosome reaction and zona pellucida binding. *Fertil Steril*, **66**, 1009-1011.
31. Fraser L and Ahuja KK. (1988) Metabolic and surface events in fertilization. *Gamete Res*, **20**, 491-519.
32. Fujino Y, Ozaki K, Yamamusi S, *et al.*, DNA fragmentation of oocytes in aged mice. *Hum Reprod*, **11**,1480-1483.
33. Gardner DK and Lane M. (1997) Culture and selection of viable blastocyst: a feasible proposition for human IVF? *Hum Reprod Update*, **3**, 367-382.
34. Harris J Hibler D Fontemot G, *et al.*, (1994) Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: The ZPA, ZPB and ZPC gene families DNA sequence. *J Sequen Map*, **4**, 361-693.
35. Hinsch E, Oehninger S and Hinsch KD. (1998) ZP3-6 peptide and acrosome reaction. In Vereecken A, De Jonge C, Franken D, Oehninger S, Ombelet W, eds. Semen Evaluation, Testing and Selection: a New Look in the 2000's. Pre-Congress training course, Andrology in the Nineties: Modern ART on the 2000's, Genk Belgium. 25-9 pp.
36. Hinsch, KD, Hinsch, E and Meinecke, B, *et al.*, (1994a) Identification if mouse ZP3 protein in mammalian oocytes with antisera against synthetic ZP3 peptides. *Biol Reprod*, **51**,193-204.
37. Hinsch KD, Hinsch E, Pfisterrer, S, *et al.*, (1994b) Anti-ZP3 antibodies binding to the human zona pellucida: effect of oocyte storage conditions. *Am J Reprod Immunol*, **32**,146-151.

38. Hinsch E Hinsch KD Aummulen G, *et al.*, (1992) Immunological identification of G protein alpha-and beta-subunits in tail membranes of bovine spermatozoa. *Biol Reprod*, **47**, 337-346.

39. Huszar G, Vigue L. (1994) Correlation between the rate of lipid peroxidation and cellular maturity as measured by creation kinas activity in human spermatozoa. *J Androl*, **15**, 71-77.

40. Jurisicova A, Varmuza S and Casper RF. (1996) Programmed cell death and human embryo fragmentation. *Mol Hum Reprod,* **2**, 93-98.

41. Kinloch R, Ruiz Seiler B, Wassaman P. (1990) Genomic organization and polypeptide primary structure of zona pellucida glycoprotein HZP3, the hamster sperm receptor. *Dev Biol,* **142**, 414-421.

42. Kligman I, Benadive C and Alikani M. (1996) The presence of multinucleated blastomeres in human embryos is correlated with chromosomal abnormalities. *Hum Reprod,* **11**, 1492-1498.

43. Kopf GS. Zona pellucida-mediated signal transduction in mammalian spermatozoa. (1990) *J Reprod Fertil*, **42**, 33-49.

44. Leclere P, Lamirande E and Gagnon C. (1996) Cyclic adenosine 3', 5' monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod*, **55**, 684-692.

45. Lee, Schwoebel E, Prasad S, *et al.*, (1993) Identification and structural characterization of the 75 kD rabbit zona pellucida. *Biol Reprod*, **36**, 617-627.

46. Leyton L, Legum P, Brunch D, *et al.*, (1992) Regulation if mouse gamete interaction by sperm tyrosine kinase. *Proc Natl Acad Sci USA*, **89**,11592-11695.

47. Leyton L, Sailing P. (1989a) 95 kD sperm proteins bind ZP3 and serve as tyrosine kinase substrates un response to zona binding. *Cell*, **57**, 1123-1130.

48. Leyton L, Sailing P. (1989b) Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. *J Cell Biol*, **108**, 2163-2168.

49. Liang L, Dean J. (1993) Conservation of mammalian secondary sperm receptor genes enable promoter of human gene function in mouse oocytes. *Dev Biol,* **156**, 399-408.

50. Liang L. Chamow S, Dean J. (1990) Oocyte-specific expression of mouse ZP2: development of the zona pellucida genes. *Mol Cel Biol*, **10**, 507-1518.

51. Liu DY and Baker HWG. (1992) Tests of human sperm function and fertilization in vitro. *Fertil Steril,* **58**, 465-483.

52. Miller DJ, Macek MB, Shur BD. (1992) Complementarity between sperm surface beta-1, 4-galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. *Nature,* **357**, 589-593.

53. Nassar A, Mahony M, Blackmore P, *et al.*, (1998) Increase of intracellular calcium is not a cause of pentoxifylline-induced hyperactivated motility or acrosome reaction in human sperm. *Fertil Steril*, **69**, 748-754.

54. Oehninger S. (2001) Molecular basis of human sperm-zona pellucida interaction. *Cells Tissues Organs*, **168**, 58-64.

55. Oehninger S. Franken D and Kruger T. (1997) Approaching the next millennium: how should we manage andrology diagnosis in the intracytoplasmic sperm injection era? *Fertil Steril*, **67**, 434-436.

56. Oehninger S, Hinsch E, Pfisterer S, *et al.*, (1996) Use of a specific ZP3 antiserum as a clinical marker for human zona pellucida integrity and function. *Fertil Steril,* **65**, 13-45.

57. Oehninger S, Franken D, Alexander N, *et al.*, (1992) Hemizona assay and its impact on the identification and treatment of human sperm dysfunctions. *Andrologia,* **24**, 307-321.

58. Oehninger S, Acosta AA, Veeck LL, *et al.*, (1991) Recurrent failure of in vitro fertilization: role of the hemizona assay in the sequential diagnosis of specific sperm-oocyte defects. *Am J Obstet Gynecol*, **164**,1210-1215.

59. Oehninger S, Acosta AA, Kruger, *et al.*, (1988) Failure of fertilization in in-vitro fertilization: the 'occult' male factor. *J In Vitro Fertil Embryo Transfer,* **5**, 181.

60. Osman RA, Andria ML, Jones AD, *et al.*, (1989) Steroids induced exocytosis: the human sperm acrosome reaction. *Biochem Biophys Res Commun,* **160**, 823-833.

61. Ozgur K, Patankar MS, Oehninger S, *et al.*, (1994) Direct evidence for the involvement if carbohydrate sequence in human sperm-zona pellucida binding. *Mol Humn Reprod,* **4**, 318-324.

62. Parrington J, Swann K, Shevchenko, *et al.*, (1996) Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature* (London), **379**, 364-368.

63. Perez CI, and Tilly JL. (1997) Cumulus cells are required for an increased apoptotic potential in oocytes of aged mice. *Hum Reprod*, **12**, 2781-2783.

64. Ringuette MJ, Sobieski DA, Chomow SM, *et al.*, (1986) Oocyte-specific gene expression: molecular characterization of a cDNA coding for ZP3, the sperm receptor of the mouse and zona pellucida. *Proc Nat Acad Sci USA,* **83**, 4341-4345.

65. Roldan E, Murage T, Shi Q. (1994) Exocytosis in spermatozoa in response to progesterone and zona pellucida. *Science,* **266**, 1578-1581.

66. Sacco A, Yurewicz E, Subramaniah MG, *et al.*, (1981) Zona pellucida composition: species cross reactivity and contraceptive of antiserum to a purified pig zona antigen (PPZA). *Biol Reprod,* **25**, 997-1008.

67. Sailing PM. (1991) How the egg regulates sperm function during gamete interaction: Facts and fantasies. *Biol Reprod,* **44**, 246-251.

68. Sailing PM. (1989) Mammalian sperm interaction with extracellular matrices of the egg. *Oxf Rev Repro Biol*, **11**, 339-388.

69. Sakkas D, Shoukir Y, Chardonnens D, *et al.*, (1998) Early cleavage of human embryos to the two-cells stage after intracytoplasmic sperm injection as an indicator of embryo viability. *Hum Reprod*, **13**, 182-187.

70. Schatten G. (1994) The centrosome and its mode of inheritance: the reduclance of the centrosome during gametogenesis and its restoration during fertilization. *Dev Biol,* **165**, 299-335.

71. Scholtes MCW and Zeilmaker GH. (1996) A prospective, randomized study of embryo transfer results after 3 or 5 days of embryo culture in in-vitro fertilization. *Fertil Steril,* **65**,1245-1248.

72. Suarez SS, Vincentti L and Ceglia MW. (1987) Hyperactivated motility induced in mouse sperm by calcium A23187 is reversible. *J Exp. Zool,* **224**, 331-336.

73. Suarez SS, Wolf DP, Meizel S. (1986) Induction of the acrosome reaction in human spermatozoa by fraction of human follicular fluid. *Gamete Res,* **14**,107-121.

74. Tesarik J, Carrera A, Mendoza C. (1993a) Differential sensitivity of progesterone and zona pellucida induced acrosome reactions to pertussis toxin. *Mol Reprod Dev,* **34**, 83-89.

75. Tesarik J, Moos J, Mandoza C. (1993b) Stimulation of protein tyrosine phosphorylation by progesterone receptor on the cell surface of human sperm. *Endocrinology*, **133**, 328-335.

76. Thibault C, Gerard M and Menezo Y. (1975) Acquisition partion du noyau du spermatozoid fecondant (MPGF). *Ann Biol Biochem Biophys*, **15**, 705-714.

77. Thomas P. Meizel S (1989) Phosphatidylinositol 4.5-biophosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx. *J Biochem*, **264**, 539-546.

78. Thomas P, Meizel S (1988) An influx of extracellular calcium is required for initiation of the human sperm acrosome reaction induced by human follicular fluid. *Gamete Res*, **20**,397-411.

79. Tomes CN, Carballada R, Moses D, *et al.*, (1998) Treatment of human spermatozoa with seminal plasma inhibits protein tyrosine phosphorylation. *Mol Hum Reprod*, **4**, 17-25.

80. Van Blerkom J. (1998a) Epigenetic influenced on oocyte developmental competence: perifollicular vascularity and intrafollicular oxygen. *J Assist Reprod Genet*, **15**, 226-234.

81. Van Blerkon J and Davis PW. (1998b) DNA stands breaks and phosphatidylserine redistribution in newly ovulated and cultured mouse and human oocytes: occurrence and relationship to apoptosis. *Hum Reprod*, **13**, 317-324.

82. Van Blerkom J, Antczak M and Schrader R. (1997a) The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Hum Reprod,* **12**,1047-1055.

83. Van Blerkom J. (1997b) Can the development of competence of early human embryos be predicted effectively in clinical IVF laboratory? *Hum Reprod*, **12**, 1610-1614.

84. Visconti PE, Kopf GS (1998) Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod,* **59**, 1-6.

85. Wassarman PM. (1999) Mammalian fertilization: Molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell*, **96**, 75-83

86. Wassarman PM. Profile of mammalian sperm receptor. (1990a) *Development,* **108**, 1-17.

87. Wassarman PM. (1990b) Regulation of mammalian fertilization by zona pellucida glyoprotiens. *J Reprod Fertil,* **42**, 79-87

88. Yanagimachi R. Mammalian fertilization. (1994) In Knobil N, Neill JD, eds. *The Physiology of Reproduction*. New York: Raven Press, 189-317.

Figure 1

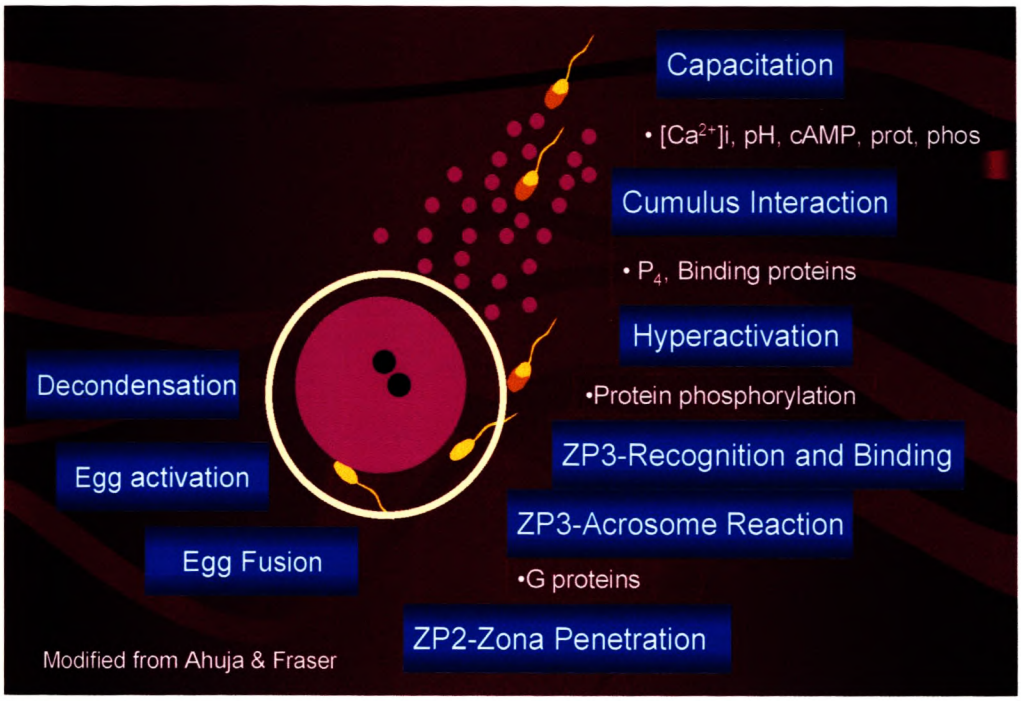


Figure 2

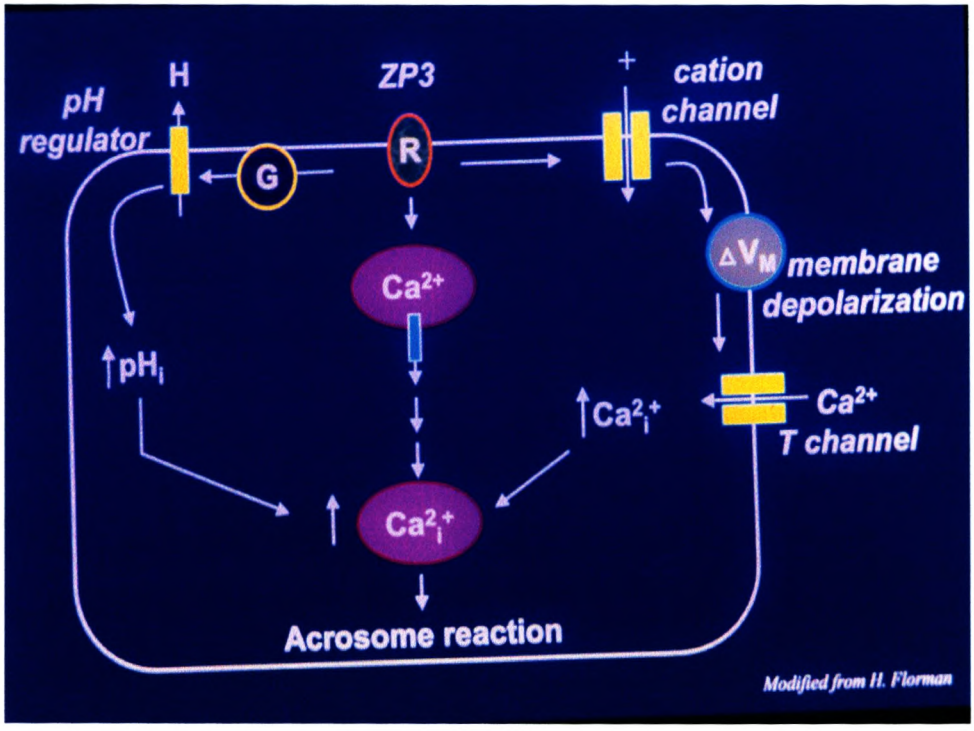


Figure 3

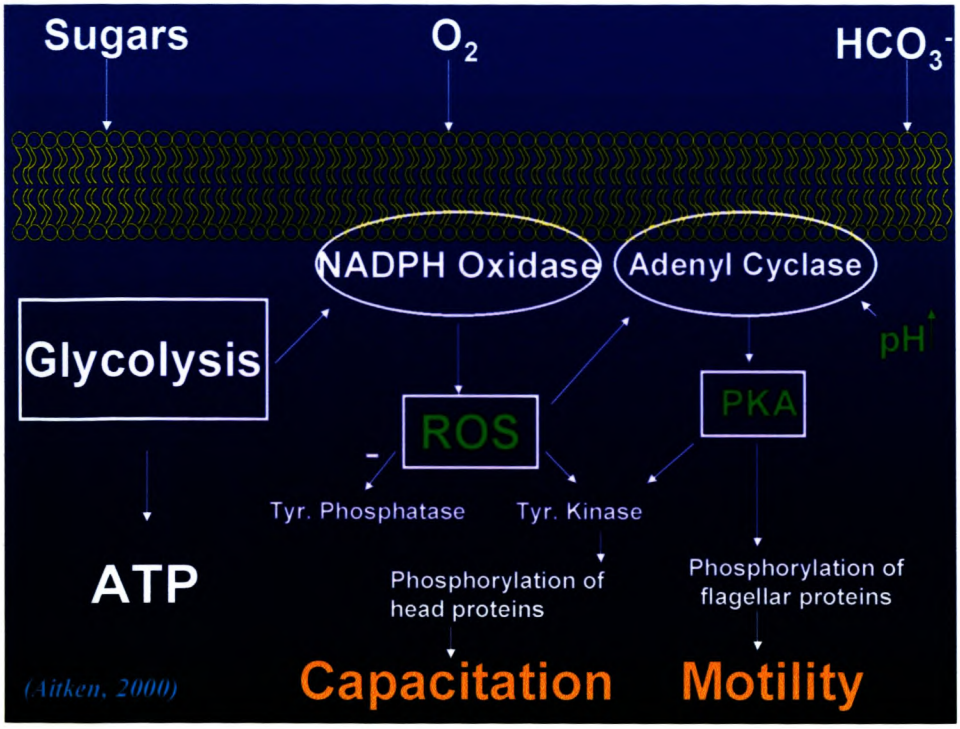
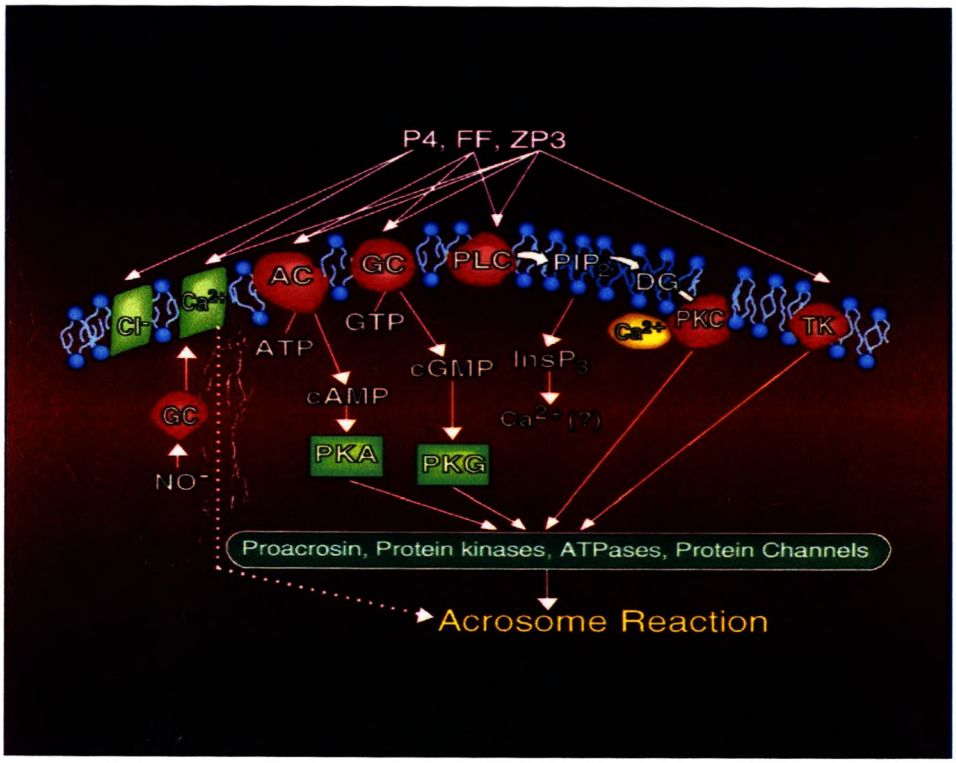


Figure 4



Legends to figures

Figure 1. Principal steps of sperm capacitation and sperm-oocyte interaction and cellular mechanisms involved (modified from Frazer and Ahuja, 1988).

Figure 2. Possible regulatory mechanisms involved in the activation of sperm motility and the induction of capacitation (modified from Aitken, 2000).

Figure 3. Schematic diagram of the acrosome reaction in mammals (modified from Florman et al, 1998 and Wassarman, 1999).

Figure 4. Hypothetical signaling pathways through which biological effectors stimulate acrosomal exocytosis in human spermatozoa (from De Jonge, 1995 and Doherty *et al.*, 1995).

2b. Molecular basis of human sperm-zona pellucida interaction

The recognition of carbohydrate sequences by complimentary receptors has been shown to be a critical factor in gamete interaction in many different animal species. We have proposed the hypothesis that, in the human, sperm binding to the zona pellucida requires a "selectin-like" interaction. We have used the hemizona assay (a unique internally controlled bioassay that evaluates tight binding of human sperm to the homologous zona pellucida) and advanced methods of carbohydrate analysis to test this hypothesis. We have provided compelling evidence that demonstrates that oligosaccharide recognition is also required for specific, tight human gamete binding. Hapten-inhibition tests, zona pellucida lectin-binding studies and removal/ modification of functional carbohydrates by chemical and enzymatic methods provide evidence for the involvement of defined carbohydrate moieties in initial binding. Our studies suggest the existence of distinct zona-binding proteins on human sperm that can bind to selectin ligands. Additionally, results suggest a possible convergence in the types of carbohydrate sequences recognized during initial human gamete binding and immune/inflammatory cell interactions. Full characterization of the glycoconjugates that manifest selectin-ligand activity on the human zona pellucida will allow for a better understanding of human gamete interaction in physiologic and pathologic situations.

Clinical aspects

Fertilization involves a complex sequence of events leading to embryogenesis. Obligatory requirements for the successful completion of normal fertilization include a mature, metaphase II oocyte and spermatozoa that have completed the process of capacitation. The newly formed human zygote undergoes early cleavage divisions depending upon the oocyte's endogenous machinery and at the 4 to 8-cell stage initiates transcription of the embryonic genome (Braude *et al.*, 1988). In vivo, these processes are synchronized with the preparation of the endometrial mucosa (window of implantation) thereby ensuring an adequate milieu receptive of the blastocyst. In vitro conditions can successfully mimic these physiological events.

Advances in basic science and the clinical application of such knowledge have allowed many infertile couples achieve their reproductive goals through the therapeutic modalities of assisted reproduction. These techniques will be, undoubtedly, the basis for future improved treatments that will help alleviate and even prevent human disease.

Why is it important for the discipline of reproductive medicine to unveil the mechanisms that operate during normal gamete interaction? The answer to this question can be readily obtained from the lessons of in vitro fertilization therapy. Failure of fertilization under in vitro conditions points out to one of three problems: (a) defective spermatozoa, (b) poor quality oocytes, and/or (c) overall poor laboratory conditions (Oehninger *et al.*, 1988). We have been promoters of a sequential, multi-step diagnostic approach to assess those critical sperm functions involved in fertilization (Oehninger *et al.*, 1991 a; 1997). An elusive goal, so far, has been that of being able to diagnose specific pathophysiological defects in cases of fertilization failure. Sperm-zona pellucida binding is a crucial step and reflects multiple sperm functions. Many patients that are unable to fertilize oocytes under IVF conditions have a severe impairment of this functional step.

A defective capacity to undergo the acrosome reaction is probably also a significant factor in some patients (Liu and Baker, 1992). It has been recently shown that acrosomal exocytosis of human spermatozoa can be studied in vitro using small volumes of solubilized human zonae pellucidae and that G-proteins are involved as mediators (Franken *et al.*, 1996). This confirms previous studies that demonstrated the involvement of heterotrimeric G proteins in the induction of the acrosome reaction in other species (Kopf, 1990).

The structure of the human zona pellucida can be examined using antisera generated against synthetic ZP3 peptides (Hinsch *et al.*, 1994a). Human metaphase II oocytes studied under a variety of conditions normally reveal a strong immuno-reactivity to the specific anti-ZP3 antisera (Hinsch *et al.*, 1994b). Of interest, in cases of failed fertilization with either a poor ovarian response or morphologically defective oocytes (and in the presence of normal sperm parameters), we observed a marked alteration in the immuno-reactivity to the antisera (Oehninger *et al.*, 1996). This finding demonstrates the presence of structural defects of the protein backbone of the zona pellucida and suggests that this defect may be a possible cause of fertilization failure. There is virtually no information about putative abnormalities that could operate at the level of receptor-ligand interaction during human gamete binding.

Since the bulk of clinical evidence highlights the impact of disorders of sperm-zona pellucida interaction, it is our goal is to unravel the relative contribution of the structural (carbohydrates and proteins) and functional (gamete binding and induction of the acrosome reaction) components of this process.

Physiologic/biochemical aspects

The early events that occur during fertilization may be viewed as a special form of highly complex cell-to-cell recognition. Cell-cell recognition mechanisms in many somatic cell systems involve carbohydrate side chains of membrane glycoproteins and several observations indicate that similar molecules may have a role in spermatozoa-oocyte binding in mammals. Compelling evidence has now demonstrated that carbohydrate-binding proteins on the sperm surface mediate gamete recognition by binding with high affinity and specificity to complex glycoconjugates of the zona pellucida (Macek and Shur, 1988; Yanagimachi, 1994; Chapman and Barratt, 1996) (Figure 1). In the initial interactions between spermatozoa and zona pellucida, both non-enzymatic (lectin-like) and enzymatic events may be involved depending upon the species, and may have in common the ability to recognize carbohydrate moieties. Four different approaches have been used to investigate the role of surface carbohydrates in gamete interactions: (1) the use of specific saccharide moieties in hapten-inhibition tests; (2) lectin binding studies; (3) the use of enzymes to remove functional carbohydrates from the cell surface; and (4) the use of inhibitors of glycoprotein synthesis.

The zona pellucida is a transparent, extracellular matrix constructed from only three glycoproteins, which build its typical fibrogranular structure by noncovalent interactions. Each of the glycoproteins is heterogeneous with respect to molecular weight due to the extensive glycosylation of a unique polypeptide with both asparagine- and serine-threonine-linked oligosaccharides. The oligosaccharides are both sulfated and sialylated with additionally adds to heterogeneity and makes all three glycoproteins relatively acidic. Reports concerning the properties of the zona in the mouse provided insight into the molecular organization of the matrix. According to their electrophoretic mobility the glycoproteins are denoted ZP1, ZP2 and ZP3. In the mouse, these glycoproteins are exclusively secreted by the growing oocyte. Two of the glycoproteins, ZP2 and ZP3, interact with each other forming hetrodimeric units that are periodically arrenage in long filaments. These filaments appear to be interconnected by ZP1. The architecture of the zona pellucida changes during maturation of the oocyte and fertilization (for review see Sinowatz *et al.*, 2000).

In the mouse, the best characterized species so far; tight binding is achieved through interaction of the zona pellucida protein 3 (ZP3) and complementary sperm-binding protein(s) present in the plasma membrane. ZP3 triggers the acrosome reaction which is then followed by

a secondary binding process involving the zona pellucida protein 2 (ZP2) and the inner acrosomal sperm membrane leading to zona penetration (Bleil and Wassarman, 1980; 1983; 1988). Glycosylation appears mandatory for ZP3-ligand function. It has been demonstrated that O-glycosylation, and particularly terminal galactose residues of O-linked oligosaccharides, are essential for maintaining mouse gamete interaction. Others have provided evidence that the amino sugar N-acetylglucosamine (NAG) is the key terminal monosaccharide involved in sperm-zona interaction in the mouse (reviewed in Wassarman, 1990; Chapman and Barratt, 1996). In contrast, acrosome reaction-triggering activity of ZP3 seems to depend upon the integrity of the protein backbone (Wassarman, 1990; Saling, 1991). Peptides synthesized based upon the published DNA sequence of ZP3 proteins are able to induce acrosomal exocytosis in some species (Hinsch *et al.*, 1998).

Although several components have been suggested as putative sperm receptors, the identity and unambiguous function of these molecules remains to be elucidated. For example, in the mouse, several sperm surface proteins with high affinity for the extracellular matrix of the egg have been proposed as sperm receptors. In the human, there is so far unequivocal data regarding the nature and specific function(s) of the zona binding protein(s) on the sperm surface.

We have been interested in determining the carbohydrate dependence of human sperm-zona pellucida interaction. For this purpose, we exploited a unique test for the assessment of the tight binding of human sperm to the homologous zona pellucida, a binding interaction that leads to triggering of the acrosome reaction under in vitro conditions. This test, the hemizona assay (HZA), provides distinctive advantages: (1) oocytes are microbisected into identical, matching zona hemispheres (hemizonae) thereby allowing for an internally controlled assay of sperm binding under identical conditions; (2) splitting of the oocyte allows to obtain statistically valid results with a limited number of oocytes compared to whole-oocyte assays; and (3) because the oocyte is split microsurgically, ethical concerns about inadvertent fertilization are eliminated (Burkman *et al.*, 1988).

Here I summarize recently published studies (Oehninger *et al.*, 1990, 1991 b, 1992, 1995; Mahony, *et al.*, 1991; Patankar, *et al.*, 1993; Clark, *et al.*, 1995; Dell, *et al.*, 1995; Koistinen, *et al.*, 1996; Morris, *et al.*, 1996; Patankar, *et al.*, 1997; Ozgur *et, al.*, 1998; Oehninger, 2000) where we have utilized the HZA and advanced methods of carbohydrate analysis to investigate glycobiological aspects of human sperm binding to its homologous zona pellucida. These

experiments were built on the work of previous investigators that had begun to address this issue in other animal species (Huang *et al.*, 1982; Ahuja, 1985; Lopez *et al.*, 1985; Macek and Shur, 1988). The results presented herein provide evidence in support of our novel hypothesis that initial human sperm-zona pellucida binding requires a selectin-like interaction between the human gametes (Patankar *et al.*, 1993; Clark *et al.*, 1995; Oehninger *et al.*, 1998).

Studies of the glycobiology of human sperm-zona pellucida interaction

All carbohydrates and charged polymers tested in theses studies were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. Monosaccharides examined were d-fucose, l-fucose, d-glucose and d-mannose. The following complex saccharides were evaluated: fucoidan, dextran sulfate, dextran, heparin, heparin sulfate, chondroitin sulfates A and B and hyaluronic acid. Charged polymers and salts tested included: polyglutamic acid, polyphosphates types 5 and 15, and sodium phosphate. Other saccharide moieties analyzed were: Le^x-, Le^b-, Le^a- active oligosaccharides, lacto-N-fucopentaose II and III, 3-fucosyllactose, lactodi-N-fucohexaose I, and highly purified mannose-containing oligosaccharides and high mannose-type glycopeptides derived from ovoalbumin (Oxford Glycosystems, UK). Human α1-acid glycoprotein (orosomucoid) and fetuin were obtained from Sigma. Sialyl Le^x was obtained from Dextra Biochemicals (UK). Glycodelin-A (previously called placental protein 14 or PP14, obtained from amniotic fluid)) and glycodelin-S (obtained from seminal plasma) were purified by antibody affinity and ion exchange chromatography as previously described (Riittinen *et al.*, 1991; Koistinen *et al.*, 1996). FITC-conjugated mannose-BSA (Bovine Serum Albumin), glucose-BSA, galactose-BSA and fucose-BSA were obtained from Sigma. Monoclonal antibodies directed against P-, E- and L-selectins were obtained from Becton-Dickinson (USA).

Carbohydrate and protein analysis

Methods of carbohydrate analysis for determination of fucoidan's structure included infrared analysis, sulfation and methylation, desulfation, synthesis of methylated derivatives, paper chromatography and gas chromatography-electron impact mass spectrometric analysis (Patankar *et al.*, 1993). Analysis of glycodelins involved peptide mapping by fingerprinting, tryptic peptide mapping by high-performance liquid chromatography, isoelectric focusing, lectin immunoassays, immunoblotting, N-terminal amino acid sequence analysis and sugar analysis by fast atom

bombardment- and electrospray mass spectrometry (Julkunen, *et al.*, 1988; Dell, *et al.*, 1995; Morris, *et al.*, 1996; Koistinen, *et al.*, 1996).

Lectins and enzymatic-chemical treatment of the human zona pellucida

Flourescein isothiocyanate (FITC) - labelled erythroagglutinating phytohemagglutinin (E-PHA), Sambucus nigra agglutinin (SNA), Wisteria floribunda agglutinin (WFA), Maakia amurensis agglutinin (MAA), Wheat germ agglutinin (WGA) and Pokeweed mitogen (PWM) were purchased from E-Y Labs (San Mateo, CA, USA). Endo-β-galactosidase was obtained from V-Labs (Covington, LA, USA); neuraminidase and sodium m-periodate were obtained from Sigma.

Human gametes and HZA

Semen was obtained from fertile men and the motile sperm fractions were prepared by wash/swim-up effected in Ham's F-10 medium (Gibco Lab., Grand Island, NY, USA) supplemented with 0.3% HSA (Human Serum Albumin, Irvine Sci., Santa Anna, CA, USA). Human oocytes were obtained from surgically removed ovarian tissue or from IVF following protocols described elsewhere and after Institutional Review Board consent was obtained. Before the HZA, the salt-stored oocytes were washed and microbisected into matching hemizonae using Narishige micromanipulators (Narishige, Tokyo, Japan) mounted on a phase-contrast inverted microscope (Nikon Diaphot, Garden City, NY) as previously described (Burkman *et al.*, 1988). The sperm were preincubated with the sugar moieties for 60 min at 37°C, in 5% CO2 in air, then washed and coincubated with the hemizonae. HZA results were expressed as hemizona index (HZI) calculated as follows: number of sperm bound for test/control x 100. For any given sugar concentration tested, a paired t-test was used to examine statistical significance. Dose- dependent studies were evaluated by analysis of variance.

Hapten-inhibition assays

Results demonstrated that different monosaccharides (at a maximum dose of 1 mg/ml) manifested various effects in the HZA. Whereas d-galactose and d-glucose had no effect on sperm binding to the zona, l-fucose and d-glucose showed a moderate stimulatory activity. Although d-

mannose had a slight inhibition, mannose-containing oligosaccharides and high-mannose glycopeptides were not inhibitory. Chondroitin sulfates A and B, hyaluronic acid, heparin and dextran did not affect binding. Fucoidan (a complex polymer of l-fucose), dextran sulfate and heparin sulfate produced significant inhibition. Fucoidan showed a potent, fast and the strongest inhibition of binding. Different charged polymers and salts tested had only a slight inhibitory effect or no effect at all. When analyzed by immunofluorescence, FITC-BSA-monosaccharides (fucose, glucose, galactose and mannose) were shown to bind to the acrosomal region of capacitated sperm. Fucose-BSA depicted maximum binding to spermatozoa under these conditions.

Further studies revealed a new, revised molecular arrangement of fucoidan indicating that this fucan is a close structural analogue of the sulfated carbohydrate ligands that bind to selectins. Fucoidan is also a potent inhibitor of L- and P-selectin mediated adhesions. Since oligosaccharides with fucose branches and sulfate groups are ligands to selectins we sought to determine whether a relationship existed between selectin-mediated adhesions of leukocytes and human sperm-zona pellucida binding. This relationship would support our hypothesis that initial gamete binding in the human involves a selectin-like interaction. This was further proven by the confirmation of inhibitory effects of sialyl Le^x and α1-glycoprotein, other blockers of selectin-mediated adhesions, and the abolition of this activity by desialylation of this glycoprotein. On the other hand, antibodies directed against selectins did not bind to human spermatozoa. This observation was not surprising since expression of selectins is not detected in human testicular cDNA libraries. Thus, our results suggested that human sperm-zona pellucida binding has a selectin-like specificity (selectin-like ligands present may be present on the zona) but does not involve the known selectin molecules as complementary receptors on the surface of the sperm.

Glycodelyn-A, an endometrial epithelial protein, produced a potent and dose-dependent inhibition in the HZA. This turned out not to be surprising since it was found that the oligosaccharides associated with this glycoprotein (fucosylated lacdiNAc antennae among others) also potently block selectin-mediated adhesions. Our present results also indicate the possibility that the carbohydrate binding specificity of the receptors mediating gamete recognition and lymphocyte/leukocyte adhesion have converged, at least to some extent. This concept is further supported by the demonstration of contraceptive (inhibition of sperm-zona binding) and immunosuppressive properties of Glycodelin-A and their carbohydrate dependence. Of significance, purified Glycodelin-A suppresses the lysis of K562 cells by natural killer (NK) cells

within the same concentration dependence of its HZA effects, possibly due to its expression of unusual N-linked glycans (Figure 2).

Lectin binding studies

It has been shown that lectins that recognize specific polylactosaminoglycan structures such as WGA (Wheat germ agglutinin), Con-A (Concanavalin A), LCA (Lens culinaris) and Ricinus communis agglutinin, have the ability to bind to the human zona pellucida. Following this approach, we investigated whether other defined lectins that recognize carbohydrates present in Glycodelin-A might bind to the human zona pellucida. Sambucus nigra agglutinin (SNA, a lectin that recognizes 6'-sialyllactosomine sequences) and Wisteria floribunda agglutinin (WFA, a lectin that recognizes terminal fucosylated lacdiNAc type sequences) showed no detectable binding to the human zona pellucida although both were shown to react with Glycodelin-A in a carbohydrate-dependent manner. Conversely, E-PHA (Erythroagglutinating phytohemagglutinin, a lectin that recognizes biantennary and triantennary N-linked glycans bearing a bisecting GlcNAc linked β1-4 to the β-linked mannose) showed strong binding to the human zona pellucida. These types of glycans are known to suppress NK cell activity. This interaction was also carbohydrate specific since specific glycopeptide mixtures could block it. Human spermatozoa also bind E-PHA.

Therefore, our data demonstrated that the human oocyte (as well as the sperm) is coated with a carbohydrate sequence that is associated with the inhibition of NK cell function. Since the innate immune response in the uterus is primarily mediated by NK cells our results indicate that human gametes may be protected from this response by expressing specific bisecting type N-linked glycans on their surfaces. In addition, these results support the concept of the possible convergence in the types of carbohydrate sequences that are recognized during initial human gamete binding and immune/inflammatory cell reactions (Clark, *et al.*, 1996 a and b).

Chemical treatment and use of enzymes to remove functional carbohydrates from the cell surface

We have performed specific chemical and enzymatic treatments of the human zona pellucida to further assess the specific, functional role of carbohydrate sequences on human gamete binding. Results have demonstrated, for the first time, that there are sialic acid dependent binding

sites in the surface of the human zona pellucida. Periodate oxidation of the human zona under very mild conditions that attack only terminal sialic acid resulted in a 30% loss of human sperm binding in the HZA. Periodate oxidation under mild conditions caused a 40% decrease in binding.

On the other hand, treatment of the human zona pellucida with neuraminidase caused a substantial increase in sperm binding. These findings indicated that there are sialic acid dependent sites coexisting with binding sites that are obscured by sialic acid. Sequential zona treatment with neuraminidase and periodate oxidation reduced sperm binding by 79%. Human sperm-zona pellucida interaction was also increased by digestion of the zona by endo-β-galactosidase indicating that potential binding for spermatozoa is also obscured by lactosaminoglycan sequences. All these treatments were monitored by lectin-binding studies using specific probes for the carbohydrates being analyzed.

Interpretation of presented studies

Here we have provided compelling evidence to support the hypothesis that human sperm-zona pellucida binding involves a selectin-like interaction (Patankar, *et al.*, 1993). This evidence can be summarized as follows: (1) fucoidan, a polymer of α1-3 linked fucose with sulfate groups substituted at the 4 position on some of the fucose residues and with fucose also attached to form branch points, inhibits sperm-zona binding in a fast, potent and dose-dependent fashion; (2) fucoidan's range of inhibition (10 to 50 μg/ml) in the HZA is similar to the one that blocks selectin-like mediated adhesions (Varki, 1994); (3) fucoidan blocks human, solubilized zona pellucida-induced acrosome reaction; (4) sialyl-Lewis^x and α1-glycoprotein (that carries sialyl-Lewis^x epitopes) show a moderate inhibitory effect in the HZA; (5) glycodelin-A (which carries fucosylated lacdiNac antennae previously shown to potently block selectin-mediated adhesions) inhibits sperm-zona pellucida binding in the same range of concentration of 10 to 100 μg/ml; and (6) glycodelin-S (which is a differentially glycosylated form of a similar protein and that carries Lewis^x and Lewis^y epitopes known not to react with selectin-mediated processes) does not interfere with sperm-zona pellucida binding.

The inhibitory effects of defined glycoconjugates such as fucoidan, sialyl-Lewis^x and glycodelin-A on human sperm-zona pellucida binding certainly suggests that oligosaccharide recognition is required for initial human gamete binding. Our data strongly suggest that the egg

binding protein(s) on human spermatozoa recognize selectin ligands. If selectin ligands can bind to the sperm's egg binding protein, it is possible that the zona pellucida oligosaccharides that mediate initial human gamete binding interact with these "selectin-like" receptors to ensure recognition and attachment (Clark, *et al.*, 1995; 1996a). This system of oligosaccharide recognition need not be limited to the "selectin-like" receptors nor does it negate the co-participation of peptidic structures in initiating and maintaining binding.

The concentration dependence of fucoidan's inhibitory effect on lymphocyte homing and human sperm-zona pellucida binding is nearly identical (Yednock, *et al.*, 1989; Oehninger, *et al.*, 1990). Based on this correlation and the structural similarity of fucoidan to the natural sulfated ligands for the selectins, we initially presented our hypothesis on the involvement of a "selectin-like" interaction for human gamete binding (Patankar, *et al.*, 1993; Clark, *et al.*, 1995). Our present results also indicate to us the possibility that the carbohydrate binding specificity of the receptors mediating gamete recognition and lymphocyte/leukocyte adhesion have converged, at least to some extent (Monroy and Rosati, 1979; Clark, *et al.*, 1966 a and b). This concept is further supported by the demonstration of contraceptive (inhibition of sperm-zona pellucida binding) and immunosuppressive properties of glycodelin-A and their carbohydrate dependence (Oehninger, *et al.*, 1995; Dell, *et al.*, 1995; Seppala, *et al.*, 1997). Glycodelyn-A manifests several biological activities when tested in immunological systems. Of significance, purified glycodelin-A suppresses the lysis of K562 cells by natural killer (NK) cells within the same concentration dependence of its HZA effects, possibly due to its expression of unusual N-linked glycans (Okamoto, *et al.*, 1991; Oehninger, *et al.*, 1995; Dell, *et al.*, 1995). Also, it is possible that the oligosaccharides bearing sialylated lacNac or lacdiNac antennae may manifest immunosuppressive effects by specifically blocking adhesive and activation-related events mediated by CD22, the human B cell associated receptor (Powell and Varki, 1994; Dell, *et al.*, 1995).

Our initial approach to study the involvement of surface carbohydrates on cell-to-cell recognition was to use specific saccharide moieties in hapten-inhibition tests (Ahuja, 1985). Thereafter, we performed lectin-binding studies and utilized enzymes to remove functional carbohydrates from the zona pellucida and sperm surface. We have demonstrated that the outer surface of the human zona pellucida potently binds a lectin probe specific for bisecting type glycans in a carbohydrate dependent manner (Patankar, *et al.*, 1997). These sugar epitopes may protect the egg from cytotoxic responses mediated by NK cells (Clark, *et al.*, 1996 a and b). Additionally, chemical and enzymatic treatments of the human zona pellucida have provided results which are

the first unequivocally direct evidence that human gamete binding is at the very least partially dependent upon glycoconjugate dependent interactions (Ozgur, *et al.*, 1998).

In summary, our studies indicate that: (1) the binding protein(s) on human spermatozoa recognize selectin ligands or molecules alike on the zona pellucida to ensure recognition and attachment. This system of oligosaccharide recognition need not be limited to the selectin-like receptors nor does it negate the co-participation of peptidic structures in initiating and maintaining binding. (2) The human zona pellucida expresses glycans linked to NK cell inhibition. (3) Enzymatic and chemical treatment of the human zona pellucida have provided direct evidence for the presence of specific carbohydrate moieties that are involved in primary binding. In current studies we plan to define more specifically those carbohydrate and protein sequences involved in human sperm-zona pellucida binding.

Acknowledgement

The studies presented herein were the result of collaborative work carried out with Drs. Gary F. Clark and Manish Patankar (from the Department of Biochemistry at Eastern Virginia Medical School, Norfolk, Virginia, USA), Dr. Markku Seppala (from the Department of Obstetrics and Gynecology, University Central Hospital, Helsinki, Finland) and Drs. Anne Dell and Howard Morris (from the Department of Biochemistry, Imperial College, London, UK).

References

1. Ahuja, K.K. (1985) Carbohydrate determinants involved in mammalian fertilization. *Am J Anat*, **179**, 207-223.
2. Braude, P., Bolton, V., Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature*, **332**, 459-461.
3. Bleil, J.D., Wassarman, P.M. (1988) Galactose at the nonreducing terminus of O-linked oligosaccharides of the mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. *Proc Natl Acad Sci USA,* **85**, 6778-6782.
4. Bleil, J.D., Wassarman, P.M. (1983) Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev Biol,* **95**, 317-324.
5. Bleil, J.D., Wassarman, P.M. (1980) Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zona pellucida possessing receptor activity for sperm. *Cell*, **20**, 873-882.
6. Burkman, L.J., Coddington, C.C., *et al.,* (1988) The hemizona assay: development of a diagnostic test for the binding of spermatozoa to the human zona pellucida to predict fertilization potential. *Fertil Steril,* **49**, 688-697.
7. Chapman, N.R., Barratt, C.L.R. (1996) The role of carbohydrate in sperm-ZP3 adhesion. *Mol Hum Reprod*, **2**, 767-774.
8. Clark, G.F., Oehninger S., *et al.,* (1996a) A role for glyconjugates in human development: the human feto-embryonic defense system hypothesis. *Hum Reprod*, **11**, 467-473.
9. Clark, G.F., Oehninger, S., Seppala, M. (1996b) Role for glycoconjugates in cellular communication in the human reproductive system. *Mol Hum Reprod*, **2**, 513-517.
10. Clark, G.F., Oehninger, S., *et al.,* (1995) New concepts in human sperm-zona pellucida interaction. *Hum Reprod*, **10**, 31-37.
11. Dell, A., Morris, H.R., *et al.,* (1995) Structural analysis of the oligosaccharides derived from glycodelin, a human glycoprotein with potent immunosuppressive and contraceptive activities. *J Biol Chem*, **270**, 24116-24126.
12. Franken, D.R., Morales, P.J., Habenicht, U.F. (1996) Inhibition of G protein in human sperm and its influence on acrosome reaction and zona pellucida binding. *Fertil Steril*, **66**, 1009-10011.

13. Hinsch, E., Oehninger, S., Hinsch, K.D. (1998) ZP3-6 peptide and acrosome reaction. In Vereecken, A., De Jonge, C., Franken, D., Oehninger, S., Ombelet, W., (eds.) Semen evaluation, testing and selection: a new look in the 2000s, pp 25-29. Pre-Congress training course, Andrology in the Nineties: Modern ART in the 2000s, Genk, Belgium.
14. Hinsch, K.D., Hinsch, E., *et al.,* (1994a) Identification of mouse ZP3 protein in mammalian oocytes with antisera against synthetic ZP3 peptides. *Biol Reprod,* **51**, 193-204.
15. Hinsch, K.D., Hinsch, E., *et al.,* (1994b) Anti-ZP3 antibodies binding to the human zona pellucida: effect of Oocyte-storage conditions. *Am J Reprod Immunol,* **32**,146-151.
16. Huang, T.T.R., Ohzu, E., Yanagimachi, R. (1982) Evidence suggesting that L-fructose is part of a recognition signal for sperm-zona pellucida attachment in mammals. *Gamete Res,* **5**, 355-361.
17. Julkunen, M., Seppala, M., Janne, O. (1988) Complete amino acid sequence of human placental protein 14: A progesterone-related uterine protein homologous to β-lactoglobulin. *Proc Natl Acad Sci USA*, **85**, 8845-8849.
18. Koistinen, H., Koistinen, R., Dell, *et al.,*(1996) Glycodelin from seminal plasma is a differentially glycosylated form of contraceptive glycodelin-A. *Mol Hum Reprod,* **2**, 759-765.
19. Kopf, G.S. (1990) Zona pellucida-mediated signal transduction in mammalian spermatozoa. *J Reprod Fertil*, **42**, 33-49.
20. Liu, D.Y., Baker, H.W.G. (1992) Tests of human sperm function and fertilization in vitro. *Fertil Steril*, **58**, 465-483.
21. Lopez, L.C., Bayna, E.M., et *al.,*(1985) Receptor function of mouse sperm surface galactosyltransferase during fertilization. *J Cell Biol*, **101**, 1501-1510.
22. Macek, M.B., Shur, B.D. (1988) Protein-carbohydrate complementarity in mammalian gamete recognition. *Gamete Res*, **20**, 93-109.
23. Mahony, M.C., Oehninger, S., Clark GF, *et al.,*(1991) Fucoidan inhibits the zona pellucida-induced acrosome reaction in human spermatozoa. *Contraception*, **44**, 657-665.
24. Monroy, A., Rosati, F. (1979) The evolution of the cell: cell recognition system. *Nature*, **278**, 165-166.
25. Morris, H.R., Dell, A., *et al.,*(1996) Gender-specific glycosylation of human glycodelin affects its contraceptive activity. *J Biol Chem*, **271**, 32159-32167.

26. Oehninger, S. (2001) Molecular basis of human sperm-zona pellucida interaction. *Cell Tissues Organs*, **168**, 58-64

27. Oehninger, S., Patankar, M., *et al.*, (1998) Involvement of selectin-like carbohydrate binding specificity in human gamete interaction. *Andrologia*, **30**, 269-274.

28. Oehninger, S., Franken, D., *et al.*, (1997) Approaching the next millennium: how should we manage andrology diagnosis in the intracytoplasmic sperm injection era? *Fertil Steril*, **67**, 434-436.

29. Oehninger S., Hinsch, E., *et al.*, (1996) Use of a specific ZP3 antiserum as a clinical marker for human zona pellucida integrity and function. *Fertil Steril*, **65**, 139-145.

30. Oehninger, S., Coddington, *et al.*, (1995) Factors affecting fertilization: endometrial placental protein 14 reduces the capacity of human spermatozoa to bind to the human zona pellucida. *Fertil Steril*, **63**, 337-383.

31. Oehninger, S., Clark, G., *et al.*, (1992) Effect of fucoidan on human sperm-zona pellucida interactions. *J Androl*, **13**, 519-525.

32. Oehninger, S., Acosta, A., Veeck, *et al.*, (1991a) Recurrent failure of in vitro fertilization: role of the hemizona assay in the sequential diagnosis of specific sperm-oocyte defects. *Am J Obstet Gyneol*, **164**, 1210-1215.

33. Oehninger, S., Clark, G.F., *et al.*, (1991b) Nature of the inhibitory effect of complex saccharide moieties on the tight binding of human spermatozoa to the human zona pellucida. *Fertil Steril*, **55**, 165-169.

34. Oehninger, S., Acosta, A.A., *et al.*, (1990) Antagonistic and agonistic properties of saccharide moieties in the hemizona assay. *Fertil Steril*, **53**, 143-149.

35. Oehninger, S., Acosta, A.A., *et al.*, (1988) Failure of fertilization in vitro fertilization: the "occult" male factor. *J In Vitro Fertil Embryo Trans*, **5**, 181.

36. Okamoto, N.A., Uchida, A., *et al.*, (1991) Suppression by human placental protein 14 of natural killer cell activity. *Am J Reprod Immunol*, **26**, 137-142.

37. Ozgur, K., Oehninger, S., *et al.*, (1998) Direct evidence for the involvement of carbohydrate sequences in human sperm-zona pellucida binding. *Mol Hum Reprod*, **4**, 316-324.

38. Patankar, M.S., Oehninger, S., *et al.*, (1997) Expression of glycans linked to natural killer cell inhibition on the human zona pellucida. *Mol Hum Reprod*, **6**, 501-505.

39. Patankar, M.S., Oehninger, S., *et al.*, (1993) A revised structure for fucoidan may explain some of its biological activities. *J Biol Chem*, **268**, 21770-21776.

40. Powell, L.D., Varki. (1994) The oligosaccharide binding specificity of CD22b, a sialic acid-specific lectin of B cells. *J. Biol Chem*, **269**, 0628-10636.

41. Riittinen, L., Narvanen, O., *et al.,* (1991) Monoclonal antibodies against endometrial protein PP14 and their use for purification and radioimmunoassay of PP14. *J Immunol Meth*, **136**, 85-90.

42. Saling, P.M. (1991) How the egg regulates sperm function during gamete interaction: facts and fantasies. *Biol Reprod*, **11**, 339-388.

43. Seppala, M., Koistinen, H., *et al.,* (1997) Glycodelins as regulators of early events of reproduction. *Clin Endocrinol*, **46**, 381-386.

44. Sinowatz, F., Kolle, S., *et al.,* (2001) Biosynthesis and expression of zona pellucida glycoproteins in mammals. *Cell Tissues Organs*, **168**, 24-35

45. Varki, A. (1994) Selectin ligands. *Proc Natl Acad Sci USA*, **91**,7390-7397.

46. Wassarman, P.M. (1990) Profile of a mammalian sperm receptor. *Development*, **108**, 1-17.

47. Yanagimachi, R. (1994) Mammalian fertilization. In Knobil, N. and Neill, J.D. (eds.) *The Physiology of Reproduction*, pp 189-317. (New York: Raven Press, Ltd.)

48. Yednock, T.A., Rosen, S.D. (1989) Lymphocyte homing. *Adv Immunol*, **44**, 313-378.

Figure 1

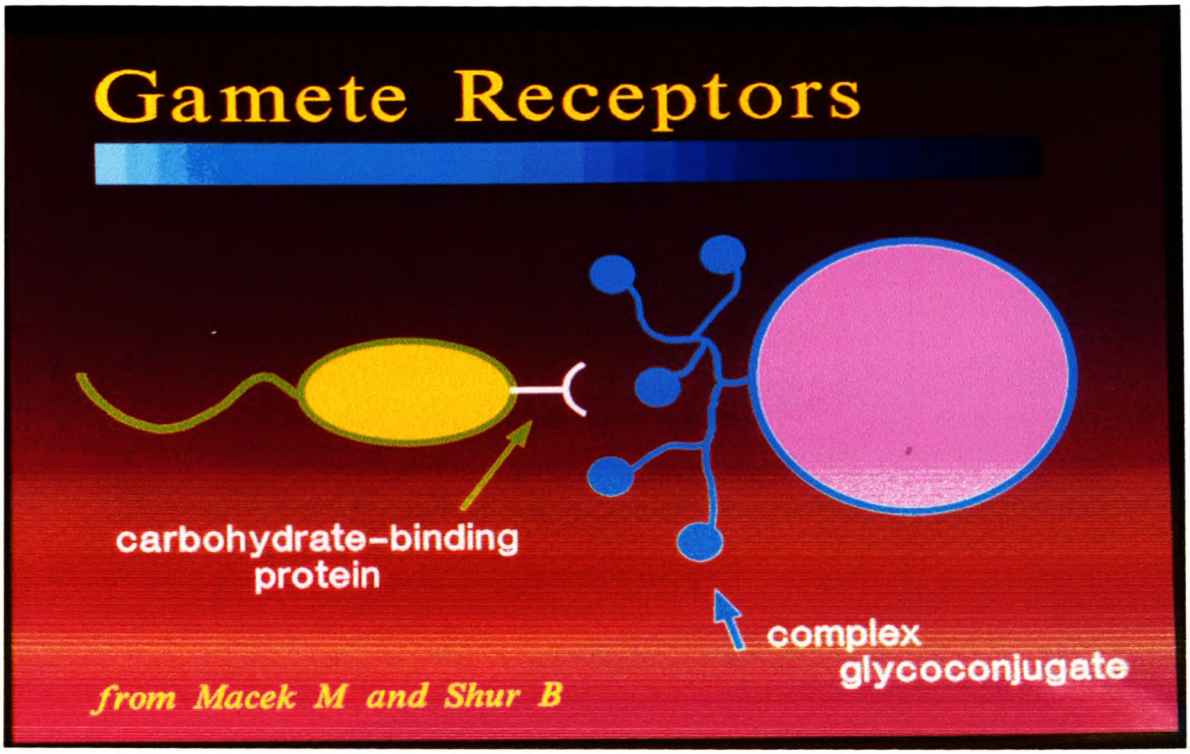
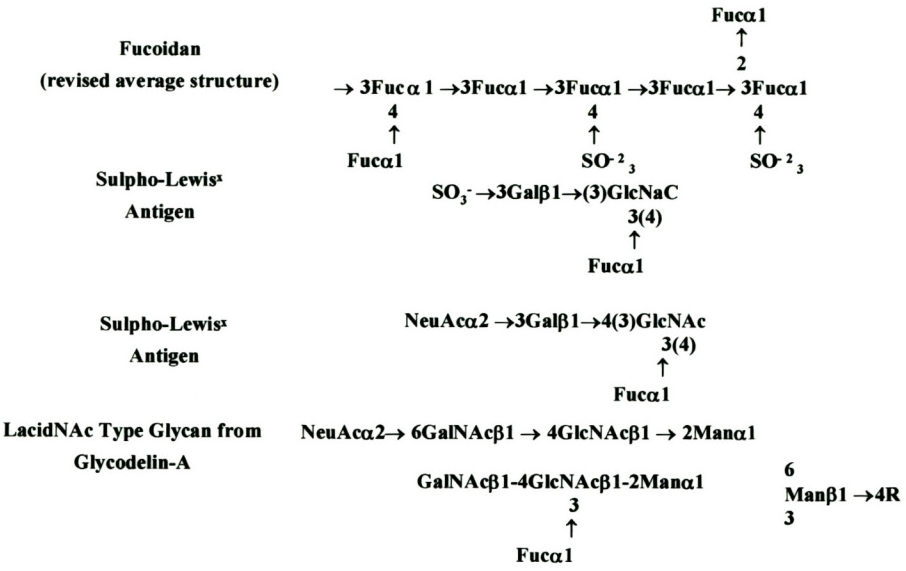


Figure 2



Legends to figures

Figure 1. Schematic diagram of sperm-zona pellucida interaction at a molecular level. A putative sperm carbohydrate binding protein binds with high affinity and specificity to a complex glycoconjugate (probably a carbohydrate motif of ZP3) present in the extracellular zona pellucida matrix (from Macek and Shur, 1988).

Figure 2. Carbohydrate sequences present in fucoidan, Sulpho-Lewis^x antigen, Syalil-Lewis^x antigen and LacdiNAc-Type Glycan from Glycodelin A. R is fucosylated di-N-acetylchitobiose (GlcNAcβ1-4 [Fucα1-6] GlNAc) (from Clark *et al.*, 1996a).

Chapter 3.

Use of the newly developed bioassays to address physiological questions

3a. *Specific aim 3*: To examine the capacitation-dependency of the spontaneous and agonist-induced acrosome reaction of human spermatozoa.

Spontaneous and agonist-induced acrosome reaction in human sperm: roles of capacitation time and conditions, protein tyrosine phosphorylation and plasma membrane translocation of phosphatidylserine

Alessandro Schuffner[1], Hakan E. Duran, Zhi-Yong Lin, Mahmood Morshedi, Daniel R. Franken[2] and Sergio Oehninger

The Jones Institute for Reproductive Medicine, Department of Obstetrics and Gynecology, Eastern Virginia Medical School

Present address: Androlab – Clinica de Reprodução Humana e Andrologia, Curitiba, Brazil

[2]Reproductive Biology Unit, Department of Obstetrics and Gynecology, University of Stellenbosch, Cape Town South Africa

Abstract

The objective of these studies was to assess the relationships between capacitation time and conditions, plasma membrane translocation of phosphatidylserine (PS) and protein tyrosine phosphorylation, and the spontaneous and agonist-induced acrosome reaction of human spermatozoa. Purified populations of highly motile sperm were incubated in human tubal fluid (HTF) supplemented with varying doses of human serum albumin (HSA) and examined at 0, 3, 6, 12 and 24 hours. Acrosome reaction of live sperm was detected by fluorescein isothiocyanate-labelled *Pisum Sativum Agglutinin*/Hoechst; annexin V binding was used to monitor PS translocation; tyrosine phosphorylation was examined by immunoblotting. The percentage of live, acrosome reacted sperm and the percentage of live sperm depicting PS translocation increased significantly over time, both under spontaneous- and calcium ionophore- treated conditions. Under spontaneous conditions there was a significant positive correlation between the percentage of live, acrosome reacted sperm and the percentage of live sperm with PS translocation. Tyrosine phosphorylation increased over time and with higher doses of HSA, and was associated with increased PS externalization. Taken together, these results suggest that the spontaneous acrosome reaction of human spermatozoa incubated under conditions that support capacitation is time dependent, is positively associated with changes in plasma membrane lipid distribution resulting in externalization of PS, and is accompanied by tyrosine phosphorylation. The responses to the agonistic effect of a calcium ionophore appear to result in different membrane dynamics.

Key words: acrosome reaction / capacitation / phosphatidylserine translocation / tyrosine phosphorylation

Introduction

Human spermatozoa must undergo the process of capacitation in order to respond to the physiologic stimuli that trigger the acrosome reaction, a prerequisite for successful fertilization. Such agonists include follicular fluid, the steroid progesterone and the zona pellucida (Tesarik, 1985; Cross *et al.*, 1988; Thomas and Meizel, 1989; Kopf, 1990; Blackmore *et al.*, 1990, 1991; Lee *et al.*, 1992; Yanagimachi, 1994; Franken *et al.*, 1996; Florman *et al.*, 1998).

There is also a variety of pharmacological stimuli reported to trigger the acrosome reaction, either by driving extracellular calcium into the sperm cells (such as calcium ionophores) or by acting on intracellular second messengers involved in cascade reactions that couple the calcium entry with the activation of the effectors of the exocytotic response, such as pentoxifylline and others (Zaneveld *et al.*, 1991; Cummins *et al.*, 1991; Tesarik *et al.*, 1993; Nassar *et al.*, 1998).

Capacitation is a priming process that programmes the spermatozoa to arrive at the site of fertilization in a timely fashion and to rapidly undergo the acrosome reaction upon making contact with specific components of the zona pellucida (de Lamirande *et al.*, 1997; Aitken, 1997). During capacitation, sperm undergo a variety of metabolic, functional and membrane structure modifications, eventually preparing the cell for membrane fusion and acrosomal exocytosis. *In vitro* studies suggest that some of these changes include alterations in sperm energy metabolism, an increase in sperm membrane calcium permeability leading to a rise in intracellular calcium levels, an efflux of cholesterol from the plasma membrane to a protein acceptor decreasing the cholesterol-phospholipid ratio, and a rise in the intracellular pH (Langlais and Roberts, 1985; Yanagimachi, 1994; Visconti *et al.*, 1998; Visconti and Kopf, 1998; Cross, 1998).

Capacitation is also associated with a global increase in tyrosine phosphorylation, probably as a consequence of reactive oxygen species-induced changes in the redox status of the cells (Aitken *et al.*, 1995, Aitken, 1997, 2000) and an increase in cAMP generation (Visconti *et al.*, 1995). Recent studies have established a correlation between capacitation and phosphorylation on tyrosine residues of various proteins in human spermatozoa (Aitken *et al.*, 1995; Carrera *et al.*, 1996; Leclerc *et al.*, 1996; Luconi *et al.*, 1996; Emmiliozi and Fenichel, 1997; Brewis *et al.*, 1998; Tomes *et al.*, 1998, Osheroff *et al.*, 1999).

Addition of cholesterol and an actively maintained asymmetric transmembrane phospholipid distribution characterize the membrane of mature spermatozoa during epididymal storage. In spermatozoa, as in somatic cells, the two leaflets of the plasma membrane bilayer differ in composition (Muller et al., 1994; Nolan et al., 1995; Gadella et al., 1999). The aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine are concentrated in the inner leaflet whereas the choline phospholipids sphingomyelin and phoshatidylcholine are preferentially distributed in the outer leaflet. In somatic cells, this asymmetric distribution is maintained by the action of a variety of enzymes, including flippases, floppases and scramblases (Bevers et al., 1998). An amino phospholipid translocase (also known as flippase) transfers PS from the outer to the inner lipid leaflet. Scramblases, on the other hand, transfer the various lipid species in both directions (Gadella and Harrison, 2000).

During capacitation, efflux of cholesterol, phospholipid movement and an enhanced calcium permeability, lead to an increase of intracellular calcium and a depletion of ATP concentration. These effects, in turn, cause a decrease in translocase activity preparing the plasma membrane for the fusion events that characterize the acrosome reaction (Nolan et al., 1995). The cholesterol-poor, lipid symmetric plasma membrane has a destabilized inner leaflet that facilitates membrane fusion upon receiving appropriate stimuli. As a result, conditions become therefore favorable for sperm to undergo the acrosomal exocytosis (Nolan and Hammerstedt, 1997).

In the present studies we aimed to further characterize the process of acrosomal exocytosis in human spermatozoa. For this purpose, we obtained purified sperm populations of high motility from fertile men (donors) and examined the spontaneous and agonist-induced acrosome reaction and their relationships with various capacitation time and conditions, plasma membrane translocation of PS and protein tyrosine phosphorylation.

Material and Methods

Patients inclusion criteria and study design

These studies were performed under approval of the Institutional Review Board at Eastern Virginia Medical School. Ejaculates from fertile men participating in our artificial insemination donor program were studied. Semen specimens were collected after a 2-4 day sexual abstinence period. All men were healthy, had a normal physical examination and were non-smokers. All samples had less than 1×10^6 leukocytes/mL (peroxidase staining). In order to provide sufficient

number of cells for all tests, ejaculates with a concentration of motile spermatozoa ≥ 30 x 10^6/mL in the original sample were included in the study.

Sperm preparation, motion analysis and morphology assessment

After liquefaction for 30 minutes at 37°C, a basic semen analysis was performed. Sperm concentration and motion parameters (i.e., the percentage progressive motility, the percentage rapid cells defined as >50 μm/s, the average path velocity or VAP, straight [VSL] and curvilinear [VCL] velocity, and linearity) were objectively evaluated using the HTM-IVOS semen analyzer (Hamilton Thorne Research, Beverly, MA, USA, version GS 771) with fixed parameter settings (Oehninger *et al.*, 1990; Oehninger, 1995, 2000). Sperm concentration and motility readings were manually monitored and corrections were made as appropriate. Sperm concentration and motility were assessed according to the World Health Organization criteria (WHO, 1999) and sperm morphology was examined according to strict criteria after Diff-Quik staining (Kruger *et al.*, 1986). The semen analysis was immediately followed by separation of the motile sperm fraction by density gradient separation.

The sperm fractions with high motility were isolated using discontinuous Percoll (Sigma Chemical Co, St. Louis, MO, USA) gradient separation (90% and 40% layers) and Human Tubal Fluid (HTF, Irvine Scientific, Santa Anna, CA) as diluent. Up to 2 mL of semen was carefully placed on Percoll layers, centrifuged at 380xg for 20 min and the pellet of the 90% layer was mixed with HTF and then centrifuged at 380xg for 10 min. The supernatant was discarded, the pellet was resuspended in 1 mL of capacitating medium and the sperm concentration was adjusted to 2-10 x 10^6/mL depending on the experiment (see below).

Assessment of acrosome reaction of live spermatozoa

The proportion of acrosome-reacted spermatozoa from initial suspensions of 2 x 10^6/mL motile spermatozoa was determined with the fluorescent probe fluorescein isothiocyanate-labeled *Pisum Sativum Agglutinin* (FITC-PSA) (Sigma) as published earlier (Cross *et al.*, 1986; Cummins *et al.*, 1991; Mahony et al, 1991; Tesarik *et al.*, 1993; Oehninger *et al.*, 1993; Nassar *et al.*, 1998). The supravital stain Hoechst 33258 (Sigma) was used to simultaneously assess sperm viability (Cross *et al.*, 1986). Under capacitating conditions, the percentage of spermatozoa undergoing spontaneous acrosome reaction was evaluated. To assess the agonist-induced acrosome reaction, the calcium ionophore A23187 (Sigma) was added to the sperm suspension for 60 min at a final concentration of 5 μM from a stock solution of 10 mM ionophore in DMSO.

At least 200 sperm per sample were evaluated in a blind fashion at a magnification of x 1,000 using an epifluorescent microscope equipped with phase-contrast optics (Eclipse 600, Nikon, Melville, NY, USA), and using a digital camera with a high pressure mercury lamp power supply (SPOT RT, software version 3.2, Diagnostic Instruments, Augusta, GA). The following staining patterns were evaluated as live, acrosome reacted spermatozoa: (i) distinct staining in the equatorial region occurring as an equatorial bar; (ii) no staining observed over the entire sperm surface; and (iii) patchy staining on the acrosomal region (Cross *et al.*, 1986, 1988; Mahony et al, 1991; Oehninger *et al.*, 1993; Franken *et al.*, 1996; 2000).

Detection of Membrane PS Translocation

We used annexin V Cy3.18 (Annexin V-Cy3; Sigma) for detection of PS externalization with simultaneous assessment of cell viability as per manufacturer's instructions. This technique was described previously (Duru *et al.*, 2000, 2001; Schuffner et al, 2001). Briefly, in order to differentiate between live cells with and without PS translocation and necrotic cells, we used 6-carboxyfluorescein diacetate (6-CFDA) in combination with Ann V-Cy3. By microscopy, Cy3 fluoresces more brightly than the FITC conjugate. The non-fluorescent 6-CFDA enters the cell and is converted to the green fluorescent compound 6-carboxylfluorescein (6-CF). This conversion is a function of esterases present only in living cells. Thus, no fluorescence is observed in necrotic cells.

Three patterns of fluorescence are typically observed: 1. Live, normal cells that stain only with 6-CF (green, annexin V^-, live cells); 2. Live cells with translocation of membrane PS that stain with both 6-CF (green) and Ann V-Cy3 (red) (annexin V^+, live cells); and 3.Dead cells that stain only with Ann V-Cy3 (red, necrotic).

A fifty µL aliquot of sperm suspension (10 x 10^6/mL) was placed on a poly-L-lysine-coated slide and stained with the 6-CFDA/Ann V-Cy3 solution. After incubation in the dark for 10 min, the slide was covered with a 24x50 mm cover slip and immediately read blindly by two investigators at a magnification of 1,000x by epifluorescence microscopy as mentioned above (excitation filter: 450-490 nm, barrier filter: 515 nm). At least one hundred spermatozoa were counted per slide. The intra-observer and inter-observer variability for this technique in our laboratory are <7% and <5%, respectively (Duru *et al.*, 2000, 2001).

Electrophoresis and protein immunobloting

Sperm proteins under different incubation conditions were prepared according to Osheroff *et al.*, (1999). Briefly, after incubation, spermatozoa from the sperm fractions with high motility were centrifuged at 20,000 g for 2 min at room temperature and then washed with phosphate-buffered saline (PBS) at room temperature. The sperm pellet was then resuspended in sample buffer (Laemmli, 1970) without mercaptoethanol and boiled for 5 min. After centrifugation at 20,000 x g for 2 min, the supernatant was removed, 2-mercaptoethanol was added to a final concentration of 5%, the sample boiled for another 5 min, and then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was performed on 10% gels according to Laemmli (1970). Electrophoresis was carried out at 200 constant voltage and was continued until the tracking dye run out of the gel after loading each lane of the gel with a sperm lysate (originally containing 1 x 10^6 spermatozoa).

After the samples were resolved by gels the sperm protein on the gel was electrically transferred to nitrocellulose membrane by using the Trans-Blot SD Semi-Dry Transfer Cell column (Bio-Rad, Mercules, CA). Membranes were blocked with blocking buffer (5% non-fat dry milk, 0.1% Tween-20 in PBS, pH 7.4) overnight at 4 °C with shaking. The primary antibody (a monoclonal anti-phosphotyrosine antibody, P3300 catalog number, Sigma) was diluted in the blocking buffer and incubated with the membranes for 1 hour at room temperature. The membrane was then washed with 0.4% Tween-20 in PBS for three times, each time 15 minutes. The membranes were then incubated with a secondary antibody (anti-mouse IgG) conjugated with Horse-Radish Peroxidase (Pierce, Rockford, IL) diluted in blocking buffer (1:3,000) for 1 hour at room temperature. After three time 15 min washes with PBS/0.3% Tween-20 and three time 5 min washes with PBS/0.1% Tween-20, the blot was developed using enhanced chemiluminescence detection with ECL kit (Amersham, NJ, USA) according to the manufacturer's instructions.

Experimental design

Experiment I. In the first experiment the purified fractions of spermatozoa high motility from 5 different donors were incubated under capacitating conditions in HTF supplemented with 0.3% HSA (Irvine) for up to 24 hours at 37°C under 5% CO_2 in water-saturated air in order to examine the time-dependency of the spontaneous and agonist-induced acrosome reaction and their relationship with PS translocation.

At 3, 6 and 24 hours, an aliquot of sperm suspension was removed and divided into two parts: (i) the first one was kept under the same capacitating conditions for 60 min and then analyzed for CASA, spontaneous acrosome reaction and annexin V binding assay results; (ii) the other one was treated with the calcium ionophore A23187 (5 μM) for 60 min under similar capacitating conditions and then subjected to CASA, (induced-) acrosome reaction and annexin V binding assay testing.

Experiment II. In the second experiment, we examined the time dependency of protein tyrosine phosphorylation under different capacitating conditions. In experiment IIa, the purified motile fractions from 3 different donors were incubated for up to 24 hours at 37°C under 5% CO_2 in water-saturated air under 2 different capacitating conditions: (i) HTF supplemented with 0.3% HSA; and (ii) HTF supplemented with 3% HSA, in order to further assess the impact of two different doses of protein supplementation on tyrosine phosphorylation. At 3, 6, 9,12 and 24 hours an aliquot of sperm suspension was removed, washed and lysed for preparation of SDS-PAGE and protein immunoblots.

In experiment IIb, we simultaneously assessed the time dependency of PS externalization and tyrosine phosphorylation using the same capacitating conditions described in experiment I. The purified motile fractions from 3 different donors were incubated in HTF supplemented with 0.3% HSA for up to 24 hours at 37°C under 5% CO_2 in water-saturated air. At 3, 6, 12 and 24 hours two aliquots of sperm suspension were removed; one was used for assessment of PS externalization by annexin V binding, and the other for protein tyrosine phosphorylation by immunoblotting.

Statistical analysis

The data were analyzed by using a linear mixed effects model for repeated measures. The analyses included examination of relationships between treatment groups (spontaneous conditions and after calcium ionophore), proportion of acrosome reacted live spermatozoa, proportion of sperm depicting PS translocation (annexin V positive, live spermatozoa or green-red cells, see above), and incubation time. Analysis of variance and Pearson's correlation coefficient were used as appropriate. Data were analyzed independently by a bio-statistician (see acknowledgement). All results are presented as mean±standard deviation. P values < 0.05 were considered significant.

Results

Experiment I

Table 1 shows the changes in motion parameters and viability (mean±standard deviation) throughout the 24-hour incubation period. Motility, the percentage of rapid cells, VAP, VSL, VCL and linearity declined significantly over time ($p< 0.001$ for all). Values for calcium ionophore-treated conditions were significantly lower than for spontaneous conditions ($p< 0.002$ for all motion parameters), but the rate of decline over time was statistically the same ($p> 0.1$ for all). Viability declined significantly over time for both spontaneous and calcium ionophore conditions ($p< 0.01$) as can be observed by the increase in the percentage of dead sperm assessed by Hoechst staining.

Acrosome reaction results are shown in Figure 1. There was a time-dependent, significant increase in the percentage of live, acrosome reacted sperm throughout the 24 hour period ($p< 0.002$) for both spontaneous and calcium ionophore-treated conditions. Calcium ionophore responses were significantly higher that the spontaneous rates at any time point ($p< 0.001$).

Results of time-dependent changes of the annexin V binding assay for spontaneous (panel A) and calcium ionophore-treated conditions (panel B) are shown in Figure 2. The figure presents results of live sperm depicting PS translocation (PS translocation), live cells without PS translocation (live, normal) and dead (necrotic) cells. There was a significant increase of live spermatozoa showing PS translocation over time for both spontaneous and calcium ionophore-treated conditions ($p< 0.001$). However, there was no significant difference between spontaneous and calcium ionophore-treated results at any point ($p> 0.1$).

Under spontaneous conditions, there was a significant positive correlation between the percentage of live, acrosome reacted sperm and the percentage of live sperm with PS translocation ($r= 0.67$, $p< 0.005$). However, such a correlation was not observed following calcium ionophore stimulation ($r= 0.45$, $p= 0>0.5$).

Experiment II

Experiment IIa showed that there was a time- and HSA-dependency of protein tyrosine phosphorylation profiles of highly motile spermatozoa. A representative immunoblot (panel A: 0.3% HSA; panel B: 3% HSA) is shown in figure 3. As can be observed, a subset of phosphorylated proteins of M_r (molecular mass ratio) ranging from 40,000 to 101,000 was observed

at time 0 and throughout 24 hours of incubation. A time-dependent increase of phosphorylation was observed for most proteins, but particularly for the subset of proteins of M_r 95-97,000, 80-83,000 and 50-55,000. In addition, 3% HSA resulted in increased protein tyrosine phosphorylation when compared to 0.3% HSA.

Experiment IIb showed similar temporal changes observed for PS externalization (over time increase in the percentage of live cells with PS translocation, $p<0.05$) that was associated with increased protein tyrosine phosphorylation. Figure 4, panel A, depicts the results of annexin V binding assay (average results of the three studied donors), whereas panel B presents a representative immunoblot from one of the donors.

Discussion

We hypothesized that the rate of spontaneous acrosome reaction of purified populations of highly motile spermatozoa from normal, fertile men is (i) time- and capacitation conditions-dependent, and (ii) associated with changes in membrane lipid symmetry. Our first experiment (experiment I) validated such hypothesis in that the non-stimulated acrosome reaction increased with time of incubation under capacitating conditions and acrosomal exocytosis was significantly and positively correlated with the degree of plasma membrane PS externalization. Furthermore, results of the second experiments (experiments IIa and IIb) confirmed that identical capacitation conditions resulted in a time-dependent enhancement of PS externalization and tyrosine phosphorylation of a subset of proteins, and furthermore, that such effect was HSA-dependent.

Capacitation conditions promote efflux of cholesterol from the sperm plasma membrane and destabilization of the phospholipid bilayer (Nolan and Hammerstedt, 1997; Gadella and Harrison, 2000). Here, capacitating conditions that lead to cholesterol efflux to a protein acceptor (i.e., HSA) were associated with increased annexin V binding indicating that live spermatozoa had increased membrane PS externalization. We speculate that such phenomena resulted in facilitation of membrane fusogenic effects and increased the rate of spontaneous acrosomal exocytosis.

This loss of membrane phospholipid asymmetry may be due to changes in the activities of intracellular sperm flippases and/or scramblases (Gadella and Harrison, 2000). These authors have demonstrated that bicarbonate levels, a major determinant of capacitation (Boatman and Robbins, 1991; Visconti *et al.*, 1995) alters greatly the steady-state distribution of aminophospholipids in

boar sperm as examined by labeled phospholipid probes and flow cytometric analysis (Gadella and Harrison, 2000). Such methodology obviates a possible "overestimation" of outer leaflet phospholipid distribution as a consequence of the acrosome reaction resulting in exposition of previously sequestered membranes.

We and others have also demonstrated that under other "stress-induced" conditions (i.e., cryopreservation) ram and human spermatozoa also show evidence of increased PS externalization providing indirect evidence for modification of the activity of such regulatory enzymes (Muller *et al.*, 1999; Glander and Schaller, 1999; James *et al.*, 1999 Duru *et al.*, 2001; Schuffner et al, 2001). The present results also extended a recent communication from our laboratory showing that prolonged incubation of human spermatozoa from fertile and infertile men at body temperature was associated with significant motility loss and membrane changes as revealed by PS translocation (Schuffner et al, 2002). Those observations as well as the results of the present studies show that although the temporal pattern of PS translocation is similar among fertile men there is inter-individual variability.

Our results also extended previous contributions that indicated that capacitation of human spermatozoa is associated with phosphorylation of multiple proteins at tyrosine residues (Aitken *et al.*, 1995; Carrera *et al.*, 1996; Leclerc *et al.*, 1996; Emiliozzi and Fenichel, 1997; Tomes *et al.*, 1998; Osheroff *et al.*, 1999). Further, we have corroborated that the presence of a higher concentration of serum albumin in the culture medium results in enhanced tyrosine phosphorylation of such proteins. Osheroff *et al.*, (1999) previously demonstrated that maximum phosphorylation of human sperm under capacitating conditions was also achieved with 3 mg/ml of bovine serum albumin and that this requirement for albumin correlated with the ability of sperm to undergo the acrosome reaction. Moreover, these authors elegantly showed that capacitation-dependent cholesterol release is associated with the activation of protein kinase A-tyrosine kinase second messenger systems resulting in protein tyrosine phosphorylation.

The ability of sperm to undergo acrosomal exocytosis in response to the agonistic effect of the calcium ionophore A23187 was also time-dependent under the capacitating conditions studied herein. However, the lack of a significant correlation with PS translocation suggests that the plasma membrane changes of the spontaneous and calcium ionophore-induced acrosome reaction may be different. These results need to be further validated by larger studies and of interest, should be compared to the effects of the physiological agonists of the acrosome reaction. Studies using either

solubilized human zona pellucida and/or a biologically active recombinant human ZP3 may provide further insight into such mechanisms (Van Duin *et al.*, 1994; Whitmarsh *et al.*, 1996; Franken *et al.*, 2000; Dong *et al.*, 2001).

Acknowledgement. We acknowledge Paul Kolm, PhD, Bio-statistician at Portsmouth Naval Hospital, Portsmouth, Virginia, USA, for providing independent statistical analysis.

References

1. Aitken RJ (2000) Possible redox regulation of sperm motility activation. *J Androl*, **21**, 491-496.
2. Aitken RJ (1997) Molecular mechanisms regulating human sperm function. *Mol Hum Reprod*, **3**, 169-173.
3. Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M (1995) Redox regulation of tyrosine phosphorylation in human spermatozoa is involved in the control of human sperm function. *J Cell Sci*, **108**, 2017-2025.
4. Bevers EM, Comfurius P, Dekkers DW, Harmsma M, Zwaal RF (1998) Transmembrane phospholipid distribution in blood cells: control mechanisms and pathophysiological significance. *Biol Chem*, **379**, 973-986.
5. Blackmore PF, Neulen J, Lattanzio F, Beebe SJ (1991) Cell surface receptors for progesterone mediate calcium uptake in human sperm. *J Biol Chem*, **266**, 18655-18659.
6. Blackmore PF, Beebe SJ, Danforth DR, Alexander N (1990) Progesterone and 17a-Hydroxyprogesterone. *J Biol Chem*, **265**, 1376-1380.
7. Boatman DE, Robbins RS (1991) Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biol Reprod*, **44**, 806-813.
8. Brewis IA, Clayton R, Browes CE, Martin M, Barratt CL, Hornby DP, Moore HD (1998) Tyrosine phosphorylation of a 95 kDa protein and induction of the acrosome reaction in human spermatozoa by recombinant human zona pellucida glycoprotein 3. *Mol Hum Reprod*, **4**, 1136-1144.
9. Carrera A, Moos J, Ning XP, Gerton GL, Tesarik J, Kopf GS, Moss SB (1996) Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: Identification of A kinase anchor proteins as major substrates for tyrosine phosphorylation. *Dev Biol*, **180**, 284-296.
10. Cross NL (1998) Roles of cholesterol in sperm capacitation. *Bio Reprod*, **59**, 7-11.
11. Cross NL, Morales P, Overstreet JW, Hanson FW (1988) Induction of the acrosome reaction by the human zona pellucida. *Biol Reprod*, **38**, 235-244.
12. Cross NL, Morales P, Overstreet JW, Hanson FW (1986) Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res*, **15**, 213-226.
13. Cummins JM, Pember SM, Jequier AM, Yovich JL, Hartmann PE (1991) A test of the human sperm acrosome reaction following ionophore challenge. *J Androl*, **12**, 98-103.

14. de Lamirande E, Leclerc P, Gagnon C (1997) Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod*, **3**, 175-194.

15. Dong KW, Chi TF, Juan YW, Chen CW, Lin Z, Xiang XQ, Mahony M, Gibbons WE, Oehninger S (2001) Characterization of the biologic activities of a recombinant human zona pellucida protein 3 expressed in human ovarian teratocarcinoma (PA-1) cells. *Am J Obstet Gynecol*, **184**, 835-844.

16. Duru NK, Morshedi MS, Schuffner A, Oehninger S (2001) Cryopreservation-thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. *J Androl*, **22**, 646-651.

17. Duru NK, Morshedi M, Schuffner A, Oehninger S (2001) Cryopreservation-thawing of fractionated spermatozoa and plasma membrane translocation of phosphatidylserine. *Fertil Steril*, **75**, 263-268.

18. Emiliozzi C, Fenichel P (1997) Protein tyrosine phosphorylation is associated with capacitation of human sperm in-vitro but is not sufficient for its completion. *Biol Reprod*, **56**, 674-679.

19. Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CM (1998) A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. *Biol Reprod*, **59**, 12-16.

20. Franken, D.R., Hadley, S.B., Oehninger, S. (2000) Physiological induction of the acrosome reaction in human sperm: validation of a microassay using minimal volumes of solubilized, homologous zona pellucida. *J Assist Reprod Genet*, **17**, 374-378.

21. Franken DR, Morales PJ, Habenicht UF (1996) Inhibition of G Protein in human sperm and its influence on acrosome reaction and zona pellucida binding. *Fertil Steril*, **66**, 1009-1011.

22. Gadella BM, Harrison RA (2000) The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development*, **127** (11), 2407-2420.

23. Gadella BM, Miller NG, Colenbrander B, van Golde LM, Harrison RA (1999) Flow cytometric detection of transbilayer movement of fluorescent phospholipid analogues across the boar sperm plasma membrane: elimination of labeling artifacts. *Mol Reprod Dev*, **53**, 108-125.

24. Gadella BM, Lopes-Cardozo M, van Golde LM, Colenbrander B, Gadella TW Jr (1995) Glycolipid migration from the apical to the equatorial subdomains of the sperm head

plasma membrane precedes the acrosome reaction. Evidence for a primary capacitation event in boar spermatozoa. *J Cell Sci*, **108**(3), 935-946.

25. Glander HJ, Schaller J (1999) Binding of annexin V to plasma membrane of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. *Mol Hum Reprod*, **5**(2), 109-115.
26. James PS, Wolfe CA, Mackie A, Ladha S, Prentice A, Jones R (1999) Lipid dynamics in the plasma membrane of fresh and cryopreserved human spermatozoa. *Hum Reprod*, **14**, 1827-1832.
27. Kopf GS (1990) Zona pellucida-mediated signal transduction in mammalian spermatozoa. *J Reprod Fertil Suppl*, **42**, 33-49.
28. Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA, Smith K (1986) Sperm morphologic features as a prognostic factor in in-vitro fertilization. *Fertil Steril*, **46**, 1118-1123.
29. Laemmli, U.K. (1970) Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature USA*, **227**, 680-685.
30. Langlais J, Roberts KD (1985) A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. *Gamete Res*, **12**, 183-224.
31. Leclerc P, de Lamirande E, Gagnon C (1996) Cyclic adenosine 3',5' monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod*, **55**, 684-692.
32. Lee MA, Check JH, Kopf GS (1992) Guanine nucleotide-binding regulatory protein in human sperm mediates acrosomal exocytosis induced by the human zona pellucida. *Mol Reprod*, **31**(1), 78-86.
33. Luconi M, Krausz C, Forti G, Baldi E (1996) Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biol Reprod*, **55**(1), 207-216.
34. Mahony MC, Oehninger S, Clark GF, Acosta AA, Hodgen GD (1991) Fucoidin inhibits the zona pellucida-induced acrosome reaction in human spermatozoa. *Contraception*, **44**(6), 657-65.
35. Muller K, Pomorski T, Muller P, Herrmann A (1999) Stability of transbilayer phospholipid asymmetry in viable ram sperm cells after cryotreatment. *J Cell Sci*, **112**, 11-20.
36. Muller K, Pomorski T, Muller P, Zachowski A, Herrmann A (1994) Protein-dependent translocation of aminophospholipids an asymmetric transbilayer distribution of

phospholipids in the plasma membrane of ram sperm cells. *Biochemistry*, **33**(33), 9968-9974.

37. Nassar A, Mahony M, Blackmore P, Morshedi M, Ozgur K, Oehninger S (1998) Increase of intracellular calcium is not a cause of pentoxifylline-induced hyperactivated motility or scrosome reaction in human sperm. *Fertil Steril*, **69**, 748-754.
38. Nolan JP, Hammerstedt RH (1997) Regulation of membrane stability and the acrosome reaction in mammalian sperm. *FASEB J*, **11**(8), 670-682.
39. Nolan JP, Magargee SF, Posner RG, Hammerstedt RH (1995) Flow cytometric analysis of transmembrane phospholipid movement in bull sperm. *Biochemistry*, **34**(12), 3907-3915.
40. Oehninger S (2000) Clinical and laboratory management of male infertility: An opinion on its current status. *J Androl*, **21**, 814-821.
41. Oehninger, S. (1995) An update on the laboratory assessment of male fertility. *Hum Reprod*, **10**, 38-45.
42. Oehninger S, Mahony MC, Swanson JR, Hodgen GD (1993) The specificity of human spermatozoa under hemizona assay conditions. *Mol Reprod Dev*, **35**(1), 59-61.
43. Oehninger S, Acosta R, Morshedi M, Philput C, Swanson RJ, Acosta AA (1990) Relation between morphology and motion characteristics of human spermatozoa in semen and in the swim-up sperm fractions. *J Androl*, **11**, 446-452.
44. Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Alvarez J, Kopf GS (1999) Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Mol Hum Reprod*, **5**(11), 1017-1026.
45. Schuffner A, Morshedi M, Oehninger S (2001) Cryopreservation of fractionated, highly motile human spermatozoa: Effect on membrane phosphatidylserine externalization and lipid peroxidation. *Hum Reprod*, **16**, 2148-2153.
46. Schuffner A, Morshedi M, Vaamonde D, Duran EH, Oehninger S (2002) Effect of different incubation conditions on phosphatidylserine externalization and motion parameters of purified fractions of highly motile human spermatozoa. *J Androl*, **23**, 194-201.
47. Tesarik J, Carreras A, Mendoza C (1993) Differential sensitivity of progesterone and zona pellucida-induced acrosome reactions to pertussis toxin. *Mol Reprod Dev*, **34**, 183-189.
48. Tesarik J (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability in vitro. *J Reprod Fertil*, **74**, 383-388.

49. Thomas P, Meizel S (1989) Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca2+ influx. *Biochem J*, **264**, 539-546.

50. Tomes CN, Carballada R, Moses DF, Katz DF, Saling PM (1998) Treatment of human spermatozoa with seminal plasma inhibits protein tyrosine phosphoryation. *Mol Hum Reprod*, **4**(1), 17-25.

51. van Duin M, Polman JE, De Breet IT, van Ginneken K, Bunschoten H, Grootenhuis A, Brindle J, Aitken RJ (1994) Recombinant human zona pellucida protein ZP3 produced by Chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. *Biol Reprod*, **51**(4), 607-617.

52. Visconti PE, Kopf GS (1998a) Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod*, **59**(1), 1-6.

53. Visconti PE, Galantino-Homer H, Moore GD, Bailey JL, Ning X, Fornes M, Kopf GS (1998b) The molecular basis of sperm capacitation. *J Androl*, **19**, 242-248.

54. Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS (1995) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development*, **121**, 1139-1150.

55. Whitmarsh AJ, Woolnough MJ, Moore HD, Hornby DP, Barratt CL (1996) Biological activity of recombinant human ZP3 produced in vitro: potential for a sperm function test. *Mol Hum Reprod*, **2**, 911-919.

56. World Health Organization, (1999) WHO Laboratory manual for examination of human semen and semen-cervical mucus interaction. 2nd ed., Cambridge, United Kingdom: Cambridge University Press, 14-17.

57. Yanagimachi R (1994) Mammalian fertilization. Knobil E, Neil JD (eds.), The physiology of reproduction, New York: Raven Press, Ltd, 189-317,

58. Zaneveld LJ, De Jonge CJ, Anderson RA, Mack SR (1991) Human sperm capacitation and the acrosome reaction. *Hum Reprod*, **6**, 1265-1274.

Table I: Experiment 1: Time-dependent changes of motion parameters (CASA) and sperm viability (Hoechst staining) of human sperm incubated in HTF and 0.3% HSA under spontaneous conditions and following incubation with the calcium ionophore A23187

	Hour			
	0[a]	3	6	24
Concentration ($\times 10^6$/ml)				
Spontaneous	20.0±0.0	19.2±0.8	18.8±1.3	16.4±3.6
CaI[b]	19.0±1.7	17.2±1.9	17.8±3.3	16.0±2.5
Motility (%)[c]				
Spontaneous	94.2±4.5	82.0±4.2	79.0±6.0	17.6±20.1
CaI	79.6±5.9	73.8±6.7	66.6±14.0	9.2±17.9
Rapid cells (%)[c]				
Spontaneous	91.2±2.8	76.6±3.4	73.4±7.1	12.6±16.3
CaI	76.0±6.8	62.0±7.1	56.6±17.5	6.0±11.8
VAP (μm/s)[c]				
Spontaneous	69.8±5.8	54.6±6.6	50.0±5.8	33.4±8.5
CaI	55.2±9.3	49.6±9.7	41.8±5.9	16.6±15.7
VSL (μm/s)[c]				
Spontaneous	60.0±5.1	47.0±7.2	42.4±7.1	26.0±9.0
CaI	51.8±10.2	41.8±10.4	34.6±7.2	13.0±12.0
VCL (μm/s)[c]				
Spontaneous	102.2±5.7	84.2±4.4	81.2±5.8	58.4±8.3
CaI	83.4±8.9	77.0±8.1	70.0±8.9	26.2±27.7
Linearity (%)[c]				
Spontaneous	60.2±4.0	54.4±6.1	51.6±7.4	42.8±9.3
CaI	54.0±7.6	50.6±8.3	48.6±5.2	20.0±20.4
Dead (%)[d]				
Spontaneous	5.0±2.7	5.0±2.1	8.0±3.5	14.0±6.1
CaI	5.0±2.7	7.4±3.2	10.8±5.8	12.6±6.5

[a] Time 0= following separation of the motile sperm fraction and resuspension.

[b] CaI= calcium ionophore A23187.

[c] For spontaneous- and CaI-treated conditions all motion parameters had a significant decline over time $p < 0.001$

[d] For spontaneous- and CaI-treated conditions viability had a significant decline over time ($p < 0.01$)

Figure 1

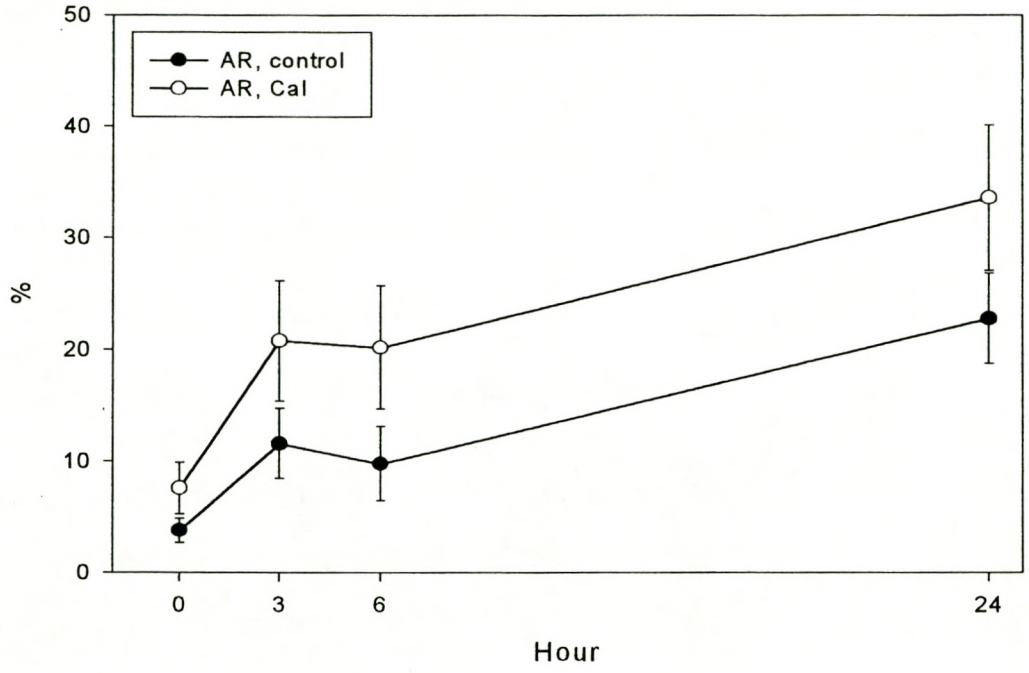


Figure 2

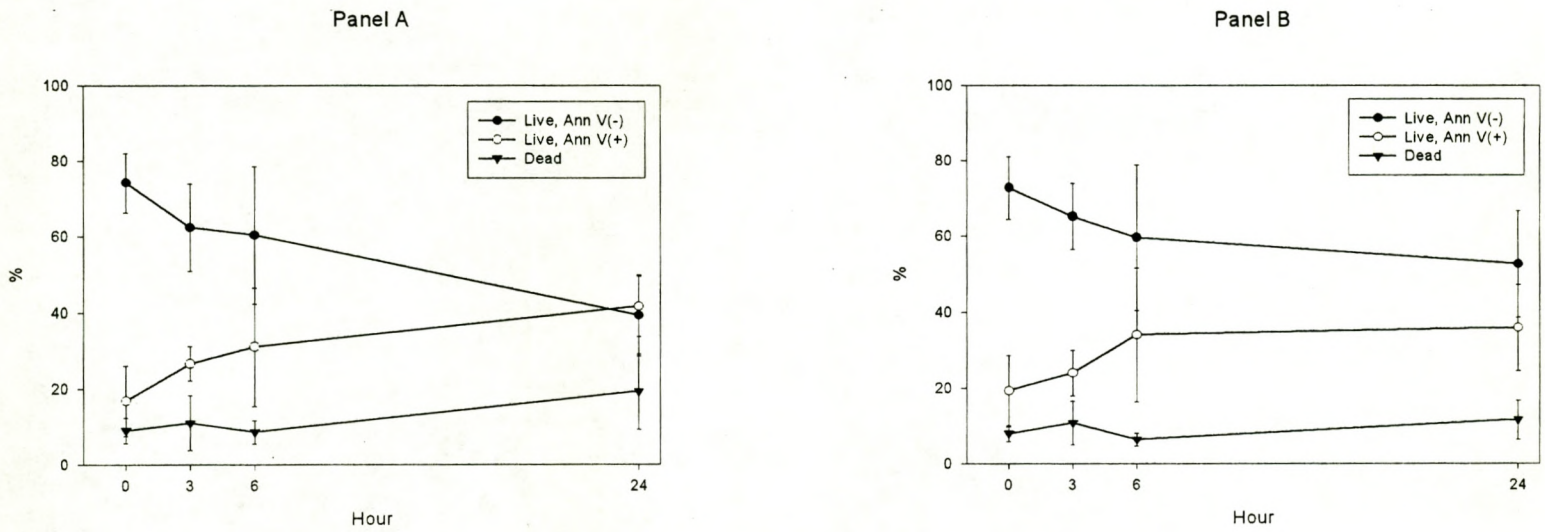


Figure 3

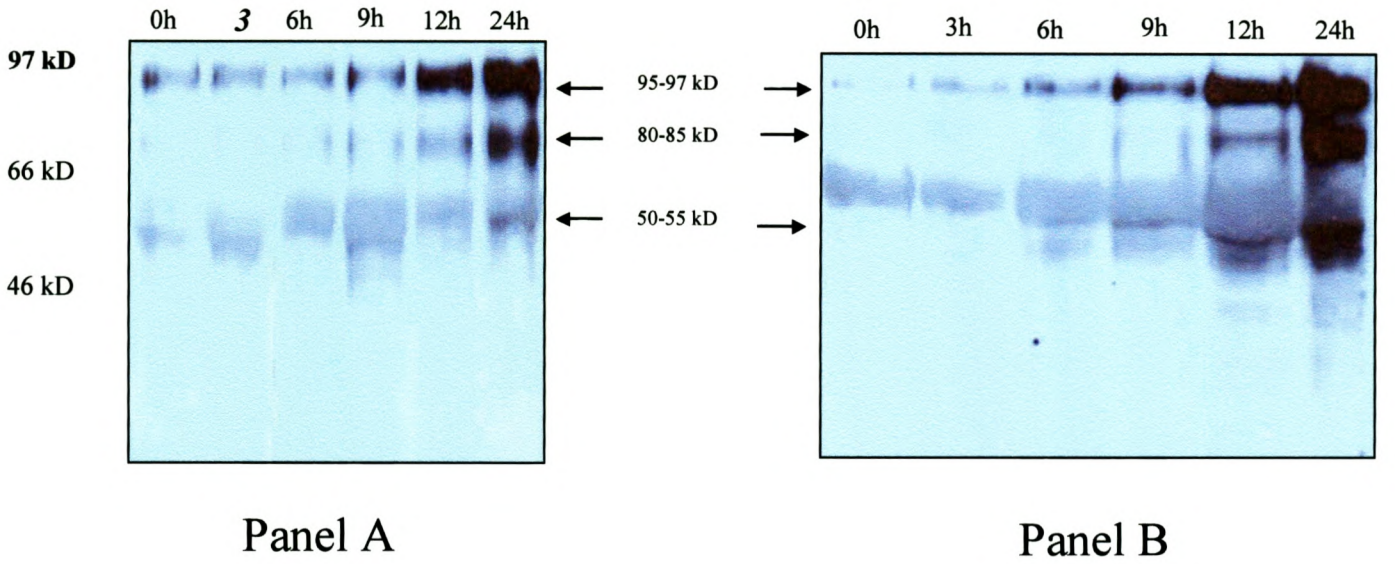
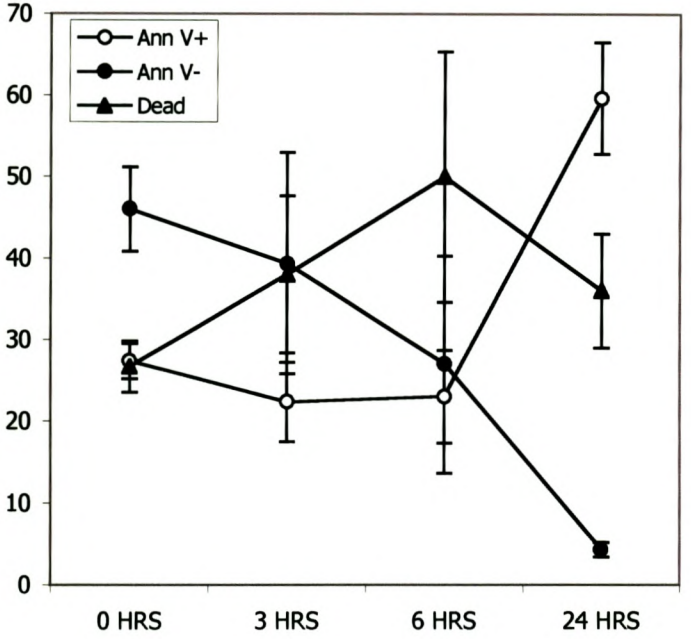
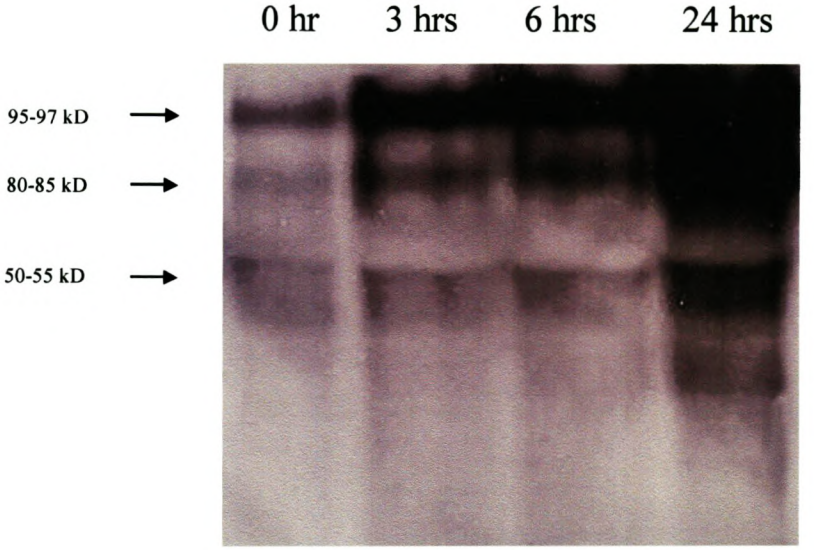


Figure 4





Legends to figures

Figure 1. Experiment I: Time-dependency of the spontaneous (control) and calcium ionophore-induced (CaI) acrosome reaction of human sperm incubated in HTF and 0.3% HSA (results of 5 fertile donors).

Figure 2. Experiment I: Time dependency of annexin V binding assay results of human sperm incubated in HTF and 0.3% HSA. Panel (A): spontaneous conditions; panel (B): calcium ionophore-treated conditions (Ca I) (results of same 5 fertile donors shown in figure 1).

Figure 3. Experiment IIa: Time course of protein tyrosine phosphorylation of human spermatozoa incubated under capacitating conditions with two different HSA doses. Panel (A): HTF and 0.3% HSA; panel (B) HTF and 3% HSA. Numbers on the left of the figure represent markers of molecular weight (results of a representative fertile donor).

Figure 4. Experiment IIb. Time course of simultaneously assessed (A) annexin V binding and (B) protein tyrosine phosphorylation by immunoblotting. Spermatozoa were incubated under capacitating conditions with HTF and 0.3% HSA (results of 3 fertile donors assessed for annexin V binding and a representative fertile donor for immunoblotting).

3b. *Specific aim 4*: To examine the intracellular cascades involved in the zona pellucida-induced acrosome reaction.

Zona pellucida-induced acrosome reaction in the human: dependency on activation of pertussis toxin-sensitive G_i protein and calcium influx, and priming effect of progesterone and follicular fluid.

Alessandro Schuffner A. [1,2], *Hadley S. Bastiaan* [3], *Hakan E. Duran* [1], *Zin-Yong Lin* [1], *Mahmood Morshedi* [1], *Daniel R. Franken* [3] *and Sergio Oehninger* [1]

[1]The Jones Institute for Reproductive Medicine, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, Virginia, USA; [2]Androlab-Reproductive Medicine and Andrology Clinic, Curitiba, Brazil; and [3]Reproductive Biology Unit, Department of Obstetrics and Gynecology, University of Stellenbosch, Cape Town, Republic of South Africa

Abstract

In these studies we aimed to characterize the effects of the physiological, homologous agonists of the acrosome reaction, i.e., the zona pellucida and progesterone/follicular fluid, on human spermatozoa. The specific aims of our studies were: (1) to examine the dependency of the solubilized zona pellucida-induced acrosome reaction on G_i protein activation and presence of extracellular calcium; and (2) to determine whether progesterone/follicular fluid exert a priming or synergist effect on the solubilized zona pellucida-induced acrosome reaction. Highly motile spermatozoa from fertile donors were exposed to the agonists in a micro assay and acrosomal status of live spermatozoa was determined by indirect immunofluorescence using PSA-FITC/Hoechst double staining. Pre-treatment with pertussis-toxin (100 ng/mL) and EGTA (2.5 mM) significantly inhibited the zona pellucida-induced acrosome reaction without affecting the spontaneous rate of exocytosis. Progesterone (1.25 μg/mL) and human follicular fluid (10%) exerted a priming, time-dependent effect on the zona-induced acrosome reaction. These studies demonstrated that: (i) acrosomal exocytosis of capacitated human spermatozoa triggered by the homologous zona pellucida is dependent on activation of G_i-proteins (pertussis toxin sensitive) and presence of extracellular calcium; and (ii) progesterone and follicular fluid exerted a priming role on the zona pellucida-induced acrosome reaction.

Key words: **acrosome reaction/calcium/follicular fluid/G protein/human spermatozoa/progesterone/solubilized zona pellucida**

Introduction

The acrosome reaction is a pre-requisite for fertilization in mammalian spermatozoa (Yanagimachi, 1994). In the mouse, one of the species best characterized so far, acrosomal exocytosis is physiologically induced by components of the zona pellucida (ZP), particularly the zona pellucida protein 3 (ZP3) (Bleil and Wassarman, 1980, 1983; Florman and Storey, 1982; Florman and Wassarman, 1985; Ward, et al, 1992). Binding of ZP3 to putative complementary receptor(s) on the sperm surface activates transmembrane signals that trigger cellular cascades resulting in the acrosome reaction (Wassarman, 1990a and b; Saling, 1991; Wassarman, 1999).

Several cellular pathways are involved in the stimulation of the acrosome reaction. It has been demonstrated that activation of pertussis toxin-sensitive heterotrimeric G proteins (G_i-class) is necessary for the ZP-induced acrosome reaction in the murine model (Kopf et al, 1986; Kopf, 1990). G_i -protein acts as a signal transducing element downstream of ZP3-receptor interactions and couples receptor occupancy to changes in ionic conductance and/or a variety of intracellular second messenger cascade systems whose activation in turn results in release of acrosomal contents (Kopf, 1990). One of such elements is likely to be a pH regulator, resulting in a transient alkanization of intracellular pH (Florman, et al, 1989, 1998; Kopf, 1990). Second messengers include the adenylate cyclase-cAMP system resulting in activation of protein kinase A (PKA) leading to phosphorylation of specific, putative proteins resulting in exocytosis. Also, the activation of phospholipase C (PLC) may lead to 1,2 diacylglycerol (DAG) and inositol 1,4-5 trisphosphate (IP3) formation. Diacylglycerol may stimulate protein phosphorylation through protein kinase C (PKC) whereas IP3 may activate intracellular calcium release through modulation of IP3- sensitive intracellular calcium stores (Kopf, 1990; Florman, et al, 1998; Wassarman, 1999).

It has also been proposed that the ZP may alternatively activate a low voltage- activated T type calcium channel that is pertussis toxin-insensitive (Florman, et al, 1992; Florman, et al, 1998; O'Toole, et al, 2000). Activation of pertussis toxin-sensitive and -insensitive mechanisms leads to significant and sustained changes in intracellular calcium levels, a prerequisite for the acrosome reaction (Kopf, 1990; Florman, et al, 1998).

Progesterone, present in high concentrations in the follicular fluid, is also a known stimulator of the acrosome reaction. It has been shown that progesterone exerts a priming effect on the ZP-stimulated acrosome reaction in the mouse (Roldan, et al, 1994). In such studies, treatment

with progesterone followed by ZP led to maximal generation of DAG and maximal breakdown of phosphatidylinositol-4,5-bis-phosphate (PIP2) signaling a priming role for progesterone in the initiation of exocytosis.

Relatively few studies have addressed the role of the physiologic, homologous inducer of the acrosome reaction, the ZP, in human spermatozoa. This is probably due to the difficulty in obtaining human material (oocytes) to perform such experiments. Zona pellucida can be obtained from oocytes recovered from ovarian tissue (post-surgical or post-mortem) or from *in vitro* fertilization treatment following appropriate patients' consent for donation.

Cross et al (1988) were the first to report that treatment of human spermatozoa in suspension with acid-disaggregated human ZP (2 to 4 zona pellucida -ZP/μL) increased the incidence of acrosome reacted spermatozoa. Lee et al (1992) demonstrated that pertussis toxin treatment of human spermatozoa inhibited the (solubilized) ZP-induced acrosome reaction. In contrast, acrosomal exocytosis induced by the calcium ionophore A-23187 was not inhibited by pertussis toxin pre-treatment. Studies by Franken et al (1996) showed a dose-dependent effect of solubilized human ZP on the acrosome reaction in the range of 0.25 to 1 ZP/μL and also confirmed the involvement of G_i-protein during ZP-induced acrosome reaction of human spermatozoa.

Capacitated human spermatozoa also respond to a progesterone stimulus *in vitro* by a rapid increase in intracellular free calcium due to promotion of calcium influx (Thomas and Meizel, 1989; Blackmore, et al, 1990, 1991). Progesterone activates a calcium channel that has yet to be defined at the molecular level in the human (Blackmore and Eisoldt, 1999). Recent findings have revealed the molecular structure of such ion channel in the murine species (Ren et al, 2001). The entire increase in intracellular calcium levels induced by progesterone is abolished when the extracellular calcium is removed by the addition of the calcium chelator EGTA to the extracellular medium (Blackmore et al, 1990). There is evidence for both L- and T-type calcium channels in mouse and human spermatozoa (Blackmore, et al, 1990; 1991; Benoff, et al, 1994; Arnoult, et al, 1996, 1997; Shiomi, et al, 1996; Florman, et al, 1998; Blackmore and Eisoldt, 1999). It has been proposed that progesterone reacts with a multireceptor system on the sperm surface and that this system cooperates with that used by the zona pellucida to control the physiological acrosome reaction (Mendoza et al, 1995; ESHRE Andrology Special Interest Group, 1996). In human spermatozoa, progesterone effects are not associated with G_i protein activation (Tesarik, et al, 1993).

The first signal transduction-second messenger pathway demonstrated to have a role in human sperm acrosome reaction involved the cAMP/PKA system (De Jonge, et al, 1991a). Also, the PLC-PKC system was demonstrated to play a role in human sperm exocytosis (De Jonge, et al, 1991b; Bielfeld, et al, 1994; Doherty, et al, 1995). However, it is unclear which of such mechanisms is the most significant under physiologic conditions and how the various systems cross talk.

Although transmission electron microscopy still represents the gold standard for the evaluation of acrosomal status, this method is expensive and laborious and cannot be therefore used routinely (Zaneveld, et al, 1991; Kohn, et al, 1997). Other well-established methods are currently being used to assess the acrosome reaction in the human. The most widely used method involves lectins (i.e., *Pisum Sativum agglutinin* -PSA- and others) labelled with fluorescence (i.e., fluorescein isothiocynate -FITC-). The inducibility of the acrosome reaction following calcium ionophore challenge (ARIC) using PSA-FITC has been recommended as the presently available optimal way to assess this physiologic event under *in vitro* conditions to test human spermatozoa (Tesarik, 1985, 1989; Cummins, et al, 1991; ESHRE Andrology Special Interest Group, 1996; Kohn, et al, 1997; Oehninger, et al, 2000a and b).

Recently, Franken et al (2000) reported on the validation of a new micro assay using minimal volumes of solubilized, human ZP to test the physiological induction of the acrosome reaction in human spermatozoa. In such studies, a dose-dependent effect of solubilized ZP on acrosomal exocytosis was observed reaching maximal induction using 1.25-2.5 ZP/μL for both the micro assay and the standard (macro) assay. Furthermore, the inducibility of the acrosome reaction by a calcium ionophore was similar in both assays.

Here, we aimed to further characterize the effects of the physiological, homologous agonists of the acrosome reaction, i.e., the ZP and progesterone/follicular fluid on human spermatozoa. The specific aims of our studies were: (1) to examine the dependency of the ZP-induced acrosome reaction on G_i protein activation **and presence of extracellular calcium**; and (2) to determine whether progesterone or follicular fluid exerts a priming or synergist effect on the ZP-induced acrosome reaction. Purified populations of highly motile spermatozoa were recovered from the ejaculates of fertile donors and exposed to the agonists in a micro volume assay. Acrosomal exocytosis of live spermatozoa was determined with indirect immunofluorescence using PSA-FITC/Hoechst double staining.

Materials and methods

Preparation of sperm samples

Ejaculates from fertile men participating in our artificial insemination donor program were used in these studies. Approval for the studies was obtained from the Institutional Review Board of Eastern Virginia Medical School, where most experiments were performed. Sperm concentration and the percentage progressive motility were objectively evaluated using the HTM-IVOS semen analyzer version GS 771 (Hamilton Thorne Research, Beverly, MA, USA) with fixed parameter settings (Oehninger et al, 1995). Sperm concentration and motility readings were manually monitored and corrections were made as appropriate. Sperm concentration and motility were assessed according to the World Health Organization criteria (WHO, 1999) and sperm morphology was examined according to strict criteria after Diff-Quik staining (Kruger et al, 1986).

The sperm fractions with high motility were isolated by discontinuous Percoll (Sigma Chemical Co, St. Louis, MO, USA) gradient separation (90% and 40% layers) using Human Tubal Fluid (HTF, Irvine Scientific, Santa Anna, CA) as diluent. Up to 2 mL of semen was carefully placed on Percoll layers, centrifuged at 380xg for 20 min and the pellet of the 90% layer was mixed with the HTF and then centrifuged at 380xg for 10 min. The supernatant was discarded and the pellet was resuspended to achieve a sperm concentration of 10 x 10^6/mL.

The original sperm parameters of samples used were as follows (mean±standard deviation): concentration, 101±10 x10^6/ml; sperm motility, 60±5 %, and normal morphology (strict criteria), 15±3%. The motile sperm fractions recovered form the 90% Percoll layers (10x10^6 cells/ml, >90% motility) were allowed to capacitate for 3 hours at 37°C under 5% CO_2 in water saturated air in HTF supplemented with 3% Human Serum Albumin (HSA, Irvine).

Preparation of solubilized zona pellucida

Human oocytes were retrieved from post-mortem derived ovarian tissue following approval by the local ethics committee at Stellenbosch University. Oocytes were stored in HTF using DMSO/sucrose at -196°C in liquid nitrogen (Franken et al, 1989). Oocytes were shipped to Norfolk and twelve hours prior to each experiment were removed from storage and thawed at 37°C. The oocytes were placed in 0.25 M sucrose and 3% HSA in modified HTF medium for 20 minutes at room temperature, after which they were placed under mineral oil (Sigma) until used.

Prior to each experiment, oocytes were vigorously pipetted with a small-bore glass pipette (inner diameter 80 microns) to separate the zona pellucida from the ooplasm. The separated ZP were then placed in a plastic Eppendorf tube containing modified HTF medium supplemented with 3% HSA. The tubes were centrifuged for 15 minutes at 1,800 x g, after which the HTF medium was carefully removed under microscopic vision, leaving only the ZP at the bottom of the tube. Solubilization of the ZP was performed in a micro-volume and microscopically controlled following addition of 10mM HCl after which 10mM NaOH was added to the zona to render a final ZP concentration of 2.5 ZP/µl. The final pH of the ZP solution was 7.2 to 7.4 (Franken et al, 1996, 2000).

Calcium ionophore

The calcium ionophore A23187 (Sigma) was prepared in a stock solution with dimethylsulfoxide (Sigma) and then diluted in modified HTF to be tested at a final concentration of 5 µM in the acrosome reaction micro assay (Franken et al, 2000).

Progesterone

Progesterone (Sigma) was prepared in a stock solution with ethanol and then diluted with PBS to be tested at a final concentration of 1.25 µg/mL (Sueldo, et al, 1993; Oehninger, et al, 1994).

Human follicular fluid

A pool of follicular fluid was obtained from women participating in our IVF program and receiving gonadotropin stimulation after obtaining approval from the Institutional Review Board. Individual fluids were used following oocyte identification and only fluids from follicles containing a mature metaphase II oocyte were studied. Each tube of follicular fluid was centrifuged at 4°C for 15 min at 1,500 x *g* and stored at –20°C until used (Marin-Briggiler, et al, 1999). For acrosome reaction the follicular fluid was tested at a final concentration of 10% in PBS.

Acrosome Reaction

The assessment of the acrosome reaction was performed using a micro assay as described by Franken et al (2000) and modified as described below. Briefly, 1µl of ZP solution (concentration, 2.5 ZP/µl) (or 1 µl of the agonists A23187, progesterone or human follicular fluid) was aspirated into a Teflon pipette tip (Hamilton Pipette-tip, Separations, Cape Town, South Africa), fitted to a micro-syringe (Hamilton 702, Separations) with 1µl of sperm, to render a final

zona pellucida concentration of 1.25 ZP/µl (or 5 µM A23187, 1.25 µg/mL of progesterone or 10% human follicular fluid). Prior to aspiration into Teflon tips, all sperm/ZP suspensions were gently mixed in a well of a micro-titer plate (Laboratory and Scientific, Cape Town South Africa). To prevent evaporation from the Teflon tips, HTF was aspirated into both sides of the Teflon tip to seal off sperm-ZP suspensions. Each sperm-ZP suspension was separated from the HTF droplets by air bubbles on both sides.

Control and treated sperm samples were carefully removed from the teflon tips and placed on separate spots on the spotted slide and immediately evaluated for motility under inverted phase contrast microscope (Nikon, Garden City, New York). The percentage live cells were recorded by adding 1µl (0.3µg/ml) Hoechst-dye (Bis-Benzimide supravital stain, Hoechst 33258, B-2883; Sigma) for 5 minutes to each spot.

Following motility assessment, the sperm droplets were allowed to air dry, then fixed in 70% ethanol for 20 minutes, and then simultaneously evaluated for percentage live cells and acrosomal status by Hoechst/FITC-PSA (Sigma) with epi-fluorescence microscopy at a magnification of x 1,000 using a phase-contrast microscope (Eclipse 600, Nikon, Melville, NY, USA) equipped with a digital camera with a high pressure mercury lamp power supply (SPOT RT, software version 3.2, Diagnostic Instruments, Augusta, GA). Two hundred cells were counted in a blinded fashion in each well of the spotted slide and results were expressed as % live, acrosome-reacted sperm. The following staining patterns were evaluated as acrosome reacted spermatozoa; (i) distinct staining in the equatorial region occurring as an equatorial bar; (ii) no staining observed over entire sperm surface; and (iii) patchy staining on acrosomal region (Cross et al, 1988; Cummins et al, 1991; Mahony, et al., 1991; Franken et al, 1996; 2000).

Experimental design

In the first experiment (experiment 1) we compared the acrosome reaction-inducing ability of solubilized ZP and the calcium ionophore, and also examined the impact of inactivation of G protein by pertussis toxin and calcium chelating from the extracellular medium by EGTA. An ejaculate from each of 5 different donors was subjected to a separation of the motile fraction

followed of a 3-hour capacitation period as described above. Each sample was then aliquoted into 5 parts and incubated under the following different conditions at 37°C under 5% CO_2 in water saturated air: (i) control medium (HTF supplemented with 3% HSA for 60 min; (ii) calcium ionophore at a final concentration of 5 μM for 60 min; (iii) solubilized zona pellucida at a final concentration of 1.25 ZP/ μL for 60 min; (iv) pre treatment with the calcium chelator EGTA [ethylene (oxyethylenenitrilo) tetraacetic acid] (Sigma) at a final concentration of 5 μM for 30 min, followed by solubilized ZP at a final concentration of 1.25 ZP/μL for 60 min; and (v) pretreatment with the functional inactivator of G protein, pertussis toxin (Calbiochem, San Diego, CA) at a final concentration of 100 ng/mL for 30 min, followed by solubilized ZP at a final concentration of 1.25 ZP/μL for 60 min. After incubation, each condition was tested for the percentage of live, acrosome reacted spermatozoa in the micro assay as detailed above.

In the second experiment (experiment 2) we compared the acrosome reaction-inducing ability of solubilized ZP and progesterone, and also examined the impact of sequential treatment of ZP and progesterone in reversed order. Twenty-seven ejaculates from 5 different donors were subjected to a separation of the motile fraction followed of a 3-hour capacitation period as described above. Each sample was then aliquoted into 5 parts and incubated under the following different conditions at 37°C under 5% CO_2 in water saturated air: (i) control medium (HTF supplemented with 3% HSA) for 30 min; (ii) solubilized ZP at a final concentration of 0.5 ZP/ μL for 30 min; (iii) progesterone at a final concentration of 1.25 μg/mL for 30 min; (iv) solubilized ZP at a final concentration of 0.5 ZP/μL for 15 min followed by progesterone at a final concentration of 1.25 μg/mL for an additional 30 min; and (v) progesterone at a final concentration of 1.25 μg/mL for 15 min followed by solubilized ZP at a final concentration of 0.5 ZP/μL for 30 min. After incubation, each condition was tested for the percentage of live, acrosome reacted in the micro assay as detailed above.

In the third experiment (experiment 3a) we compared the acrosome reaction-inducing ability of solubilized ZP and follicular fluid, and also examined the impact of sequential treatment with follicular fluid followed by solubilized ZP. An ejaculate from each of 3 different donors was subjected to a separation of the motile fraction followed of a 3-hour capacitation period as described above. Each sample was then aliquoted into 5 parts and incubated under the following different conditions at 37°C under 5% CO_2 in water saturated air: (i) control medium (HTF supplemented with 3% HSA) for 60 min; (ii) calcium ionophore at a final concentration of 5 μM for 60 min; (iii) solubilized ZP at a final concentration of 1.25 ZP/μL for 60 min; (iv) human

follicular fluid (10%) for 60 min; and (v) pretreatment with human follicular fluid at 10% for 30 min followed by solubilized ZP at a final concentration of 1.25 ZP/μL for 60 min. After incubation, each condition was tested for the percentage of live, acrosome reacted spermatozoa.

In a subsequent follow-up experiment (experiment 3b) we examined the kinetics (time dependency) of the acrosome reaction-inducing ability of solubilized ZP following pretreatment with follicular fluid. An ejaculate from each of 3 different donors was subjected to a separation of the motile fraction followed of a 3-hour capacitation period as described above. Each sample was then incubated for 30 min with human follicular fluid (10%) at 37°C under 5% CO_2 in water saturated air. Thereafter, the sample was aliquoted into 4 parts and further incubated with human solubilized ZP (final concentration of 1.25 ZP/μL) for 4 different time periods: 15, 30, 45 and 60 min. An aliquot exposed to follicular fluid under the same conditions served as control. After incubation, each condition was tested for the percentage of live, acrosome reacted spermatozoa.

Statistical Analysis

Comparisons of the percentage of live, acrosome reacted spermatozoa under the different experimental conditions were performed using one-way or repeated measures analysis of variance (ANOVA) as appropriate. The Tukey-Kramer multiple comparisons test was used to assess individual differences among conditions tested. P values < 0.05 were considered significant. Results are expressed as mean±standard error.

Results

The results of experiment 1 are depicted in Figure 1. The overall results analyzed by ANOVA had a $p<0.0001$, exhibiting an extremely significant difference. The calcium ionophore A23187 significantly increased acrosome reaction from 12±2% (control conditions) to 29±3% ($p<0.001$). The solubilized ZP produced an increase in acrosomal exocytosis (49±5%) that was significant higher than control and calcium ionophore treatment conditions ($p<0.001$ versus both conditions). Pre-treatment with EGTA followed by ZP resulted in a significantly lower acrosome reaction (22±2%) than ZP alone ($p< 0.001$), but this percentage of acrosomal exocytosis was significantly higher than control conditions ($p< 0.01$). Pre-treatment with pertussis toxin followed by ZP also resulted in a significantly lower acrosome reaction (25±3%) than ZP alone ($p< 0.001$), but this percentage of acrosomal exocytosis was also significantly higher than control conditions ($p< 0.001$). The results of this experiment demonstrated that (i) the calcium ionophore and

solubilized ZP are powerful stimulators of acrosomal exocytosis under micro assay conditions; and (ii) that the functional inactivation of G_i proteins (pertussis toxin-sensitive) and the absence of extracellular calcium are capable of inhibiting the solubilized ZP-induced acrosome reaction.

Figure 2 shows the results of experiment 2. The overall results analyzed by ANOVA had a $p< 0.0001$, exhibiting an extremely significant difference. Both progesterone (27±3%) and ZP (28±2%) resulted in a significant enhancement of the acrosome reaction when compared to control conditions (12±1%; $p< 0.001$ for both comparisons). Pre-treatment with ZP followed by progesterone (28±3%) resulted in a significant increase in acrosomal exocytosis versus control ($p< 0.001$). Pre-treatment with progesterone followed by ZP (36±3%) also resulted in a significant increase in acrosome reaction versus control ($p< 0.001$). However, pre-treatment with progesterone followed by ZP resulted in a significant increase in acrosome reaction when compared to pre-treatment with ZP followed by progesterone ($p <0.01$). Pre-treatment with progesterone followed by ZP increased acrosome reaction significantly when compared to progesterone alone ($p <0.01$) and ZP alone ($p< 0.01$). On the other hand, pre-treatment with ZP followed by progesterone had a non-significant effect when compared to progesterone or ZP alone ($p>0.05$ for both comparisons). The results of this experiment demonstrated that (i) progesterone, as the solubilized ZP, enhances acrosomal exocytosis under micro assay conditions; and (ii) the sequential treatment with progesterone followed by ZP results in a significantly higher increase in acrosome reaction than the reverse order, providing evidence for a priming effect of progesterone (and not a synergistic one) on the ZP-induced exocytosis.

The results of experiment 3a are shown in Figure 3. The overall results analyzed by ANOVA had a $p<0.0001$, exhibiting an extremely significant difference. The calcium ionophore A23187 significantly increased acrosome reaction from 9±1% (control conditions) to 23±1% ($p<0.001$). The solubilized ZP produced an increase in acrosomal exocytosis (47±1%) that was significantly higher than control and calcium ionophore treatment conditions ($p<0.001$ versus both conditions). Human follicular fluid -stimulated acrosome reaction (21±1%) was significantly higher than that of control ($p< 0.001$), but was not different than that of calcium ionophore ($p> 0.5$), and was significantly lower than that of ZP ($p< 0.001$). Pre-treatment with follicular fluid followed by ZP (53±1%) resulted in a significantly higher acrosome reaction than control ($p< 0.001$), calcium ionophore ($p< 0.001$), follicular fluid alone ($p< 0.001$) and ZP alone ($p< 0.01$). The results of this experiment demonstrated that (i) follicular fluid, as progesterone, is a potent inducer of the

acrosome reaction under micro assay conditions; and (ii) pre-treatment with follicular fluid also results in a priming effect on the solubilized ZP-induced acrosomal exocytosis.

The results of experiment 3b are shown in Figure 4. The overall results of the subsequent treatment with follicular fluid and solubilized ZP analyzed by repeated measures ANOVA had a $p<0.0002$, exhibiting an extremely significant difference. Upon pre-treatment with follicular fluid for 30 min hour, a time-dependent effect of ZP was observed. The induction of acrosome reaction at 45 and 60 min was significantly higher than that at 15 min ($p< 0.01$ and $p<0.001$, respectively). The induction of acrosome reaction at 45 and 60 min was also significantly higher than that at 30 min ($p< 0.01$ and $p< 0.001$, respectively). There were no significant differences observed for the effect of follicular fluid at the different time points. This experiment examined the kinetics of the solubilized ZP-induced acrosome reaction after exposure to human follicular fluid. Results demonstrated that upon exposure to follicular fluid there is a time-dependent effect of solubilized ZP on acrosomal exocytosis, with increased exocytosis after 45-60 min incubation.

Discussion

Franken et al (2000) devised a new micro assay that is easy and rapid to perform, and facilitates the use of minimal volumes of solubilized ZP (even a single zona) for assessment of the human sperm acrosome reaction. The micro assay has been validated as compared to the standard macro assay and consequently offers a unique arena to test for the physiological induction of acrosomal exocytosis by the homologous ZP. Moreover, preliminary clinical studies using the micro assay have demonstrated that the zona pellucida-induced acrosome reaction (ZIAR) can predict fertilization failure in the IVF setting. The micro assay ZIAR can therefore refine the therapeutic approach for male infertility prior to the onset of therapy (Esterhuizen, et al, 2001a and b).

We hypothesized that, in human spermatozoa, solubilized ZP triggers acrosomal exocytosis via a transmembrane signaling cascade involving heterotrimeric G proteins (pertussis toxin-sensitive); and that an alternative or complementary pathway may involve regulation of intracellular calcium levels by modulation of calcium influx. To test this hypothesis, human capacitated spermatozoa were treated with pertussis toxin (that functionally inactivates heterotrimetric G_i protein by ADP-ribosylating its α-subunit) (Casey and Gilman, 1988) or EGTA (a calcium chelator), prior to induction of acrosome reaction with solubilized zona pellucida.

Results of experiment 1 confirmed that under the micro assay conditions, the solubilized ZP induced a high level of acrosomal exocytosis. Importantly, pre-treatment with pertussis toxin significantly inhibited the ZP-induced acrosome reaction (although it did not affect basal or spontaneous levels of exocytosis) (Franken et al, 1996). These results confirmed and extended those of Lee et al (1992), Tesarik, et al (1993) and Franken, et al (1996, 2000), who reported on the G_i protein-dependency of human acrosome reaction triggered by the homologous ZP.

Calcium appears to be essential for several sperm functions. It has been shown that elevated intracellular free calcium concentrations and protein tyrosine phosphorylation are determinants of sperm capacitation and that extracellular calcium modulates tyrosine phosphorylation and tyrosine kinase activity in human spermatozoa (Luconi, et al, 1996; Osheroff, et al, 1999; Visconti, et al, 1999a and b). The acrosome reaction necessary for fertilization in many species also requires an increase in intracellular calcium levels. Incubation of human sperm in a calcium-depleted medium inhibited or delayed capacitation resulting in fewer spontaneous or A23187-induced acrosome reacted sperm (Perry et al, 1997). In the mouse, ZP3 produced a sustained increase in intracellular calcium leading to acrosome reaction, probably due to the persistent activation of a calcium influx mechanism during the late stages of ZP3 signal transduction (O'Toole et al, 2000).

Here, the calcium chelator EGTA significantly inhibited the ZP-induced acrosome reaction. These results demonstrated that extracellular calcium is required for signal transduction resulting in agonist-induced acrosome reaction. But as such, the design of these experiments does not allow discriminating between cell surface requirements for calcium (i.e., calcium might be required for sperm-ZP initial attachment, that subsequently leads to induction of exocytosis) or a role of calcium influx itself in the cascade of events leading to the acrosome reaction. Experiments performed in other species have demonstrated the presence and significance of calcium channels and calcium influx in the activation of the acrosome reaction (see above). In addition, two recombinant human ZP3 products have been reported to trigger calcium influx in human spermatozoa (Whitmarsh et al, 1996; Bray et al, 2002). We therefore conclude that our data provide further indirect evidence for the role of calcium influx on the induction of human acrosome reaction. In our experiments, pre-treatment with EGTA did not inhibit the basal or spontaneous acrosome reaction, suggesting that intracellular sources of calcium may be sufficient for sustaining basal levels of exocytosis.

The results of experiments 2 and 3 confirmed that human follicular fluid and progesterone (which is present at high concentrations in follicular fluid) are potent stimulators of the acrosome reaction (Blackmore, et al, 1990). Controversy surrounds the precise time at which the physiologic acrosomal exocytosis occurs, i.e., during exposure to follicular fluid-cumulus cell products or at the level of the zona pellucida. We hypothesized that a population of spermatozoa may undergo exocytosis before reaching the zona, but that the most functional acrosome reaction leading to zona penetration takes place after interaction with ZP3, maybe primed by follicular fluid constituents.

In the mouse, Roldan, et al (1994) have elegantly shown that progesterone exerts a priming effect on ZP-induced exocytosis. Here, we provided further data in support of this priming effect as related to progesterone and follicular fluid in human spermatozoa. Progesterone priming effect was evident even after 15 min of preincubation. Moreover, experiments revealed that the priming effect produced by follicular fluid on the ZP-induced acrosome reaction was time-dependent, with maximal results observed in the range of 45-60 min zona incubation following steroid exposure.

In conclusion, our studies demonstrated that: (i) acrosomal exocytosis of capacitated human spermatozoa triggered by homologous solubilized ZP is dependent on both the activation of G_i-protein (pertussis toxin sensitive) and on the presence of extracellular calcium; and (ii) progesterone and human follicular fluid exert a priming, time-dependent effect on the ZP-induced acrosome reaction. The operative mechanisms downstream to G_i-.protein activation (ZP-dependent) and increase in intracellular calcium levels (ZP and progesterone/follicular fluid-dependent), and cross talk between such pathways leading to acrosomal exocytosis in human spermatozoa remain to be further characterized.

References

1. Arnoult, C., Cardullo, R.A., Lemos, J.R., Florman, H.M. (1996) Activation of mouse sperm T-typeCa^{2+} channels by adhesion to the egg zona pellucida. *Proc Natl Acad Sci*, **93**, 13004-13009.
2. Benoff, S., Cooper, G.W., Hurley, I., Mandel, F.S., Rosenfeld, D.L., Scholl, G.M., Gilbert, B.R., Hershlag, A. (1994) The effect of calcium ion channel blockers on sperm fertilization potential. *Fertil Steril*, **62**, 606-617.
3. Bielfeld, P., Faridi, A., Zaneveld, L., De Jonge,C.J. (1994) The zona pellucida-induced acrosome reaction of human spermatozoa is mediated by protein kinases. *Fertil Steril*, **61**, 536-541.
4. Blackmore, P.F., Eisoldt, S. (1999a) The neoglycoprotein mannose-bovine serum albumin, but not progesterone, activates T-type calcium channels in human spermatozoa. *Mol Hum Reprod*, **5**, 498-506.
5. Blackmore, P.F., Eisoldt, S. (1999b) The neoglycoprotein mannose-bovine serum albumin, but not progesterone, activates T-type calcium channels in human spermatozoa. *Mol Hum Reprod*, **5**, 498-506.
6. Blackmore, P.F., Neulen, J., Lattanzio, F.A., Beebe, S.J. (1991) Cell surface receptors for progesterone mediated calcium uptake in human sperm. *J Biol Chem*, **266**, 18655-18659.
7. Blackmore, P.F., Beebe, S.J., Danforth, D.R., Alexander, N. (1990) Progesterone and 17α-Hydroxyprogesterone. *J Biol Chem*, **265**, 1376-1380.
8. Bleil, J.D., Wassarman, P.M. (1983) Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev Biol*, **95**, 317-324.
9. Bleil, J.D., Wassarman, P.M. (1980) Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell*, **20**, 873-882.
10. Bray, C., Son, J.H., Kumar, P., Harris, J.D., Meizel, S. (2002) A role for human sperm glycine receptor/Cl^- channel in the acrosome reaction initiated by recombinant ZP3. *Biol Reprod*, **66**, 91-97
11. Casey, P.J., Gilman, A.G. (1988) G protein involvement in receptor-effector coupling. *J Biol Chem*, **263**, 2577-2580.
12. Cross, N.L., Morales, P., Overstreet, J., Hanson, F.W. (1988) Induction of acrosome reaction by the human zona pellucida. *Biol Reprod*, **38**, 235-244.

13. Cummins, J., Pember, S., Jequier, A., Yovich, J.L., Hartmann, P.E. (1991) A test of the human sperm acrosome reaction following ionophore challenge. *J Androl*, **12**, 98-103.
14. De Jonge, C.J., Han, H., Lawrie, H., Mack, S.R., Zaneveld, L.J. (1991a) Modulation of the human sperm acrosome reaction by effectors of the adenylate cyclase/cyclic AMP second messenger pathway. *J Exp Zool*, **258**, 113-125.
15. De Jonge, C.J., Han, H., Mack, S.R., Zaneveld, L.J. (1991) Effect of phorbol diesters, synthetic diacylglycerols, and a protein kinase inhibitor on the human sperm acrosome reaction. *J Androl*, **12**, 62-70.
16. Doherty, C., Tarchala, S., Radwanska, E., De Jonge,C.J. (1995) Characterization of two second messenger pathways and their interactions in eliciting the human sperm acrosome reaction. *J Androl*, **16**, 36-46.
17. ESHRE Andrology Special Interest Group (1996) Consensus Workshop on Advance Diagnostic Andrology Techniques. *Hum Reprod*, **11**, 1463-1479.
18. Esterhuizen, A.D., Franken, D.R., Lourens, J.G.H., Van Rooyen, L.H. (2001a) Clinical importance of a micro-assay for the evaluation of sperm acrosome reaction using homologus zona pellucida. *Andrologia*, **33**, 87-93.
19. Esterhuizen, A.D., Franken, D.R., Lourens, J.G.H van Rooyen, L.H. (2001b) Clinical importance of zona pellucida-induced acrosome reaction and its predictive value for IVF. *Hum Reprod*, **16**, 138-44
20. Florman, H.M., Arnoult, C., Kazam, I.G., Li, C., O'Toole, C.M. (1998a) A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: A tale of two channels. *Biol Reprod*, **59**, 12-16.
21. Florman, H.M., Corron, M.E., Kim, T.D., Babcock, D.F. (1992) Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. *Dev Biol*, **152**, 304-314.
22. Florman, H.M., Tombes, R.M., First, N.L., Babcock, D.F. (1989) An adhesion-associated agonist from the zona pellucida activates G protein-promoted elevations of internal Ca and pH that mediate mammalian sperm acrosomal exocytosis. *Dev Biol*, **135**, 133-146.
23. Florman, H.M., Wassarman, P.M. (1985) O-linked oligosaccharides of mouse eggs ZP3 account for its sperm receptor activity. *Cell*, **41**, 313-324.
24. Florman, H.M., Storey, B.T. (1982) Mouse gamete interactions: the zona pellucida is the site of the acrosome reaction leading to fertilization in vitro. *Dev Biol*, **91**, 121-130.

25. Franken, D.R., Bastiaan, H.S., Oehninger, S. (2000) Physiological induction of the acrosome reaction in human sperm: Validation of a microassay using minimal volumes of solubilized, homologous zona pellucida. *J Assist Reprod Genet*, **17**, 374-378

26. Franken, D.R., Burkman, L.J., Oehninger, S., *et al.*, (1989) Hemizona assay using salt-stored human oocytes: evaluation of zona pellucida capacity for binding human spermatozoa. *Gamete*, **22**, 15-26.

27. Franken, D.R., Morales, P.J., Habenicht, U.F. (1996) Inhibition of G protein in human sperm and its influence on acrosome reaction and zona pellucida binding. *Fertil Steril*, **66**, 1009-1011.

28. Kohn, F.M., Mack, S.R., Schill, W.B., Zaneveld, L.J. (1997) Detection of human sperm acrosome reaction: comparison between methods using double staining, Pisum sativum agglutinin, concanavalin A and transmission electron microscopy. *Hum Reprod*, **12**, 714-721.

29. Kopf, G.S. (1990) Zona pellucida-mediated signal transduction in mammalian spermatozoa. *J Reprod Fertil*, **42**, 33-49.

30. Kopf, G.S., Woolkalis, M.J., Gerton, G.L. (1986) Evidence for a guanine nucleotide-binding regulatory protein in invertebrate and mammalian sperm: identification by islet-activating protein-catalyzed ADP-ribosylation and immunochemical methods. *J Biol Chem*, **261**, 7327-7331.

31. Kruger, T.F., Menkveld, R., Stander, F.S.H, Lombard, C.J., Van der Merwe, J.P., van Zyl, J.A., Smith, K. (1986) Sperm morphologic features as a prognostic factor in in-vitro fertilization. *Fertil Steril*, **46**, 1118-1123.

32. Lee, M.A., Check, L.H., Kopf, G.A. (1992) Guanine nucleotide-binding regulatory protein in human sperm mediates acrosomal exocytosis induced by the human zona pellucida. *Mol Reprod*, **31**, 78-86.

33. Luconi, M., Krausz, C., Forti, G., Baldi, E. (1996) Extracelluar calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biol Reprod*, **55**, 207-216.

34. Mahony MC, Oehninger S, Clark GF, Acosta AA, Hodgen GD (1991) Fucoidin inhibits the zona pellucida-induced acrosome reaction in human spermatozoa. *Contraception,* **44,** 657-65.

35. Marin-Briggiler, C.I., Vazquez-Levin, M.H, Gonzalez-Echeverria, F., Blaquier, J.A., Tezon, J.G., Miranda, P.V. (1999) Strontium supports human sperm capacitation but not follicular fluid-induced acrosome reaction. *Biol Reprod*, **61**, 673-680.

36. Mendoza, C., Soler, A., Tesarik, J. (1995) Nongenomic steroid action: independent targeting of plasma membrane calcium channel and a tyrosine kinase. *Biochem Biophys Res Comm*, **210**, 518-523.

37. Oehninger, S. (2000a) Clinical and laboratory management of male infertility: An opinion on its current status. *J Androl*, **21**, 814-821.

38. Oehninger, S., Franken, D.R., Sayed, E., Barroso, G., Kolm, P. (2000b) Sperm funtion assays and their predictive value for fertilization outcome in IVF therapy: a meta-analysis. *Hum Reprod*, **6**, 160-168.

39. Oehninger, S. (1995) An update on the laboratory assessment of male fertility. *Hum Reprod*, **10**, 38-45.

40. Oehninger, S., Sueldo, C., Lanzendorf, S., Mahony, M., Burkman, L.J., Alexander, N.J., Hodgen, G.D. (1994) A sequential analysis of the effect of progesterone on specific sperm functions crucial to fertilization in vitro in infertile patients. *Hum Reprod*, **9**, 1322-1327.

41. Osheroff, J.E., Visconti, P.E., Valenzuela, J.P., Travis, A.J., Alvarez, J., Kopf, G.S. (1999) Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Mol Hum Reprod*, **5**, 1017-1026.

42. O'Toole, C., Arnoult, C., Darszon, A., Steinhardt, R.A., Florman, H.M. (2000) Ca^{2+} entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drive the acrosome reaction. *Mol Biol Cell*, **11**, 1571-1584.

43. Perry, R.L., Barratt, C.L., Warren, M.A., Cooke, I.D. (1997) Elevating intracellular calcium levels in human sperm using an internal calcium ATPase inhibitor, 2,5-di (tert-butyl) hydroqui (TBQ), initiates capacitation and the acrosome reaction but on the presence of extracellular calcium. *J Exp Zool*, **279**(3), 291-300.

44. Ren D., Navarro B., Perez G., Jackson A.C., Hsu S., Shi Q., Tilly, J.L., Clapham, D.E. (2001) A sperm ion channel required for sperm motility and male fertility. *Nature*, **413**, 603-9.

45. Roldan, E.R.S., Murase, T., Shi, Qi-Xian (1994) Exocytosis in spermatozoa in response to progesterone and zona pellucida. *Science*, **266**, 1578-1581.

46. Sailing, P.M. (1991) How the egg regulates sperm function during gamete interaction: facts and fantasies. *Biol Reprod*, **44**, 246-251.

47. Shiomi, H., Yamano, S., Shono, M., Aono, T. (1996) Characteristics of calcium ion influx induced by human follicular fluid in individual human sperm. *Arch Androl*, **37**, 79-86.

48. Sueldo, C.E., Oehninger, S., Subias, E., Mahony, M., Alexander, N.J., Burkman, L.J., Acosta, A.A. (1993) Effect of progesterone on human zona pellucida sperm binding and oocyte penetrating capacity. *Fertil Steril*, **60**, 137-140.

49. Tesarik, J., Carrera, A., Mendoza, C. (1993) Differential sensitivity of progesterone and zona pellucida-induced acrosome reactions to pertussis toxin. *Mol Reprod Dev*, **34**, 183-189.

50. Tesarik, J. (1989) Appropriate timing of the acrosome reaction is a major requirement for the fertilizing spermatozoon. *Hum Reprod*, **4**, 957-961.

51. Tesarik, J. (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability in vitro. *J Reprod Fertil*, **74**, 383-388.

52. Thomas, P., Meizel, S. (1989) Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx. *Biochem J*, **264**, 539-546.

53. Visconti, P.E., Ning, X., Fornes, M.W., Alvarez, J.G., Stein, P., Connors, S.A., Kopf, G.S. (1999a) Cholesterol efflux-mediated signal transduction in mammalian sperm: Cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Dev Biol*, **214**, 429-443.

54. Visconti, P.E., Stewart-Savage, J., Blasco, A., Battaglia, L., Miranda, P., Kopf, G.S., Tezon, J.G. (1999b) Roles of bicarbonate, cAMP, and protein tyrosine phosphorylation on capacitation and the spontaneous acrosome reaction of hamster sperm. *Biol Reprod*, **61**, 76-84.

55. Ward, C.R., Storey, B.T., Kopf, G.S. (1992) Activation of a Gi protein in mouse sperm membranes by solubilized proteins of the zona pellucida, the egg's extracellular matrix. *J Biol Chem*, **267**, 14061-14067.

56. Wassarman, P.M. (1999) Mammalian fertilization: Molecular aspects of gamete adhesion, exocytosis and fusion. *Cell*, **96**, 175-183.

57. Wassarman, P.M. (1990a) Profile of a mammalian sperm receptor. *Development*, **108**, 1-17.

58. Wassarman, P.M. (1990b) Regulation of mammalian fertilization by zona pellucida glycoproteins. *J Reprod Fertil*, **42**, 79-87.

59. Whitmarsh, A.J., Woolnough, M.J., Moore, H.D.M., Hornby, D.P., Barratt, C.L. (1996) Biological activity of recombinant human ZP3 produced *in vitro*: potential for a sperm function test. Mol Hum Reprod **2**, 911-19

60. World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction.* 2nd ed. Cambridge, United Kingdom: Cambridge University Press; 1999:14-17.

61. Yanagimachi, R. (1994) Mammalian fertilization. Knobil E, Neil JD (eds.), 189-317, The physiology of reproduction, New York: Raven Press, Ltd.

62. Zaneveld, L., De Jonge, C., Anderson, R., Mack, S.R. (1991) Human sperm capacitation and the acrosome reaction. *Hum Reprod*, **6**, 1265-1274.

Figure 1

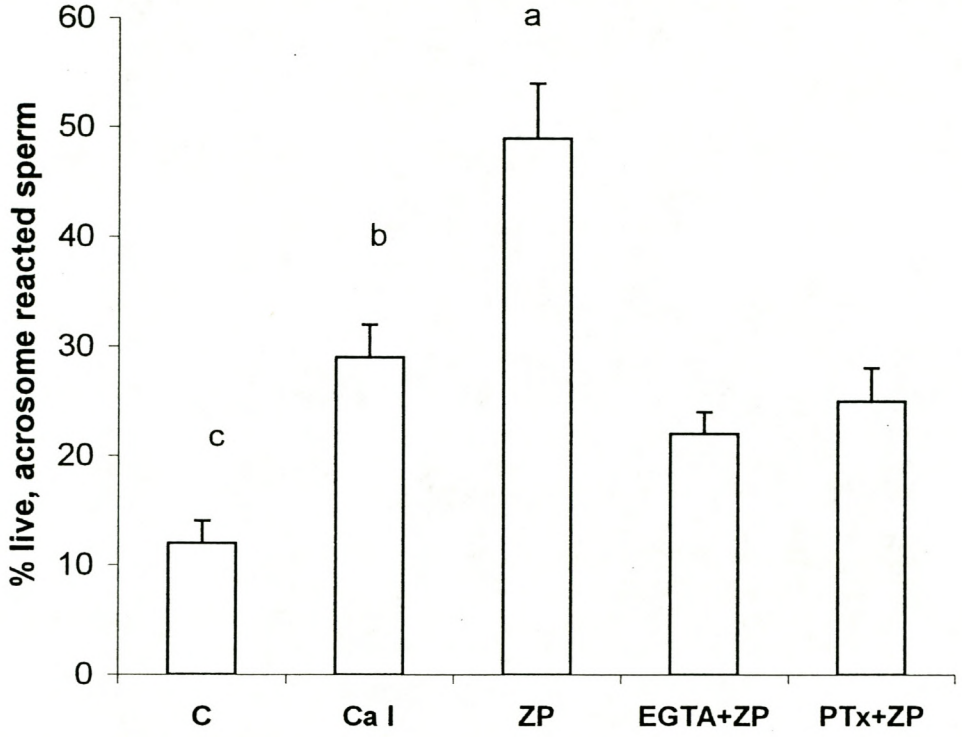


Figure 2

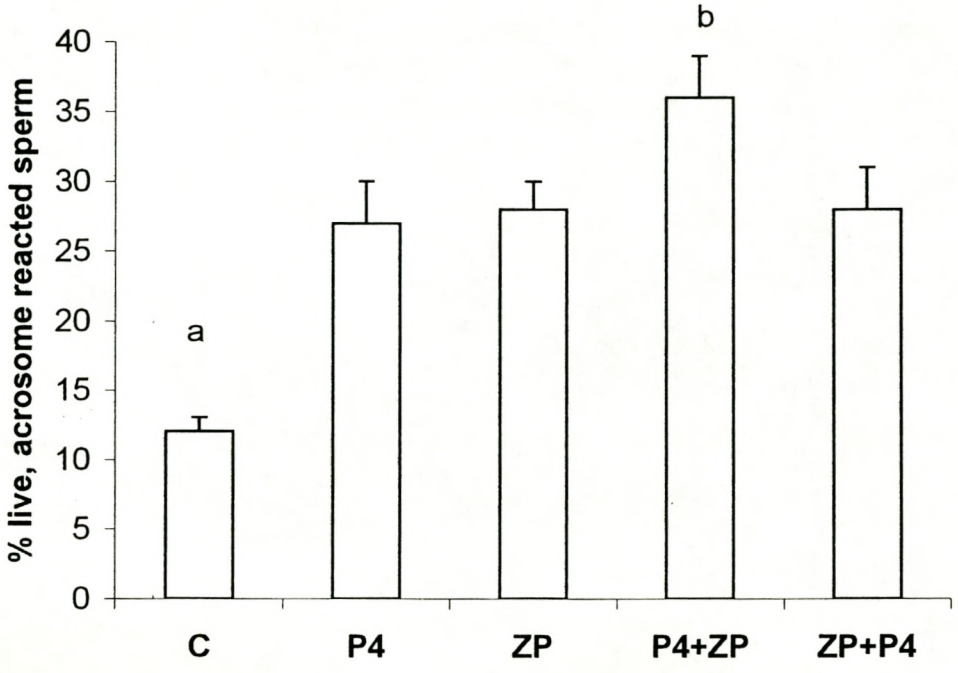


Figure 3

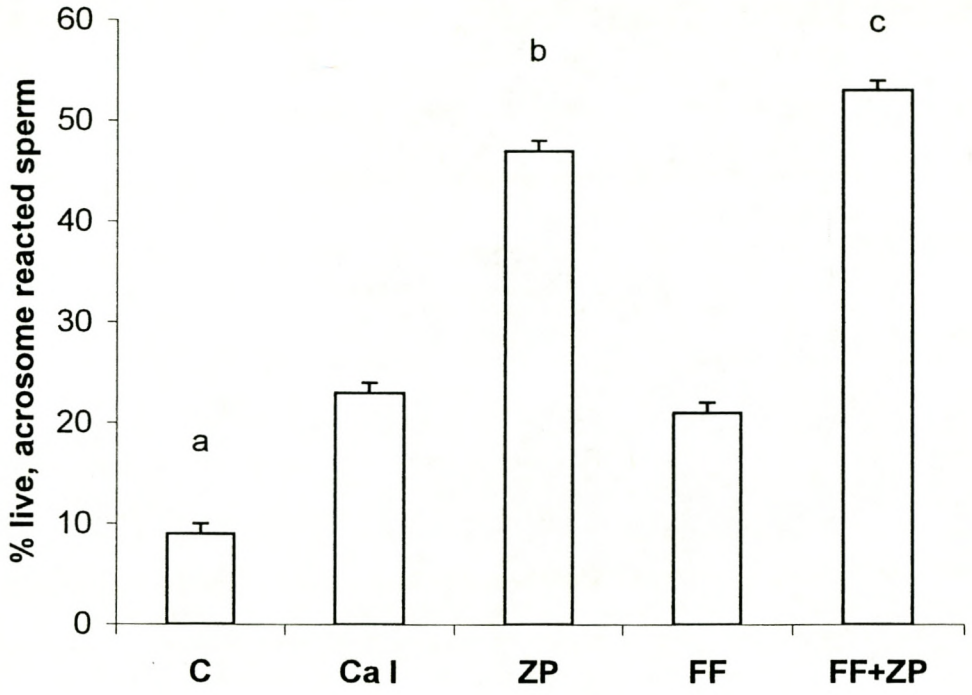
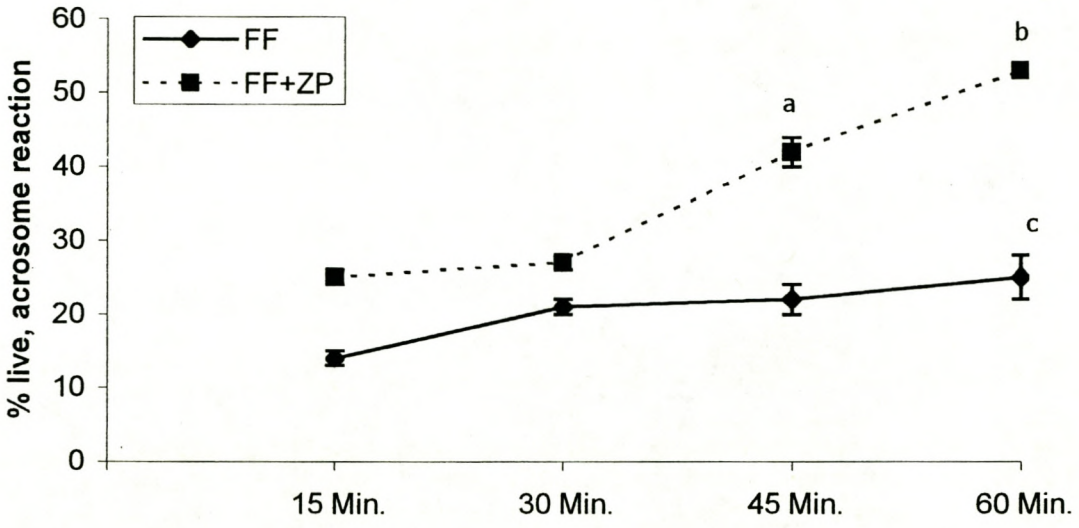


Figure 4



Legends to figures

Figure 1. Results of experiment 1. C: control conditions; CaI: calcium ionophore (5μM), ZP: solubilized zona pellucida (1.25 ZP/μL); EGTA (2.5 μM) + ZP (1.25 ZP/μL); PTx: pertussis toxin (100 ng/mL) + ZP (1.25 ZP/μL). [a]ZP vs C, CaI, EGTA + ZP and PTx +ZP: $p< 0.001$.
[b]CaI vs C: $p< 0.001$. [c]C vs EGTA + ZP and PTx + ZP: $p< 0.01$ and $p< 0.001$, respectively.

Figure 2. Results of experiment 2. C: control conditions; P4: progesterone (1.25 μg/mL); ZP: solubilized zona pellucida (0.5 ZP/μL); P4 (1.25 μg/mL) + ZP (0.5 ZP/μL); ZP (0.5 ZP/μL) + P4 (1.25 μg/mL). [a] C vs all other conditions: $p< 0.001$; [b]P4 + ZP vs ZP, P4 and ZP + P4: $p< 0.001$ $p< 0.01$ and $p< 0.01$, respectively).

Figure 3. Results of experiment 3a. C: control conditions. CaI: calcium ionophore (5μM); ZP: solubilized zona pellucida (1.25 ZP/μL); FF: follicular fluid (10%); FF (10%) + ZP (1.25 ZP/μL). [a]C vs all other conditions: $p< 0.001$. [b]ZP vs CaI and FF: $p< 0.001$. [c]FF + ZP vs FF and ZP: $p< 0.001$ and $p< 0.01$, respectively.

Figure 4. Results of experiment 3b. Spermatozoa were treated with follicular fluid (10%) for 60 min followed by solubilized zona pellucida (1.25 ZP/μL) for 15, 30 45 and 60 min. [a]45 min vs 15 and 30 min: $p< 0.01$, and vs 60 min: $p< 0.05$. [b] 60 min vs 15 and 30 min: $p< 0.001$. Control experiments were performed with incubation of spermatozoa with follicular fluid (10%) alone. [C]There were no significant differences for follicular fluid exposure at the different time points.

Chapter 4.

Role of the newly developed bioassays in the clinical ART setting

4a. *Specific aim 5*: To establish the predictive value of the HZA for fertilization outcome in IVF

Clinical significance of human sperm-zona pellucida binding

[1]Oehninger S, [1]Mahony M, [1]Ozgur K, [1]Kolm P, [2]Kruger T, [2]Franken D.

Fertility and Sterility 1997, 67:1121-1127

[1] The Jones Institute for Reproductive Medicine,
Department of Obstetrics and Gynecology,
Norfolk, Virginia 23507, USA, and
[2]Tygerberg Reproductive Unit,
University of Stellenbosch, Cape Town, South Africa

Abstract

The objective is to assess the relationship between sperm morphology and motion parameters and sperm-zona-pellucida (ZP) binding capacity under hemizona assay (HZA) conditions and to determine the discriminatory power of the HZA for the prediction of the *in vitro* sperm fertilizing ability. There were 196 couples undergoing IVF therapy participating in this study, (Hemizona assay and IVF results were determined for each couple). Each main outcome measure(s) were computerized sperm motion analyses, sperm morphology (strict criteria), and HZA results were correlated with fertilization outcomes. The results among the sperm parameters from the original ejaculates, morphology was the best predictor of sperm-ZP binding ability; hyperactivated motility was the best predictor of HZA results after swim-up separation of the motile sperm fractions. The HZA index provided the highest discriminatory power for fertilization success/failure, with an overall accuracy of 86%. In conclusion, the sperm morphology and hyperactivated motility showed a high correlation with the capacity of sperm to achieve tight binding to the ZP. The excellent positive and negative predictive values of the HZA for fertilization outcome provide additional support for the use of this functional bioassay in the decision-making process within the assisted reproduction setting.

Introduction

The prediction of the capacity of human sperm to achieve fertilization under in vitro conditions has been a major goal within the assisted reproductive technology setting. However, because fertilization entails a complex series of events leading to early embryo development, it is unlikely that the evaluation of a single sperm feature a function can be predictive in an absolute fashion of fertilization potential of the male gamete. For the correct identification of sperm dysfunctions, we have been promoters of a sequential, multistep diagnostic scheme that includes [1] the so-called "basic" semen analysis, [2] the assessment of the variety of sperm functions and biochemical features, and [3] bioassays that directly examine sperm-oocyte interaction (Oehninger *et al.*, 1991; Oehninger *et al.*, 1992, Oehninger *et al.*, 1995).

Different laboratories have highlighted the diagnostic power of variety of such assays, and the World Health Organization has incorporated some of these assays under the category of functional tests (World Health Organization, 1992). Nevertheless at a recent Consensus Workshop in Advanced Andrology (ESHRE Special Interest Group), it was agreed that better standardization of the majority of these methods should be implemented before they are introduced as routine clinical tools (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996). It was concluded and recommended that because of the powerful evidence for prediction of fertilization success/failure in the IVF arena, sperm-zona pellucida (ZP) binding tests should be incorporated in the workshop (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996; Liu, *et al.*, 1988; Liu, *et al.*, 1989; Burkman, *et al.*, 1988). Notwithstanding some practical limitations of the sperm-ZP assays (mainly the availability of human oocytes), we have included the hemizona assay (HZA) as part of our routine advanced diagnostic scheme. The HZA offers the main advantages of [1] amplification of the required number of oocytes by using a single oocyte per experiment; [2] an internally controlled assay (provided by matching hemizona surfaces); and [3] avoiding inadvertent fertilization by splitting the egg microsurgically (Oehninger, *et al.*, 1991; Oehninger, *et al.*, 1992; Burkman, *et al.*, 1988).

In our laboratories in Norfolk, Virginia and Cape Town, South Africa, we have implemented identical methods of semen analysis at both the basic and functional levels. The same techniques have been used for the performance of the semen analysis and the HZA, and similar standards are used for the identification and diagnosis of male infertility and patient selection for IVF therapy (Acosta, 1992: Franken, *et al.*, 1993).

Here, we have combined our experiences to evaluate the clinical significance of sperm-ZP binding as assessed by the HZA. Our specific objectives were [1] to determine the relationship between different sperm characteristics (from the original ejaculates and from the processed motile fractions) and sperm-ZP binding potential; and [2] to examine the role of HZA as a diagnostic tool for prognosticating fertilization in vitro. We prospectively studied a large number of infertile couples before IVF therapy; results from robust statistical analyses corroborate the discriminatory power of the HZA as predictor of fertilization and its validity to direct patients to intracytoplasmic sperm injection (ICSI) therapy.

Materials and Methods

Patients

These studies were performed under the approval of the Institutional Review Board of the Eastern Virginia Medical School and the University of Stellenbosch. A total of 196 couples who underwent 361 IVF cycles were included during the period from January 1993 through March 1995. One hundred fourteen couples (220 IVF cycles) were studied in Norfolk, whereas 82 couples (141 IVF cycles) were studied at Tygerberg. The female partners either were normal or were diagnosed as having tubal infertility.

Study Design

Semen samples were provided (n = 196) before IVF therapy and were assessed for sperm concentration, motion parameters, and morphology after liquefaction. The procedure of HZA (n = 196) was performed in the same ejaculates after separation of the motile sperm fraction wash/swim-up (Oehninger *et al.*, 1991: Oehninger, *et al.*, 1992). For a subset of patients (n = 33), motion parameters also were assessed immediately after the swim-up procedure. All 196 couples underwent IVF therapy within 3 months of the semen evaluation.

Semen Evaluation

Semen samples were obtained by masturbation after 2 to 4 days sexual abstinence and were studied after liquefaction was complete and within 1 hour of collection. Samples had a

normal seminal volume (range 1.1 to 5 mL), had <1 x 10^6 round cells (leukocytes, by peroxidase staining, and immature sperm cells), and were negative for antisperm antibodies (direct immunobead test) (Oehninger, 1995; van der Merwe, *et al.,* 1990). Fertile men (10 donors routinely used as controls in the HZA) had fathered a pregnancy within 2 years and had negative bacteriologic semen cultures. After liquefaction, samples were subjected to a computer-assisted evaluation of sperm concentration and motion parameters while a slide was prepared for morphology assessment.

Sperm motion was evaluated with the HTM-IVOS Motility Analyzer (Hamilton-Thorne Research Inc., Danvers, MA). Each sperm sample (a 3-μL fraction of liquefied semen or swim-up fraction) was loaded onto a four-chamber micro cell slide (20 μm deep) (Vitro Dynamics, Rockaway, NJ). Each chamber was transferred to the HTM-IVOS where it was maintained at 37°C for 1 minute before the start of data acquisition. Data collection was completed on randomly selected fields along the length of the microcell chamber until ≥ 100 motile sperm were analyzed (Mahony *et al.,* 1991).

The pertinent settings used during HTM-IVOS assessment were as follows: frame acquired, 30; frame rate, 60 Hz; minimum contrast, 85; minimum cell size, 4 pixels; low average path velocity (VAP) cutoff 5.0 μm/s; medium VAP cutoff, 25.0μm/s; head size, nonmotile, 12 pixels; head intensity nonmotile, 130; and straightness threshold, 80.0%. At the outset of each experiment, we verified that the settings permitted accurate differentiation of motile sperm versus nonmotile sperm debris by using "playback" option. During playback, the motions of sperm in the previous field where replayed; a red dot was located over the head of the all-motile sperm for each frame and a blue dot was positioned over the head of nonmotile spermatozoa. When errors were detected, the settings were adjusted until the spermatozoa were identified correctly as motile and nonmotile (Mahony, 1991). The parameters assessed by the computer were sperm concentration (10^6/mL), total motile concentration, (10^6); and VAP (μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCLμm/s), lateral amplitude (ALH, μm), beat frequency (Hz), straightness (%), linearity (%), and hyperactivated motility (%). To differentiate hyperactivated sperm from those that were not hyperactivated, the following settings were used in the automatic SORT program for the HTM-IVOS, as previously described by Berkman (Burkman, 1984): VCL (the velocity derived from all 20 head positions), 100 to 500 μm/s; linearity (VSL/VCL, a measure of the straightness of the trajectory), 0 to 65; maximal lateral head displacement (ALH, a measure if the side-to-side movement of the head), 7.5 to 100 μm;

VSL (the velocity based on the first and the last head positions only) 20 to 500 μm/s and VAP (five-point running average), 40 to 500 μm/s.

Morphology slides were prepared for each patient after semen liquefaction and were stained by the Quick stain technique (Kruger *et al.*, 1986). The slides were read on the same day at a magnification of x 1,000. Two independent observers each evaluated sperm morphology pattern counting 200 cells and using strict criteria (Kruger *et al.*, 1986: Kruger *et al.*, 1988). This method of evaluation had an inter-technician coefficient of variation (CV) and an intra-technical variability that are not significant in our laboratories (Spearman rank correlation coefficient, r = 0.87 and 0.97, respectively) (Kruger *et al.*, 1988).

Hemizona Assay

Salt-stored, immature human oocytes (obtained from surgically removed ovarian tissue, postmortem or after IVF) were used in the experiments after desalting and microbisection into matching hemizona following procedures extensively published elsewhere (Oehninger, *et al.*, 1991: Oehninger, *et al.*, 1992: Burkman, *et al.*, 1988: Franken, *et al.*, 1993 and Franken, *et al.*, 1990). A was/swim-up separation of the motile sperm fraction was effected in Ham's F-10 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 0.3% human serum albumin (Irvine Scientific, Santa Anna, CA) (1,2,8,10,16). Control (fertile donor) and test (patient) sperm droplets (100 μL of a final dilution of 0.5 x 10^6 motile sperm per mL) were incubated separately under heavy white mineral oil (Sigma Chemical Co., St Louis, MO) with the matching hemizona from the same pair for 4 hours at 37°C in 5% CO_2 in air. After the coincubation period, the hemizonae were washed to remove loosely attached sperm, using a finally drawn glass pipette, and the sperm tightly bound to the other zona surface were counted under phase contrast microscopy (x200) (Burkman, *et al.*, 1988: Franken, *et al.*, 1993: Franken, *et al.*, 1990).

In vitro Fertilization Procedures

Ovarian stimulation was accomplished in Norfolk using a combination of GnRH agonist (leuprolide acetate, luteal suppression) and gonadotropins (Oehninger *et al.*, 1991: Oehninger *et al.*, 1992); a combination of clomiphene citrate and gonadotropins was used at Tygerberg (van der Merwe, *et al.*, 1990). Patients were selected only on the basis of having ≥ 2 preovulatory oocytes (metaphase II at aspiration) in Norfolk and ≥ 6 preovulatory oocytes at Tygerberg (in

the latter center, four mature oocytes were allocated randomly for GIFT and the remaining ones used for IVF). For IVF oocytes were inseminated 5 hours after retrieval at a standard concentration of 1 x 10^5 motile spermatozoa per mL per oocyte after separation of the motile sperm fraction. In male infertility cases (defined a < 20 x 10^6 sperm per mL, < 30% progressive motility, and/or ≤ 4% normal morphology), the number of spermatozoa was increased (if possible) to 5 x 10^5 motile spermatozoa per mL per oocyte (Oehninger, *et al.*, 1991: Oehninger, *et al.*, 1992: Oehninger, 1995: Oehninger, *et al.*, 1998). In some cases, and because of low sperm recovery, a second semen sample, obtained 3 hours after the original one, was pooled with the initial sample (after sperm separation). Normal (diploid) fertilization was diagnosed after visualization of two pronuclei 18 hours post insemination.

Statistical Analysis

The relationship between sperm parameters (concentration, motion characteristics, and morphology from the original and processed samples) and HZA results (expressed as the absolute number of sperm tightly bound to the hemizona) were assessed by all possible subset regressions (which analyzes the contribution of all possible combination of these parameters) and stepwise regression analyses. Fertilization outcome was evaluated after division of the patients into those with ≥ 60% and < 60% fertilization rate (the mean minus two SDs of the normal fertilization rate of preovulatory oocytes averaged from Norfolk and Tygerberg programs). Logistic regression analysis was performed to identify the most statistically valid HZA results (expressed as HZA index = [number of sperm tightly bound for patients/controls] x 100) predictive of fertilization rate. Linear discriminate analysis was used to determine the sensitivity, specificity, and predictive value of the HZA index as a prognostic index of fertilization outcome. Student's t-test and paired t-test were used as appropriate. Results are presented as the means ± SE. P values < 0.05 were considered significant.

Results

Relationship between Sperm Parameters and Sperm-ZP Binding

(Table 1) presents the results of sperm motion analysis (in the original ejaculates and following swim-up separation of motile sperm function), morphology (in original samples), and HZA out come for 33 infertile patients tested against 33 ejaculates from fertile controls. To assess the best regression model of all sperm variables of the original samples as predictors of

sperm-ZP binding (HZA), all possible subsets regression and stepwise regression were used. Morphology was the obvious best single predictor of the HZA. (Figure 1) depicts the scatter diagram of normal morphology vs. HZA results with 95% confidence interval (CI) and prediction interval.

Any predictive model with morphology had essentially the same R^2 value as morphology alone. Even with all independent variables in the equation, the R^2 value increased by only 4% (R^2 of 0.71 compared with 0.67). The stepwise regression analysis resulted in the same conclusion. Morphology was the first an only variable to enter the equation ($P < 0.0001$). After adjustment for morphology, no other variable was statistically was statistically significant. The regression equation for morphology was HZA = -1.0758+1.8105 (morphology).

For the swim-up samples, none of the sperm variables analyzed were excellent predictors of HZA outcome (R^2 value of 0.32). The stepwise analysis entered hyperactivated motility and the total motile sperm concentration first. When the analysis was adjusted for these two, no other variables were strategically significant. The regression equation was HZA = 10.1590 + 0.0892 (total motile) + 0.6240 (hyperactivated motility). Although the regression equation was statistically significant ($P < 0.0001$), the model was not a good fit to the data as indicated by the multiple R^2 value of 0.27. (Figure 2) presents the scatter diagram of hyperactivated motility vs. sperm-ZP binding with the 95% CI and prediction interval.

Hemizona Assay Index as Predictor of fertilization Outcome in IVF

(Table 2) presents the semen analysis results, HZA index, and fertilization outcome of the 196 patients segregated by the diagnosis of normal (n = 138) and abnormal (n = 158) semen parameters. The relationship between sperm parameters/functions and fertilization outcome was analyzed using a cutoff fertilization level of 60% as discussed above. Stepwise regression analysis for prediction of fertilization showed HZA that the HZA index was the most predictive parameter (R^2 = 43.1%). Adding motility to the model increased the predictive power to an R^2 of 58.3%. No other variable significantly enhanced the prediction of fertilization rate.

Logistic regression analysis was preformed to identify the most statistically valid HZA index predictor of fertilization outcome. For the prediction of good or successful fertilization vs. poor or failed fertilization (≥60% vs. <60%), an HZA index of 30 provided the best predictive

power (this is a cutoff HZA index level similar to the one reported earlier by our two groups) (Oehninger *et al.*, 1991: Oehninger *et al.*, 1992: Oehninger, 1995, Franken, *et al.*, 1993,Coddington *et al.*, 1994). Linear discriminate analysis reveled a sensitivity of 93%, specificity of 73%, positive predictive value of 85% (i.e., power of an HZA index ≥30 to predict successful fertilization ≥60%), and negative predictive value of 87% (i.e., power of an HZA index <30 to predict poor fertilization <60%). The false-positive rate was 27% and the false-negative rate was only 6%; the overall correct predictive ability of the HZA for fertilization outcome was 86% (28 classification errors in 196 observations.)

Discussion

The first objective of this study was to assess the relationship between the basic sperm parameters and the capacity of sperm to achieve tight binding to the capacity of sperm to achieve tight binding to the homologous ZP. The HZA offers a unique means to assess this functional step in standardization repeatable, and previously validated homologous bioassay (Oehninger *et al.*, 1991: Oehninger *et al.*, 1992: Oehninger, 1995: Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996; Burkman, 1998: Franken, *et al.*, 1993: Franken, *et al.*, 1990: Coddington, *et al.*, 1994: Gamzu, *et al.*, 1994). A distinct definition of that main factors affecting data interpretation has been reported (i.e., precision of zona cutting, kinetics of binding, oocyte source and storage methods, influence of oocyte maturation status and prior oocyte contact with spermatozoa, and intraassay CV) (Clark, *et al.*, 1995: Oehninger, *et al.*, 1992). The specificity of gamete interaction under HZA conditions is strengthened by the fact that spermatozoa tightly bound to the ZP have undergone a zona-induced acrosome reaction (AR) (Clark, *et al.*, 1995: Oehninger, *et al.*, 1992). The specificity of gamete interaction under HZA conditions is strengthened by the fact that those spermatozoa tightly bound to the ZP undergone a zona-induced acrosome reaction (AR) (Clark, *et al.*, 1995: Oehninger, *et al.*, 1992). Moreover, results of interspecies experiments have demonstrated a high specificity of human sperm-ZP functions in the HZA (Oehninger, *et al.*, 1993). Taken together, these results provide compelling evidence in support of the use if this bioassay as a test for human gamete interaction, including sperm-ZP binding and ZP-induced AR (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996: Clark *et al.*, 1995: Oehninger, *et al.*, 1992: Oehninger, *et al.*, 1993).

To fulfill our first objective, we studied sperm parameters of both the original (liquefied) ejaculates and the sperm motile, fractions (post-swim-up). For the original samples, sperm morphology (assessed by strict criteria) was an excellent and single best predictor of sperm-ZP binding capacity. These results confirm our previous reports (Oehninger, *et al.,* 1991: Oehninger, *et al.,* 1995; Franken, *et al.,* 1993) depicting the negative impact of teratozoospermia on gamete binding and the potential use of the HZA to determine the optimal sperm insemination concentration at the time of IVF in such patients.

Among those parameters of the separated motile sperm fractions analyzed, hyperactivated motile sperm fractions analyzed, hyperactivated motility was a very good predictor of ZP binding. Although controversy still exists regarding both the definition and physiological significance of hyperactivated motility, our data demonstrated a positive relationship between hyperactivated motility and HZA results. Theoretically, both a prematurely induced hyperactivated motility and delayed or failed hyperactivated motility could contribute to a low incidence sperm-ZP collisions and/or a defective ZP penetration. Although more studies are needed to establish the exact temporal relationship between the capacitated state of sperm, hyperactivated motility, and ZP binding, these results highlight a possible use of the objective determination of hyperactivated motility in the vicinity of the prediction of sperm function in the vicinity of the oocyte. Others (Oehninger, *et al.,* 1996) also have shown that the quality if motility correlates positively with the number of sperm bound to the ZP using a different assay.

To fulfill our second objective, we correlated HZA results with fertilization outcome in a large sample of IVF patients. Here, we corroborated and extended previous reports (Oehninger, *et al.,* 1991, Oehninger, *et al.,* 1992: Oehninger, *et al.,* Franken, *et al.,* Franken, *et al.,* 1990; Gamzu, *et al.,* 1994: Oehninger, *et al.,* 1992) of the high discriminatory power of the HZA for fertilization success/failure. This also has been demonstrated using a different sperm-ZP binding assay (Liu, *et al.,* 1988: Liu, *et al.,* 1989: Liu, *et al.*, 1992). The HZA was able to predict fertilization outcome correctly in 86% of the cases. Importantly, the incidence of false-negative results (i.e., the failure of a low HZA index correctly to predict poor fertilization outcome) was extremely small. This represents an important finding, because it is clinically significant to be able it predict poor fertilization outcome correctly in 86% of the cases. Importantly, the incidence of false-negative results (i.e., the failure of a low HZA index correctly to predict poor fertilization outcome) was extremely small. This represents an important finding, because it is clinically significant to be able to establish prospectively which

patients are at high risk of fertilization failure. Because fertilization entails other processes beyond sperm-ZP interaction, it is anticipated that some false-positive results should occur (i.e., patients with a high HZA index still may not be able to fertilize successfully).

Based on the present results, and following the recommendation of the ESHRE Consensus Workshop (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996), we are using prospectively the HZA to identify patients who are at significant risk of fertilization failure in IVF. We therefore propose that the HZA may be used as a discriminating functional test in the decision making process to allocate patients to IVF vs. ICSI. Our present indications for ICSI include [1] poor "basic" semen parameters, ($1x10^6$ total motile after separation and/or an HZA index <30), [2] previous failed fertilization, and [3] presence of obstructive or non-obstructive azoospermia in cases in which ICSI is combined with sperm extraction from the testes or epididymis. A question that remains to be answered is whether ICSI will be favored in those cases in which IVF is preformed with a high insemination concentration (i.e., cases with severe teratozoospermia with an adequate motile fraction) (Oehninger, *et al.*, 1988). Preliminary evidence from our laboratories indicates that ICSI results in a superior embryo quality that leads to improved implantation rates compared with high insemination concentration in such cases (Oehninger, *et al.*, 1992).

We conclude that [1] sperm morphology assessed by strict criteria and hyperactivated motility are the most significant factors related to sperm-ZP binding and [2] the HZA is an excellent predictor of sperm fertilizing potential and should be incorporated as a functional test to direct patients to IVF or assisted fertilization.

References

1. Acosta A. (1992) Male factor in assisted reproduction. *Infertil Reprod Clin North Am*, **3**, 487-503.
2. Burkman L, Coddington C, *et al.*, (1988) The hemizona assay: development of a diagnostic test for the binding of spermatozoa to the human zona pellucida to predict fertilization potential. *Fertil Steril*, **49**, 688-693.
3. Burkman L. (1984) Characterization of hyperactivated motility by human spermatozoa during capacitation: comparison of the fertile and oligozoospermic sperm population. *Arch Androl*, **13**, 153-165.
4. Clark G, Patankar M, *et al.*, (1995) New concepts in human sperm-zona pellucida interaction. *Hum Reprod*, **10**, 31-37.
5. Coddington C, Oehninger S, *et al.*, (1994) Hemizona index (HZI) demonstrates excellent predictably when evaluating sperm fertilization capacity in *in vitro* fertilization patients. *J Androl*, **15**, 250-254.
6. Consensus Workshop on Advanced Diagnostic Andrology Techniques. (1996) ESHRE Andrology Special Interest Group. *Hum Reprod*, **11**, 1463-1479.
7. Franken D, Kruger T, *et al.*, (1993) The ability of the hemizona assay to predict human fertilization in different and consecutive in vitro fertilization in different and consecutive in vitro fertilization cycles. *Hum Reprod*, **8**, 1240-1244.
8. Franken D, Kruger T, *et al.*, (1990) Hemizona assay and teratozoospermia: increasing sperm insemination concentration to enhance zona pellucida binding. *Fertil Steril*, **50**, 497-503.
9. Gamzu R, Yogev L, *et al.*, (1994) The hemizona assay is of good prognostic value for the ability of sperm to fertilize oocytes in vitro. *Fertil Steril*, **62**, 1056-1059.
10. Kruger T, Acosta A, *et al.*, (1988) Predictive value of abnormal sperm morphology in *in vitro* fertilization. *Fertil Steril*, **49**, 112-117.
11. Kruger T, Menkveld R, *et al.*, (1986) Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril*, **46**, 1118-1123.
12. Liu D, Gordon Baker H. (1992) Sperm nuclear chromatin normality: relationship with sperm morphology, sperm-zona pellucida binding and fertilization rates in vitro. *Fertil Steril*, **58**, 1178-1184.
13. Liu D, Clarke G, *et al.*, (1989) A sperm-zona pellucida binding test and in vitro fertilization. *Fertil Steril*, **52**, 281-287.

14. Liu D, Lopata A, *et al.,* (1988) A human sperm-zona pellucida binding test using oocytes that failed to fertilize in vitro. *Fertil Steril*, **50**, 782-788.

15. Mahony M, Oehninger S. (1991) Fucoidin inhibits the pellucida-induced acrosome reaction in human spermatozoa. *Contraception*, **44**, 657-665.

16. Oehninger S, Kruger T, *et al.,* (1996) A comparative analysis of embryo implantation potential in patients with sever teratozoospermia undergoing in vitro fertilization with high insemination concentration or intra cytoplasmic sperm injection. *Human Reprod*, **11**, 1086-1089.

17. Oehninger S, Mahony M, *et al.,* (1993) The specificity of human sperm/zona pellucida interaction under hemizona assay conditions. *Mol Reprod Dev*, **35**, 57-61.

18. Oehninger S, Franken D, *et al.,* (1992a) Hemizona assay and its impact on the identification and treatment of human sperm dysfunctions. *Andrologia* , **21**, 307-321.

19. Oehninger S, Toner J, *et al.,*(1992) Prediction of fertilization in vitro with human gametes: is there a litmus test? *Am J Obstet Gynecol*, **167**, 1760-1767.

20. Oehninger S, Acosta A, *et al.,* (1991) Recurrent failure of in vitro fertilization role of the hemizona assay in the sequential diagnosis of specific sperm- oocyte defects. *Am J Obstet Gynecol*, **164,**1210-1215.

21. Oehninger S, Acosta A, *et al.,* (1988) Corrective measures and pregnancy outcome in IVF in patients with sever sperm morphology abnormalities. *Fertil Steril*, **50**, 283-297.

22. Oehninger S. An update on the laboratory assessment of male fertility. *Hum Reprod*, **10**, 38-45.

23. van der Merwe J, *et al.,* (1990) Treatment of male sperm autoimmunity by using the gamete intrafallopian transfer procedure with washed spermatozoa. *Fertil Steril*, **53**, 682-687.

24. World Health Organization. (1992) WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. *3rd Edition, Cambridge: Cambridge University Press*, **23**.

Figure 1

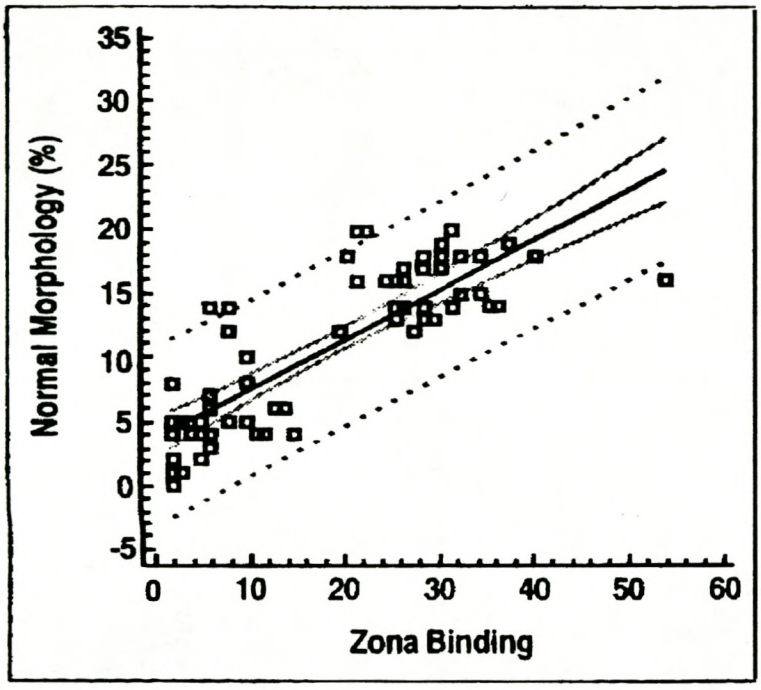
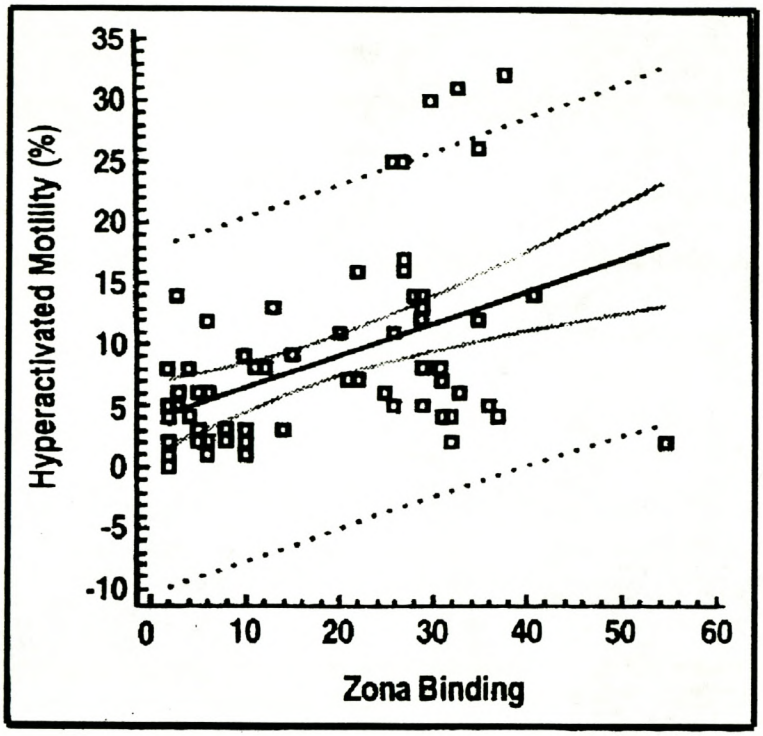


Figure 2



Legends Figures

Figure 1. Scatter diagram of normal morphology against sperm-ZP binding results with the 95% CI (this interval includes the true regression line with 95% probability) and the 95% prediction interval (these curves are parallel to and wider than the 95% CI and represent the 95% probability for the values of the dependent variable).

Figure 2. Scatter diagram of hyper activated motility against sperm-ZP binding results with the 95% CI and prediction interval.

Table I

Sperm Motion Analysis Results and HZA Outcome of 33 Infertile Men in the Original Ejaculates and After Separation of the Motile Fraction by Swim-up*

	Total motile ($\times 10^6$)	Motility	Morphology	VAP	VSL	VCL	ALH	Beat frequency	Straightness	Linearity	Hyperactivated motility	HZA†
		%	%	μm/s	μm/s	μm/s	μm	Hz	%	%	%	
Original sample												
Patients	83.3 ± 13.2	56.4 ± 4.5	5.4 ± 0.6	35.4 ± 1.6	28.6 ± 1.4	50.8 ± 2.3	4.2 ± 0.1	13.3 ± 0.3	76.3 ± 0.8	55.3 ± 1.1	2.9 ± 0.6	—
Controls	270.0 ± 16.7	78.9 ± 2.5	15.9 ± 0.4	43.7 ± 1.0	34.1 ± 0.9	60.4 ± 1.2	4.2 ± 0.1	11.8 ± 0.5	74.1 ± 0.7	55.1 ± 1.1	3.2 ± 0.5	—
P	<0.00001	<0.00001	<0.00001	<0.0001	0.002	0.001	>0.5	<0.02	>0.5	>0.5	>0.5	—
Motile fraction												
Patients	9.7 ± 1.2	69.3 ± 3.6	—	44.9 ± 1.3	39.9 ± 1.3	65.4 ± 1.4	4.5 ± 0.1	16.6 ± 0.2	83.0 ± 1.1	59.5 ± 1.5	4.8 ± 0.6	6.2 ± 0.6
Controls	48.0 ± 11.4	80.2 ± 3.4	—	55.5 ± 1.8	47.6 ± 1.7	80.1 ± 2.1	5.1 ± 0.1	16.5 ± 0.3	81.4 ± 0.9	57.7 ± 1.9	12.4 ± 1.5	30.1 ± 1.1
P	<0.001	<0.03	—	<0.002	<0.001	<0.0001	<0.0006	>0.5	>0.5	>0.5	<0.0001	<0.00001

* Values are means ± SE.

† No. of sperm bound.

Table II

Semen Parameters, HZA Outcome (Expressed as HZA Index), and Fertilization Results (in IVF) for 196 Patients Divided According to the Diagnosis of Normal and Abnormal Semen Parameters*

	Concentration	Motility	Morphology	HZA index	Fertilization rate
	(10^6/mL)	%	%		%
Normal semen parameters (n = 138)	81.0 ± 11.7	66.5 ± 4.0	12.1 ± 2.0	60.8 ± 5.9	64.2 ± 7.6
Abnormal semen parameters (n = 58)	26.0 ± 5.4	31.9 ± 6.3	3.0 ± 1.7	28.1 ± 10.4	27.6 ± 1.0
P	<0.0001	<0.0001	<0.002	<0.001	<0.0006

* Values are means ± SE.

4b. *Specific aim 6*: To integrate the HZA and induced-acrosome reaction assays into a novel, sequential diagnostic scheme in andrology and to compare results of methodologies currently available to assess sperm functional capacities by a meta-analytical approach

Sperm Function Assays And Their Predictive Value For Fertilization Outcome In IVF Therapy: A Meta-Analysis.

[1]Sergio Oehninger, [4]Daniel R. Franken, [2]Ellen Sayed, [1]Gerardo Barroso and [3]Paul Kolm

Human Reproduction Update 2000, 6:160-168

[1]The Jones Institute for Reproductive Medicine, Department of Obstetrics and Gynecology; [2]Moorman Memorial Library; [3] Bio-statistics, Eastern Virginia Medical School; and [4]Reproductive Biology Unit, Department of Obstetrics and Gynecology, University of Stellenbosch, Tygerberg, Republic of South Africa

Abstract

The prevalence of male infertility and the availability of new, highly successful therapeutic options make the testing of sperm functional competence mandatory. An objective, outcome-based examination of the validity of the currently available assays was performed based upon the results obtained from 2,906 subjects evaluated in 34 prospectively designed, controlled studies. The aim was carried out through a meta-analytical approach that examined the predictive value of four categories of sperm functional assays (computer-aided sperm motion analysis or CASA, induced-acrosome reaction testing, sperm penetration assay or SPA, and sperm-zona pellucida binding assays) for in vitro fertilization (IVF) outcome. Results demonstrated a high predictive power of the sperm-zona pellucida binding and the induced-acrosome reaction assays for fertilization outcome. On the other hand, the findings indicated a poor clinical value of the SPA as predictor of fertilization and a real need for standardization and further investigation of the potential clinical utility of CASA systems. This analysis points out to limitations of the current tests and the need for standardization of methodologies and provides objective evidence in which clinical management and future research can be directed.

Introduction

The clinical (andrological) investigation of the male partner in an infertile couple relies on a thorough history and physical examination. Additionally, urological and endocrinological investigations should be implemented as needed. Nonetheless, the semen analysis still remains the cornerstone of the diagnostic management. We and others have been promoters of a sequential, multi-step diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics when abnormalities are found in the initial evaluation (Oehninger, *et al.*, 1991; Amman and Hammerstedt, 1993; Oehninger, *et al.*, 1997a). This diagnostic scheme should include (i) assessment of the "basic" semen analysis and (ii) functional testing of spermatozoa. The "basic" semen analysis performed by the infertility specialist should include the evaluation of physical semen characteristics (volume, pH, agglutination and viscosity), the assessment of sperm concentration, progressive motility and normal morphology, the examination of sperm vitality, presence of leukospermia and immature sperm cells, detection of antisperm antibodies and a bacteriologic investigation (WHO, 1992). The investigation of the total motile sperm fraction following a separation procedure should also be implemented in this step (Oehninger, 1995).

If abnormalities are found during the basic investigation, the work-up should progress to the examination of specific sperm functions. The questions that immediately arise are (a) which sperm functions should be examined? and (b) what validated tests are available?

Numerous assays have been proposed to assess the various sperm functions. The categories of assays that are usually considered include: (1) tests that examine defective sperm functions indirectly through the use of biochemical tests (i.e., measurement of the generation of reactive oxygen species or evidence of peroxidative damage, measurement of enzyme activities such as creatine phosphokinase and others); (2) bioassays of gamete interaction (i.e., the heterologous zona-free hamster oocyte test and the homologous sperm-zona pellucida binding assays) and induced-acrosome reaction scoring; and (3) computer-aided sperm motion analysis (CASA) for the evaluation of sperm motion characteristics (Yanagimachi, *et al.*, 1976; Cross, *et al.*, 1986; Burkman, *et al.*, 1988; Liu, *et al.*, 1988; Aitken, *et al.*, 1989 and 1991; Mortimer, *et al.*, 1990a and b; WHO, 1992; Huszar, *et al.*, 1992; Huszar and Vigue, 1994; Consensus Workshop, ESHRE Andrology Special Interest Group, 1996). Different laboratories have highlighted the diagnostic power of a variety of such assays particularly related to the outcome of assisted reproductive technologies. In vitro fertilization and embryo transfer therapy (IVF), singularly, offers the

advantage of making use of fertilization as a main outcome measure of successful sperm functional capacities. Embryo implantation potential and pregnancy are obviously the ultimate desired outcome but their multi-factorial nature makes it more difficult to utilize them as specific end points.

What is the overall significance of sperm functional testing and which test(s) should be chosen? The truth of the matter is that the "basic" semen evaluation allows the diagnosis of "male infertility," usually without providing evidence for an etiologic or physiopathologic origin (except in inflammatory-infectious or immunologic cases). Furthermore, if no specific therapy is indicated (i.e., urological, hormonal, pharmacological or other) or these treatments have failed, if the diagnosis is "idiopathic" infertility, or if the degree of sperm abnormalities is severe enough to refer the couple to ART, there is a real need to determine the sperm functional capacity in order to direct treatment to IUI, IVF or ICSI. The answer then is that there is a real need to assess sperm functional competence in the extended evaluation of the infertile man.

How often do clinicians involved in reproductive medicine care confront this situation? Infertility in general and the proportion of cases where a male factor can be identified as the cause (alone or in combination with female factors) are prevalent conditions (Hull, *et al.*, 1985; Mosher and Pratt, 1990; Wilcox and Mosher, 1993; SART, American Society for Reproductive Medicine, 1995; Centers for Disease Control and Prevention of the U.S. Department of health and Human Services, 1997). Although diagnostic problems make it difficult to establish the extent of the male partner's contribution with certainty, a number of studies suggest that male problems represent the commonest single defined cause of infertility (Irvine, 1998). In addition, currently available therapeutic options (both urological and assisted reproductive technologies) are extremely successful in aiding couples achieve conception. The answer to the question, therefore, is that sperm function examination using validated tests would be extremely valuable for an improved clinical management in reproductive medicine.

Evidence-based medicine (EBM) has been defined as the judicious and conscientious use of current best evidence from medical care research for making medical decisions (Swets, 1988; Evidence-Based Medicine Working Group, 1992; Jaeschke, *et al.*, 1994; Sackett, 1995; Sackett, *et al.*, 1996; Collins, 1997; Schlesselman, 1997). EBM has now been extensively used in Gynecology and in some fields of ART, but the discipline of Andrology has been late in accepting the need for controlled trials (Evers, 1997; Nieschlag, 1997). Although various reports have recently assessed

the value of applying EBM to therapeutic modalities, to date, no studies have examined in depth the clinical and research evidence supporting the use of the most widely used sperm function tests. Moreover, only 11 meta-analyses comparing a diagnostic test against a concurrent reference standard were identified by extensive searching of the general medicine literature (Irwig, *et al.*, 1995). Consequently, EBM and meta-analysis of diagnostic sperm function testing may have the potential to assist clinical-decision making in reproductive medicine.

The objective of the present study was to perform a meta-analysis to determine the diagnostic test accuracy and predictive value of various sperm function assays for in vitro fertilization outcome. There are numerous tests presently being utilized to assess different sperm functions. Here, we elected to define the accuracy of four categories of such tests using a meta-analytic approach as a guide to the routine use of these techniques in the ART discipline. The scrutinized tests were CASA, acrosome reaction testing, the zona-free hamster egg penetration test or sperm-penetration assay (SPA) and sperm-zona pellucida binding assays. The selection of these assays for the meta-analysis does not negate the potential significance of the evaluation of other sperm functional capacities.

Materials and methods

To find the relevant studies we conducted a computerized search using MEDLINE on Silver Platter CD-ROM. We searched the English-language literature from January 1983 to December 1997 using various MeSH (medical subject) headings. The search process evolved in three steps: (1) The initial search was conducted using primarily controlled vocabulary. (2) The search strategy was modified to a combination of text words and controlled vocabulary. (3) The researcher then conducted a manual review of bibliographies based on his knowledge of the subject area ("expanded search") (Schlesselman, 1997; Oxman, *et al.*, 1993 and 1994). This approach brought numerous references not retrieved in the initial MEDLINE search (Dickerson, *et al.*, 1994; Councel, 1997).

A variety of exploded MeSH subjects were used. For test identification they were: for CASA: computer-aided sperm motion analysis, hyperactivated motility and cross-headings; for acrosome reaction: acrosome reaction, spontaneous or induced, and cross-headings; for SPA: zona-free hamster egg penetration assay, sperm penetration assay and cross-headings; and for sperm-zona pellucida binding assays: sperm-zona pellucida binding test and hemizona assay (HZA).

Sensitivity, specificity, regression analysis, likelihood ratios and probability were the headings used for selection of studies addressing the predictive value of the tests. In vitro fertilization (IVF) was the heading used to identify the main outcome measure.

Statistical analysis

The statistical methods employed in the primary studies included Spearman's rank order correlation between IVF and test parameters, logistic regression of fertilization (defined as "good' or "poor" based on various cut-off values) on tests parameters, or multiple linear regression if fertilization was analyzed as continuous. In addition, predictive statistics or sensitivity, specificity and predictive values (positive –PPV- and negative –NPV) were reported in some studies as well as odds ratios. In many cases, even if a study did not report the statistics the other studies did, an adequate analysis could be calculated from data given.

Here, the meta-analysis was carried out combining correlation coefficients (Altman, 1991; Collins, 1997; Fleiss, 1993) and computing summary odds ratios (with 95% confidence intervals - CI) and summary receiving operating characteristics (ROC) curves (Irwig, *et al.*, 1995). Computation of partial areas under the ROC curve followed the methods described by McClish (1989).

Results

(Table I) presents the number of references addressing the predictive value of the four sperm function tests for IVF outcome as retrieved from the MEDLINE and "expanded" searches and the number of references that were included in the meta-analysis. There were 47 and 75 references obtained from the MEDLINE and "expanded" searches, respectively. Adequate data could be retrieved from 34 references (half of them retrieved by the "expanded" search) in order to perform the meta-analysis (i.e., raw data on test outcome and fertilization results were directly provided or could be abstracted from two-by-two tables). Studies not providing those data could not be combined for further analysis and were not included in the analysis. Altogether, we were able to analyze the predictive value of the individual tests for IVF outcome in a total of 2,906 cycles.

Computer-aided sperm motion analysis

There were only four studies that addressed the relationship between IVF rates and data from CASA systems (Check, *et al.*, 1990; Liu, *et al.*, 1991; Sukcharoen, *et al.*, 1995 and 1996). These studies included 289 patients (IVF cycles). Overall, there were inconsistencies among the studies, including use of various sperm preparation techniques (i.e., use of liquefied semen or separation of the motile sperm fraction), incubation time, CASA equipment and parameter settings of the computers, and analysis of fertilization cut-off levels.

In the initial analysis the four studies were combined and examined with a Spearman rank-order correlation of fertilization with the various sperm motion parameters. (Table II) presents the correlations that were reported in the studies or correlations that were calculated from other information given (i.e., a contingency table of rates and parameter values). NS means that the correlation was computed but was not statistically different from zero. NR means the correlation was probably performed in the study but not reported. A blank entry (--) indicates that the correlation was not done in the particular study.

Subsequently, we calculated an odds ratio for the influence of the particular variable - motion parameter- (adjusted for all other variables in the equation) on fertilization. Three of the primary studies performed multiple logistic regression for data analysis. Exponentiation of the regression coefficient was therefore used to yield the estimated odds ratio for the influence of the particular variable on fertilization. For the fourth primary study, which did not perform multiple regression analysis, two-by-two tables were constructed and used to compute the odds ratio. The combined results of all studies demonstrated a large degree of variability indicating a poor predictive power for sperm parameters assessed by CASA and IVF results (table II). Predictive statistics demonstrated low specificity and sensitivity and a high rate of false positives (data not shown).

Acrosome reaction

There were eight studies that addressed the relationship between the induced-acrosome reaction results and IVF outcome in a total of 797 subjects (Cummins, *et al.*, 1991; Henkel, *et al.*, 1993; Pampiglioni, *et al.*, 1993; Coetzee, *et al.*, 1994; Calvo, *et al.*, 1994; Parinaud, *et al.*, 1995, Carver-Ward, *et al.*, 1996; Krausz, *et al.*, 1996). There were some inconsistencies among studies

regarding sperm capacitation conditions, methods used to induce acrosomal exocytosis (most studies used a calcium ionophore agent, but two other used human follicular fluid and low temperature conditions), as well as in the methods used to evaluate acrosome reaction (most studies used a fluorescent-labeled lectin but one use flow cytometry). There were also differences in definition of thresholds for acrosome reaction and in vitro fertilization rates.

Five of the primary studies presented results in the form of a Spearman's rank order or Pearson correlation of acrosome reaction and fertilization rates. (Table III) presents correlations for each of the five studies that could be directly analyzed. The estimated overall correlation was .458 (95% CI = .455 - .462, $p < .0001$). However, the test for homogeneity of correlations across studies was significant ($p < .0001$) indicating that correlations were not homogeneous across studies.

Predictive statistics could be analyzed for seven of the eight primary studies (see Table III). With one exception, PPV were 75% or higher. NPV tended to be more variable. Those seven studies could be combined to construct a summary ROC curve (Figure 1). The partial area under the curve was 32% and was 82% when scaled to the total area. A sensitivity of 80% was achieved with a little over 20% false positive rate. The slope of the regression line of log odds ratio on the sum of the logits of true and false positive rates was .0487 ($p = .895$) suggesting that test accuracy could be summarized by a common odds ratio. The common odds ratio was 13.97 (95% CI = 2.91 - 67.14). The odds ratios of the studies ranged from 3 to 451.

Sperm penetration assay

There were 12 studies that addressed the relationship between the SPA results and IVF outcome in a total of 842 subjects (Wolf, *et al.*, 1983; Margalioth, *et al.*, 1983; Foreman, *et al.*, 1984; Ausmanas, *et al.*, 1985; Margalioth, *et al.*, 1986; Aitken, *et al.*, 1987; Kruger, *et al.*, 1988: Coetzee, *et al.*, 1989; Ibrahim, *et al.*, 1989; Nahhas, *et al.*, 1989; McClure, *et al.*, 1989; Soffer, *et al.*, 1992). There were some inconsistencies among studies regarding capacitation conditions, gamete co-incubation times, use or not of conditions to induce acrosome reaction and cut-off levels selected for data analysis. The above-mentioned reports either provided adequate data or the counts of two-by-two tables for calculation of diagnostic accuracy statistics. Of those studies not reporting individual data, three used 20% (or a close value of 17%) as the SPA cut-off value. All other studies used 10% as the cut-off value. For those studies reporting individual data, the necessary cell counts for two-by-two tables could be determined for either cut-off. In addition, "good" fertilization

was defined differently. Some studies defined fertilization as any value greater than 0% but others defined it as greater than or equal to 50%. Two additional studies provided breakdowns to define fertilization either way.

(Table IV) presents correlations and diagnostic statistics for all the twelve primary studies. The estimated overall correlation for the studies was .396 (95% CI = .393 - .398, p< .0001). However, the test for homogeneity of correlations was significant (p< .0001) indicating that correlations were not homogeneous across studies. Further analysis indicated two homogeneous subsets, the largest one composed of nine studies (r = .458, 95% CI = .456 - .461, p< .0001) and the other one composed of three studies (r = .079, 95% CI = .066 - .092, p< .0001). With one exception, the PPV were all above 70% (range = 50% - 96%). However, the false negative rate was generally high (low specificity). The slope of the regression line of the log of the odds ratios on the sum of the logits of true and false positive rates was .0954 (p = .712) suggesting that test accuracy could be summarized by a common odds ratio. The common odds ratio for all studies was 7.61 (95% CI = 2.86 – 20.26). The odds ratios of the studies ranged from1 to 42. The summary ROC curve for all studies is plotted in (figure 1). The partial area under the curve was 53% and was 56% when scaled to the total area. As indicated by the diagnostic statistics, the summary ROC shows achievement of high sensitivity but accompanied by a very high false positive rate.

Sperm-zona pellucida binding assays

There were 10 studies that investigated the relationship between sperm-zona pellucida binding assays and fertilization in vitro (seven of them using the HZA and three of them the sperm-zona binding test) (Liu, *et al.*, 1988 and 1989; Oehninger, *et al.*, 1989, 1992a and 1997b; Franken, *et al.*, 1993a and b; Coddington, *et al.*, 1994; Liu and Gordon Baker, 1994; Gamzu, *et al.*, 1994). Those studies prospectively evaluated a total of 978 male patients (587 in the HZA and 323 in the sperm-zona binding test). Of the 10 studies selected for the meta-analysis, there were 8 from which a two-by-two table of sperm-zona binding cut-off values (either hemizona index for the HZA or binding ratio for the sperm-zona binding test) and fertilization (categorized as "good" or "poor") could be constructed. There were two reports (out of the 10 studies) that did not analyze their data in such a way to construct a two-by-two table. The latter two studies correlated the sperm-zona binding ratio with fertilization rate by the Spearman's rank order correlation. A Spearman correlation can be calculated from a two-by-two table, and therefore a meta-analysis of this statistic could also be performed for all 10 studies.

Table V presents the correlations and diagnostic statistics for all the ten primary studies. The estimated overall correlation for the studies was .641 (95% CI = .64 - .642, p< .0001). However, the test for homogeneity of correlations was significant (p< .0001) indicating that correlations were not homogeneous across studies. Further analysis indicated three homogeneous subsets. The largest subset included seven HZA studies (r = .643, CI = .64 - .645, p< .00001); the second one included two studies carried out with the sperm-zona binding assay (r = .470, CI = .465 - .470, p< .0001); and the third one was composed of a single HZA study with an unusually high correlation of .96. For eight studies that could be combined for predictive statistics (see Table V), PPV were 80% or better (range = 79% - 95%) and NPV were generally better than 70%. Importantly, the false negative rate was consistently low, ranging from 2% to 25%. The slope of the regression line of the log of the odds ratios on the sum of the logits of true and false positive rates was .6647 (p= .192). The common odds ratio of the eight studies was 23.68 although with a large 95% CI (4.83 – 115.99). A summary ROC curve could be constructed for those eight studies and is plotted in (figure 1). The partial area under the curve was 25% and was 85% when scaled to the total area.

Discussion

The present meta-analysis was based on the results of 34 different studies independently performed by 25 centers in various geographical locations throughout the world in a total of 2,906 subjects. These were all prospectively performed studies, with appropriate internal controls, addressing the predictive power of a defined functional test for fertilization outcome (outcome-based clinical research). Overall correlations and/or predictive statistics were performed for all the 34 primary studies.

It must be acknowledged, however, that there were some inconsistencies in the primary studies as related to methodologies, patient populations analyzed and clinical thresholds selected for data analysis. Some of these factors may introduce biases into the meta-analysis. Because of lack of consistency and/or data presentation among some reports, subsets of studies were reanalyzed to obtain more accurate odds ratios and ROC curves (for a total of 27 studies out of the 30 primary studies exclusive of CASA analysis). Although the meta-analytical approach, in general, may have inherent imperfections (including potential problems with the selected primary studies, the issue of combinability of data sets currently available, lack of literature reporting on negative results, discrepancies between meta-analyses and subsequent large randomized, controlled

trials), it is still a very useful method to gain objective evidence on the predictive power of diagnostic assays and efficacy of therapeutical trials (Collins, 1997; LeLorier, 1997; Comhaire, 1998). Notwithstanding these limitations, several important conclusions can be drawn from the present meta-analysis.

Results clearly demonstrate the high predictive power of the sperm-zona pellucida binding and induced-acrosome reaction assays for IVF outcome. Comparatively, the sperm-zona pellucida binding assays and the acrosome reaction testing were both better predictors of fertilization than the SPA. The areas under the summary ROC curves scaled to the total curve were 85% and 82%, (odds ratio of 23.6 and 13.97) for the zona binding and acrosome reaction assays, respectively. This compared to only 56% for the SPA (odds ratio of 7.61) that was significantly lower than the area under the curve for the former assays ($p< 0.05$) thus demonstrating the poor clinical value of the SPA as predictor of fertilization (unacceptably high false positive rate).

The interaction between spermatozoa and the zona pellucida is a critical event leading to fertilization and reflects multiple sperm functions (i.e., completion of capacitation as manifested by the ability to bind to the zona pellucida and to undergo ligand-induced acrosome reaction) (Oehninger, *et al.*, 1992b; Liu and Gordon Baker, 1992). The two most common sperm-zona pellucida binding tests currently utilized are the HZA (Burkman, *et al.*, 1988) and a competitive intact-zona binding assay (Liu, *et al.*, 1988). Although different in their methodologies, they both use assessment of tight sperm binding to the zona as the primary endpoint in an independent comparison within an internally controlled assay. As proven here, such tests can therefore be immediately applied to the clinical management of infertile patients within the assisted reproduction setting. The high positive and negative predictive values, but more importantly, the low false negative rate (i.e., robust power to identify patients at high risk for fertilization failure) underscore the predictive ability of these tests. The only present limitation to the use of the sperm-zona pellucida binding assays is the need for a constant supply of human oocytes. Because the induced-acrosome reaction assays appear to be equally predictive of fertilization outcome and are simpler in their methodologies, the former tests could be favored. However, prospective studies should be carried out to compare their predictive abilities in the same group of patients.

The induced-acrosome reaction testing, coupled with sperm vitality assessment, demonstrated an equal predictive power (similar area under the ROC curve) to that of the sperm-zona binding assays. It also has to be acknowledged that the acrosome reaction conditions and

inducing agents varied for the studies (i.e., calcium ionophore, follicular fluid, temperature, capacitation conditions, etc.). Consequently, more studies are needed for optimization and validation of such assays. The calcium ionophore induced-acrosome reaction is at the present time the most widely used methodology (Tesarik, 1989; Cummins, *et al.*, 1991). Nevertheless, the implementation of assays using small volumes of human solubilized zonae pellucidae (Franken, *et al.*, 1996), biologically active recombinant human ZP3 (Chapman and Barratt, 1996) or active, synthetic ZP3 peptides (or analogs) (Hinsch, *et al.*, 1998) will probably allow for the design of improved, physiologically oriented acrosome reaction assays. The use of such agents combined with a better understanding of the biochemistry of the carbohydrate-protein interactions that take place during gamete recognition, binding and induction of acrosomal exocytosis will undoubtedly help in their elaboration.

The SPA, although a very valuable research tool, is proven to offer little help in the clinical setting due to its low predictive power. Another meta-analysis of the predictive value of this assay also reached a similar conclusion (Mol, *et al.*, 1998). Claims that the SPA predictability can be improved by modified versions of the assay (Aitken, *et al.*, 1987; Johnson, *et al.*, 1991) need to be corroborated by more studies. Of interest, the SPA results have been positively correlated with the outcome of spontaneous pregnancy and conceptions following other interventions (Corson, *et al.*, 1988; Gwatkin, *et al.*, 1990).

More work is needed to define and validate the use of CASA systems for sperm motion analysis. First, there were too few studies related to the use of sperm motion analysis and prediction of IVF outcome to reach general conclusions. Second, results demonstrated the lack of uniform criteria applied by different laboratories. Recently, guidelines have been proposed in order to standardize these methodologies (ESHRE Andrology Special Interest Group, 1998). We adhere to such recommendations as related to equipment, computer parameter settings, semen preparation techniques and overall criteria established for clinical use. It is expected that new studies will soon be available reporting on the clinical application of these systems.

Ideally, sperm function assays should sequentially examine the various dynamic properties of the spermatozoon. These include: (a) maturation and capacitation status; (b) interaction with the female tract components; (c) interaction with the oocyte vestments; and (d) interaction with the ooplasm, oocyte activation and contribution to early embryogenesis (Sharpe, 1992; Oehninger, *et al.*, 1992a and 1997a; Amman and Hammerstedt, 1993; Fraser, 1995; Aitken, 1997). Obviously, no

single test will be able to assess those different and complex properties. The sequential, multi-step analysis of the main sperm functions examined by a combination of tests may prove to be clinically applicable (Oehninger, *et al.*, 1992a; Amman and Hammerstedt, 1993). For a more universal application of such tests, we need to have well defined methodologies, establish a defined outcome measure (i.e., fertilization in vitro or pregnancy in vivo, use in fertility evaluation, contraception or reproductive toxicology studies), have a common definition of male infertility and establish whether the tests will be applied to whole semen or to a selected sperm population (Jeyendran and Zaneveld, 1993; Mortimer, 1994; Cummins and Jequier, 1994).

It is expected that imminent advances in the cellular and molecular aspects of human gamete physiology and in biotechnology may help us develop rational, accurate and more predictive assays. At present, clinicians are usually forced to make decisions based on non-standardized techniques and imperfect studies. Or even worse, patients may be directed to efficient therapies such as ICSI without any knowledge about a physiopathologic diagnosis. The present meta-analysis provides objective evidence in which clinical management and recommendations can be based. It also points out to the limitations of the current tests and can serve as an import guide to direct future research.

References

1. Aitken, R.J. (1997) Molecular mechanisms regulating human sperm function. *Mol Hum Reprod*, **3**, 169-173.
2. Aitken, R.J., Irvine, D.S. and Wu, F.C. (1991) Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol*, **164**, 542-551.
3. Aitken, R.J., Clarkson, J.S. and Fishel, S. (1989a) Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol Reprod*, **41**, 183-187.
4. Aitken, R.J., Clarkson, J.S., Hargreave, T.B., *et al.*, (1989b) Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl.*, **10**, 214-220.
5. Aitken, R.J. (1988) Assessment of human sperm function for IVF. *Hum Reprod*, **3**, 89-95.
6. Aitken, R.J., Thatcher, S., Glassier, A.F., *et al.*, (1987) Relative ability of modified versions of the hamster oocyte penetration test, incorporating hyperosmotic medium or the ionophore A23187, to predict IVF outcome. *Hum Reprod*, **2**, 227-231.
7. Altman, D.G. (1991) Practical Statistics for Medical Research. Chapman & Hall, New York, pp. 293-296.
8. Amann, R.P. and Hammerstedt, R. H. (1993) In vitro evaluation of sperm quality: an opinion. *J Androl*, **14**, 397-406.
9. Ausmanas, M., Tureck, R.W., Blasco, L., *et al.*, (1985) The zona-free hamster egg penetration assay as a prognostic indicator in a human in vitro fertilization program. *Fertil Steril*, **43**, 433-437.
10. Burkman, L.J., Coddington, C.C., Franken, D.R., *et al.*, (1988) The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril*, **49**, 688-697.
11. Calvo, L., Dennison-Lagos, L., Banks, S.M., *et al.*, (1994) Acrosome reaction inducibility predicts fertilization success at in vitro fertilization. *Hum Reprod*, **9**, 1880-1886.
12. Carver-Ward, J.A., Jaroudi, K.A., Hollanders, J.M.G., *et al.*, (1996) High fertilization prediction by flow cytometric analysis of the CD46 antigen on the inner acrosomal membrane of spermatozoa. *Hum Reprod*, **11**, 1923-1928.
13. Centers for Disease Control and Prevention, U.S. Department of Health and Human Services (1997). Fertility, family planning and women's health: new data from the 1995 national survey of family growth, pp 1-11.

14. Chapman, N.R. and Barratt, C.L.R. (1996) The role of carbohydrate in sperm-ZP3 adhesion. *Mol Hum Reprod*, **2**, 767-774.
15. Check, J.H., Bollendorf, A., Lee, M.A., *et al.*, (1990) Correlation of computerized semen analysis with successful fertilization of oocytes in an in vitro fertilization program. *Arch Androl*, **24**, 229-234.
16. Coddington, C. C., Oehninger, S. C., Olive, D. L., *et al.*, (1994) Hemizona Index (HZI) demonstrated excellent predictability when evaluating sperm fertilizing capacity in in-vitro fertilization patients. *J Androl*, **15**, 250-254.
17. Coetzee, K., Olmedo, J. and Lombard, C.J. (1994) Induced acrosome reactions as fertility predictor. *J Assist Reprod Genet*, **11**, 470-473.
18. Coetzee, K., Kruger, T.F., Menkveld, R., *et al.*, (1989) Usefulness of sperm penetration assay in fertility predictions. *Arch Androl*, **23**, 207-212.
19. Collins, J.A. (1997) Guides for practitioners: meta-analysis. *Hum Reprod*, **12**, 1847-1850.
20. Comhaire, F.H. (1998) Pitfalls of evidence-based medicine in andrology and assisted reproduction. In Ombelet, W., Bosmans, E., Vandeput, H., *et al.*, (eds) Modern ART in the 2000s:andrology in the nineties. Parthenon Publishing Group, New York, pp. 45-54.
21. Corson, S.L., Batzer, F.R., Marmar, J., *et al.*, (1988) The human sperm—hamster egg penetration assay: prognostic value. *Fertil Steril*, **49**, 328-334.
22. Councel, C. (1997) Formulating questions and locating primary studies for inclusion in systematic reviews. *Ann Intern Med*, **127**, 380-387.
23. Cross, N.L., Morales, P., Overstreet, J.W., *et al.*, (1986) Two simple methods for detecting acrosome-reacted human sperm. *Gamete Research*, **15**, 213-226.
24. Cummins, J.M., Pember, S.M., Jequier, A.M., *et al.*, (1991) A test of the human sperm acrosome reaction following ionophore challenge. Relationship to fertility and other seminal parameters. *J Androl*, **12**, 98-103.
25. Cummings, J.M. and Jequier, A.M. (1994) Treating male infertility needs more clinical andrology, not less. *Hum Reprod*, **9**, 1214-1219.
26. Dickerson, K., Scherer, R. and Lefebvre, C. (1994) Identifying relevant studies for systematic reviews. *J Br Med*, **309**, 1286-1291.
27. ESHRE Andrology Special Interest Group (1998) Guidelines on the application of CASA technology in the analysis of spermatozoa. *Hum Reprod*, **13**, 142-145.
28. ESHRE Andrology Special Interest Group (1996) Consensus Workshop on Advanced Diagnostic Andrology Techniques. *Hum Reprod*, **11**, 1463-1479.

29. Evers, J.L.H. (1997) Evidence-based medicine in andrology. *Andrology in the Nineties* (abstract book); The Jones Institute for Reproductive Medicine, Norfolk, Virginia, pp. 14.
30. Evidence-Based Medicine Working Group (1992) *J Am Med Assoc*, **17**, 2420-2425.
31. Fraser, L.R. (1995) Ionic control of sperm function. *Reprod Fertil* Dev., **7**, 905-925.
32. Foreman, R., Cohen, J., Fehilly, C.B., *et al.*, (1984) The application of the zona-free hamster egg test for the prognosis of human in vitro fertilization. *J IVF and Embryo Transfer*, **1**, 166-171.
33. Franken, D.R., Morales, P.J. and Habenicht, U.F. (1996) Inhibition of G protein in human sperm and its influence on acrosome reaction and zona pellucida binding. *Fertil Steril*, **66**, 1009-1011.
34. Franken, D.R., Kruger, T.F., Oehninger, S., *et al.*, (1993a) The ability of the hemizona assay to predict human fertilization in different and consecutive in vitro fertilization cycles. *Hum Repro*, **8**, 1240-1244.
35. Franken, D. R., Acosta, A. A., Kruger, T. F., *et al.*, (1993b) The hemizona assay: its role in identifying male factor infertility in assisted reproduction. Fertil Steril, **59**, 1075-1080.
36. Fleiss, J. L. (1993) The statistical basis of meta-analysis. *Statistical Methods in Medical Research*, **2**, 121-145.
37. Gamzu, R., Yogev, L., Amit, A., *et al.*, (1994) The hemizona assay is a good prognostic value for the ability of sperm to fertilize oocytes in vitro. *Fertil Steril*, **62**, 1056-1059.
38. Gwatkin, R.B.L., Collins, J.A., Jarrell, J.F., *et al.*, (1990) The value of semen analysis and sperm function assays in predicting pregnancy among infertile couples. *Fertil Steril*, **53**, 693-699.
39. Henkel, R., Müller, C., Miska, W., *et al.*, (1993) Determination of the acrosome reaction in human spermatozoa is predictive of fertilization in vitro. *Hum Reprod*, **8**, 2128-2132.
40. Hinsch, E., Oehninger, S. and Hinsch, K. (1998) ZP3-6 Peptide and acrosome reaction. Semen evaluation, testing and selection: a new look in the 2000s. In: Andrology in the Nineties Precongress Training Course, Genk, Belgium, April 22, pp 25-30.
41. Hull, M.G.R., Glazener, C.M.A. and Kelly, N.J. (1985) Population study of causes, treatment, and outcome of infertility. *J Br Med*, **291**, 1693-1697.
42. Huszar, G. and Vigue, L. (1994) Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. *J Androl*, **15**, 71-77.

43. Huszar, G., Vigue, L. and Morshedi, M. (1992) Sperm creatine phosphokinase M-isoform ratios and fertilizing potential of men: a blinded study of 84 couples treated with in vitro fertilization. *Fertil Steril*, **57**, 882-888.

44. Ibrahim, M.E., Moussa, M.A.A. and Pedersen, H. (1989) Efficacy of zona-free hamster egg sperm penetration assay as a predictor of in vitro fertilization. *Arch Androl*, **23**, 267-274.

45. Irvine, D.S. (1998) Epidemiology and etiology of male infertility. *Hum Reprod*, **13** (Supplement 1), 33-44.

46. Irwig, L., Macaskill, P., Glasziou, P., *et al.*, (1995) Meta-analytic methods for diagnostic rest accuracy. *J Clin Epidemiol*, **48**, 119-130.

47. Johnson, A., Smith, R.G., Bassham, B., *et al.*, (1991) The microsperm penetration assay: development of a sperm penetration assay suitable for oligospermic males. *Fertil Steril*, **56**, 528-534.

48. Jaeschke, R., Guyatt, G. and Sackett, D. L. (1994). How to use an article about a diagnostic test. Are the results of the study valid? *Users guide to the medical literature*, III, **271**, 389-391.

49. Jeyendran, R.S. and Zaneveld, L.J.D. (1993) Controversies in the development and validation of new sperm assays. *Fertil Steril*, **59**, 726-728.

50. Kruger, T. F., Swanson, R.J., Hamilton, M., *et al.*, (1988) Abnormal sperm morphology and other semen parameters related to the outcome of the hamster oocyte human sperm penetration assay. *Int. J Androl*, **11**, 107-113.

51. Krausz, C., Bonaccorsi, L., Maggio, P., *et al.*, (1996) Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. *Hum Reprod*, **11**, 1661-1667.

52. LeLorier, J., Gregoire, G., Benhaddad, A., *et al.*, (1997) Discrepancies between meta-analyses and subsequent large randomized, controlled trials. *N Engl J Med*, **337**, 536-542.

53. Liu, D.Y. and Baker, H.W.G. (1994) A new test for the assessment of sperm-zona pellucida penetration: relationship with results of other sperm test and fertilization in vitro. *Hum Reprod*, **9**, 489-496.

54. Liu, D. Y. and Baker, H. W. G. (1992) Tests of human sperm function and fertilization in vitro. *Fertil Steril*, **58**, 465-483.

55. Liu, D.Y., Clarke, G.N. and Baker, H.W.G. (1991) Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates in vitro. *J Androl*, **12**, 231-239.

56. Liu, D.Y., Clarke, G.N., Lopata, A., *et al.*, (1989) A sperm-zona pellucida binding test and in vitro fertilization. *Fertil Steril*, **52**, 281-287.

57. Liu, D.Y., Lopata, A., Johnston, W.I.H., *et al.*, (1988) A human sperm-zona pellucida binding test using oocytes that failed to fertilize in vitro. *Fertil Steril*, **50**, 782-788.

58. Margalioth, E. J., Navot, D., Laufer, N., *et al.*, (1986) Correlation between the zona-free hamster egg sperm penetration assay and human in vitro fertilization. *Fertil Steril*, **45**, 665-670.

59. Margalioth, E. J., Navot, D., Laufer, N., *et al.*, (1983) Zona-free hamster ovum penetration assay as a screening procedure for in vitro fertilization. *Fertil Steril*, **40**, 386-388.

60. McClish, D.K. (1989) Analyzing a portion of the ROC curve. *Medical Decision Making*, **9**, 190-195.

61. McClure, R.D., Tom, R. A. and Dandekar, P.V. (1990) Optimizing the sperm penetration assay with human follicular fluid. *Fertil Steril*, **53**, 546-550.

62. Mol, B. W. J., Meijer, S., Yuppa, S. *et al.* (1998) Sperm penetration assay in predicting successful in vitro fertilization: a meta-analysis. *J Reprod Med*, **43**, 503-508.

63. Mortimer, D. (1994) The essential partnership between diagnostic andrology and modern assisted reproductive technologies. *Hum Reprod*, **9**, 1209-1213.

64. Mortimer, D. (1990a) Objective analysis of sperm motility and kinematics. In Keel, B.A. and Webster, B.W. (eds), *Handbook of the Laboratory Diagnosis and Treatment of Infertility*. CRC Press, Boca Raton, pp. 97-133.

65. Mortimer, D, Curtis, E.F. and Camenzind, A.R. (1990b) Combined use of fluorescent peanut agglutinin lectin and Hoechst 33258 to monitor the acrosomal status and vitality of human spermatozoa. *Hum Reprod*, **5**, 99-103.

66. Mosher, W.D. and Pratt, W. F. (1990) Fecundity and infertility in the United States, 1965-1988. Advance data from vital and health statistics. Public Health Service, Hyattsville, MD, (DHHA publication no. (PHS) 91-1250).

67. Nahhas, F. and Blumenfeld, Z. (1989) Zona-free hamster egg penetration assay: prognostic indicator in an IVF program. *Arch Androl*, **23**, 33-37.

68. Nieschlag, E. (1997) Scope and goals of andrology. In Nieschlag, E. and Behre, H. M. (eds) Andrology. Springer-Verlag, Berlin, pp. 1-8.

69. Oehninger, S., Sayed, E. and Kolm, P. (1998) Role of sperm function tests in subfertility treatment strategies. In Ombelet, W., Bosmans, E., Vandeput, H., *et al.*, (eds), Modern ART in the 2000s:andrology in the nineties. Parthenon Publishing Group, New York, pp. 25-36.

70. Oehninger, S., Franken, D. and Kruger, T. (1997a) Approaching the next millennium: how should we manage andrology diagnosis in the intracytoplasmic sperm injection era? *Fertil Steril*, **67**, 434-436.

71. Oehninger, S., Mahony, M., Ozgur, K., *et al.*, (1997b) Clinical significance of human sperm-zona pellucida binding. *Fertil Steril*, **67**, 1121-1127.

72. Oehninger, S.C. (1995) An update on the laboratory assessment of male fertility. *Hum Reprod* (Suppl. 1), **10**, 38-45.

73. Oehninger, S., Toner, J., Muasher, S., *et al.*, (1992a) Prediction of fertilization in vitro with human gametes: Is there a litmus test? *Am J Obstet Gynecol*, **167**, 1760-1767.

74. Oehninger, S., Franken, D., Alexander, N., *et al.*, (1992b) Hemizona assay and its impact on the identification and treatment of human sperm dysfunctions. *Andrologia*, **24**, 307-321.

75. Oehninger, S., Acosta, A., Veeck, L., *et al.*, (1991) Recurrent failure of in vitro fertilization: role of the hemizona assay in the sequential diagnosis of specific sperm-oocyte defects. *Am J Obstet Gynecol*, **164**, 1210-1215.

76. Oehninger, S., Coddington, C.C., Scott, R., *et al.*, (1989) Hemizona assay; assessment of sperm dysfunction and prediction of in vitro fertilization outcome. *Fertil Steril*, **51**, 665-670.

77. Oxman, A.D., Cook, D.J. and Guyatt, G.H. (1994) Users' guide to the medical literature. VI. How to use an overview. *J Am Med Assoc*, **272**, 1367-1371.

78. Oxman, A. D., Sackett, D. L. and Guyatt, G. H. (1993) Users' guide to the medical literature. 1. How to get started. *J Am Med Assoc*, **270**, 2093-2095.

79. Parinaud, J., Vieitez, G., Moutaffian, H., *et al.*, (1995) Variations in spontaneous and induced acrosome reaction: correlations with semen parameters and in vitro fertilization results. *Hum Reprod*, **10**, 2085-2089.

80. Pampiglione, J.S., Tan, S. and Campbell, S. (1993) The use of the stimulated acrosome reaction test as a test of fertilizing ability in human spermatozoa. *Fertil Steril*, **59**, 1280-1284.

81. Sackett, D.L., Rosenberg, W.M.C., *et al.*, (1996) Evidence based medicine: what it is and what it isn't. *J Br Med*, **312**, 56-57.

82. Sackett, D.L. (1995) Meta-analytic methods for diagnostic test accuracy: discussion. *J Clin Epidemiol*, **48**, 131-132.

83. Schlesselman, J.J. (1997) Risk of endometrial cancer in relation to use of combined oral contraceptives. A practitioner's guide to meta-analysis. *Hum Reprod*, **12**, 1851-1863.

84. Sharpe, R. M. (1992) Monitoring of spermatogenesis in man: measurement of Sertoli cell or sperm cell-secreted proteins in semen or blood. *Int J Androl*, **15**, 201-210.

85. Society for Assisted Reproductive Technology, American Society for Reproductive Medicine (1995) Assisted reproductive technology in the United States and Canada: 1993 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. *Fertil Steril*, **64**, 13-21.

86. Soffer, Y., Golan, A., Herman, A., *et al.*, (1992) Prediction of in vitro fertilization outcome by sperm penetration assay with TEST-yolk buffer preincubation. *Fertil Steril*, **58**, 556-562.

87. Sukcharoen, N., Keith, J., Irvine, D.S., *et al.*, (1996) Prediction of the in vitro fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Hum Reprod*, **11**, 1030-1034.

88. Sukcharoen, N., Keith, J., Irvine, D.S., *et al.*, (1995) Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in vitro fertilization as a functional end-point. *Hum Reprod*, **10**, 2928-2937.

89. Swets, JA. (1988) Measuring the accuracy of diagnostic systems. *Science*, **240**, 1285-1293

90. Tesarik, J. (1989) Appropriate timing of the acrosome reaction is a major requirement for the fertilizing spermatozoon. *Hum Reprod*, **4**, 957-961.

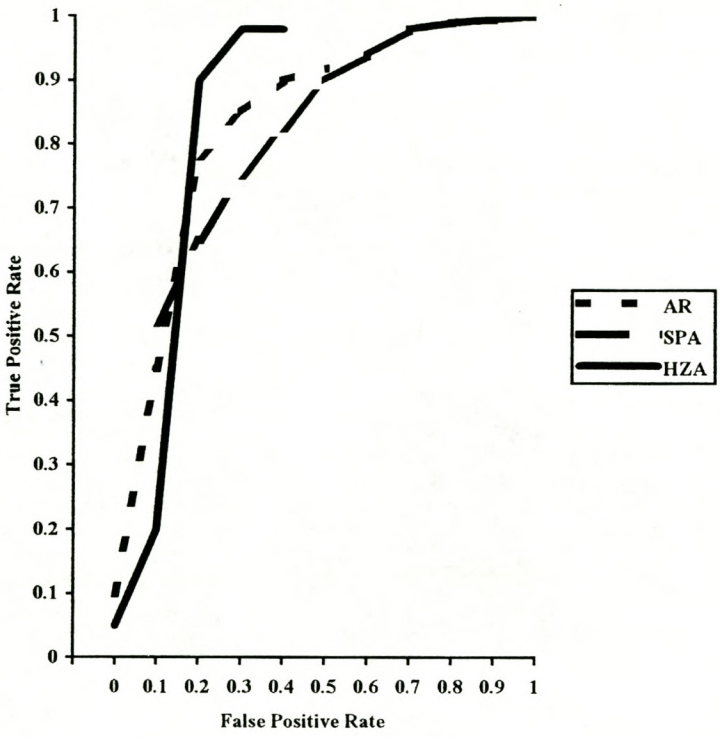
91. Wilcox, L.S. and Mosher, W.D. (1993) Use of infertility services in the United States. *Obstet Gynecol*, **82**, 122-127.

92. Wolf, D.P., Sokoloski, J.E. and Quigley, M.M. (1983) Correlation of human in vitro fertilization with the hamster egg bioassay. *Fertil Steril*, **40**, 53-59.

93. World Health Organization (1992) WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 3rd edn, Cambridge University Press, Cambridge, pp. 23-26.

94. Yanagimachi, R., Yanagimachi, H. and Rogers, B.J. (1976) The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod*, **15**, 471-476.

Figure 1



Legends of Figures

Figure 1. ROC curves analyzing the predictive value of acrosome reaction, SPA and sperm-zona pellucida binding assays for IVF outcome (* = HZA and sperm-zona pellucida binding assay).

Table I.

Number of references retrieved from searches and used in the meta-analysis.

	# of references addressing the predictive value for IVF outcome		# of references included in the meta-analysis	# of patients analyzed
	Medline Search	Expanded Search		
CASA	18	20	4	289
Acrosome Reaction	9	15	8	797
SPA	16	30	12	842
Sperm-Zona Pellucida Binding Tests	4	10	10	978

Table II.

CASA and prediction of IVF outcome.

Study	Liu, *et al.*, 1991		Sukcharoen, *et al.*, 1996		Sukcharoen, *et al.*, 1995		Check, *et al.*, 1990	
Sperm parameter	r	OR	r	**OR**	r	OR	r	OR
VCL	NS	NS	NS	NS	NS	1.0	.37	5.7
VSL	.22	1.0	NS	NS	NS	NS	.26	3.3
VAP	NR	NS	NS	1.1	NS	NS	—	—
LIN	.37	1.0	-.26	0.9	NS	NS	.32	4.7
ALH	NS	NS	.24	NS	NS	NS	NS	—
BCF	NS	NS	NS	NS	NS	1.4	NS	—
HA	—	—	—	—	.47	1.9	—	—

r = Spearman's correlation coefficient; OR = odds ratio; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; LIN = linearity; ALH = amplitude of lateral head displacement; BCF = beat-cross frequency; HA = hyperactive motility.

Table III.

Acrosome reaction and prediction of IVF outcome

Study	n	r	Fert. Rate Cut-off	Acr. Reac. Cut-off	Sen	Spe	PPV	NPV
Coetzee, *et al.*, 1994	22	.34	—	—	—	—	—	—
Carver-Ward, *et al.*, 1996	129	.68	> 30%	10%	100	82	93	100
Krausz, *et al.*, 1996	90	.31	> 50%	10%	85	52	87	53
Henkel, *et al.*, 1993	74	—	> 66%	10%	71	66	50	82
Calvo, *et al.*, 1994	232	.37	> 0%	10%	55	71	75	49
Pampiglione, *et al.*, 1993	54	—	> 0%	31%	50	99	85	100
Parinaud, *et al.*, 1995	117	.34	> 0%	20%	54	73	87	31
Cummins, *et al.*, 1991	79	—	> 50%	10%	85	54	79	64

n = number of cases; r = Spearman rank order or Pearson correlation; sen = sensitivity; spe = specificity; PPV = positive predictive value; NPV = negative predictive value.

Table IV.

SPA and IVF outcome.

Study	n	r	Fert.Rate cut-off	SPA cut-off	Sen	Spe	PPV	NPV
Wolf, *et al.*, 1983	27	.25	> 0%	> 10%	85	14	74	25
Margalioth, *et al.*, 1983	20	- .08	> 0%	> 10%	100	0	50	—
Margalioth, *et al.*, 1986	134	.56	> 0%	> 20%	94	57	85	78
Foreman, *et al.*, 1984	37	.47	> 0%	> 10%	86	53	73	73
Ausmanas, *et al.*, 1985	54	- .03	> 0%	> 10%	84	33	96	11
Kruger, *et al.*, 1988	84	.18	> 0%	> 10%	59	62	82	33
Coetzee, *et al.*, 1989	71	.37	> 0%	> 10%	65	85	95	35
Ibrahim, *et al.*, 1989	59	.54	> 0%	> 17%	84	73	90	61
McClure, *et al.*, 1989	19	.68	> 0%	> 10%	93	75	93	75
Soffer, *et al.*, 1992	241	.44	> 0%	> 20%	96	38	82	74
Nahhas, *et al.*, 1989	31	.45	> 0%	> 10%	100	22	76	100
Aitken, *et al.*, 1987	65	.28	> 50%	> 10%	85	41	80	50

n = number of cases; r = Spearman rank order or Pearson correlation; sen = sensitivity; spe = specificity; PPV = positive predictive value; NPV = negative predictive value.

Table V.

Sperm-zona pellucida binding assays and IVF outcome.

Study	n	r	Fert. rate cut-off	HZA cut-off	Sen	Spe	PPV	NPV
Oehninger, *et al.*, 1997	196	.69	> 50%	30	93	73	85	87
Franken, *et al.* 1993a	112	.55	> 50%	30	84	72	85	70
Franken, *et al.*, 1993b	48	.50	> 50%	30	75	68	81	68
Oehninger, *et al.*, 1989	28	.79	> 65%	36	95	83	95	83
Oehninger, et al., 1992	44	.70	> 65%	36	100	61	79	100
Gamzu, *et al.*, 1994	133	.96	> 0%	23	100	94	85	100
Coddington, *et al.*, 1994	94	.59	> 0%	15	82	78	88	69
Liu, *et al.*, 1988	20	.80	50%	—	90	90	90	90
Liu, *et al.*, 1989	106	.50	—	—	—	—	—	—
Liu and G. Baker, 1994	197	.45	—	—	—	—	—	—

n = number of cases; r = Spearman rank order or Pearson correlation; sen = sensitivity; spe = specificity; PPV = positive predictive value; NPV = negative predictive value.

4c. *Specific aim 7* : To present a state-of-the-ART strategy for the clinical management of male infertility. How should we manage andrology diagnosis and treatment in the new millennium?

Abstract

Male infertility is one of the most common identifiable causes of human reproductive failure. Although considerable progress has been made toward understanding sperm physiology and the biology of gamete interaction, still more work is needed to achieve objectivity and standardization of some of the andrological diagnostic methods used in the clinical setting. More information is needed to definitively establish which tests are more accurate predictors of sperm performance and how they correlate with pregnancy potential following *in vivo* and *in vitro* interventions. Infertile men can be successfully treated with defined urological and medical therapies or with assisted reproductive technologies (ARTs). Among the latter, intracytoplasmic sperm injection (ICSI) has become a validated means to overcome multiple sperm deficiencies. Nevertheless, it is expected that simplified and more cost-efficient therapeutic modalities will be developed as additional basic (cellular-molecular) and clinical knowledge is gained.

Introduction

Increased public awareness of a couple's infertility as a treatable condition and the availability of improved therapeutic options have resulted in a dramatically increased number of visits to fertility specialists in the last years. Although diagnostic problems make it difficult to establish the extent of the male partner's contribution with certainty, a number of studies suggest that male problems represent the most common single defined cause of infertility. Male-related disorders are probably present in up to 40-50% of childless couples, alone or in combination with female factors. (Hull, *et al.*, 1985: Mosher, *et al*, 1990: Wilcox, *et al*, 1993: Bhasin, *et al.*, 1994: Society for Assisted Reproductive Technology, 1995: CDC, 1997: Irvine, 1998).

Treatment options for male infertility include a large number of urological procedures (surgical and non-surgical), medical-pharmacological interventions, low complexity assisted reproductive procedures (such as intrauterine insemination therapy or IUI), and the more advanced and complex assisted reproductive technologies (ARTs). Among the latter, in vitro fertilization (IVF) and embryo transfer, augmented with intracytoplasmic sperm injection (ICSI) in moderate and severe cases, constitute validated and successful ways to assist fertilization and conception. National statistics from the U.S.A, (CDC, 1998) reported a 40% incidence of ICSI in 61,650 IVF cycles performed in a one-year period, a figure that highlights the importance of this technique.

However, in spite of the fact that contemporary therapies have enhanced the opportunities to conceive in couples suffering from male infertility, often these solutions are brought up in the absence of a defined etiological or pathophysiological diagnosis. Male infertility, unfortunately, is still considered "idiopathic" in a large proportion of cases.

Two other lines of investigation have recently provided further reasons for concern in the area of human male reproduction. First, some researchers have reported an overall decline in the quantity and quality of spermatozoa present in semen (caused by reproductive bio-hazards?) (Irvine, 1997). Second, it has been shown that spermatozoa from infertile men may be chromosomally and/or genetically abnormal. Such finding is in agreement with a slight but significant increase in the incidence of chromosomal anomalies in babies born after ICSI (Bonduelle, *et al.*, 1999). Although ICSI has become a real "boom" in the treatment of men with various degrees of sperm anomalies, it may carry a risk of transmission of chromosomal/genetic disease.

Within this clinical scenario, several practical questions arise, including [1] what are the diagnostic steps that we should take to direct the infertile man to a defined therapeutic modality, and [2] which is the most cost-effective therapy for each particular case?

Semen analysis

The cornerstone of the andrological evaluation in all cases is an exhaustive history and a physical examination followed by repeated semen analyses. A urological, endocrine, and/or imaging work-up should be implemented as appropriate.

A comprehensive semen analysis following the World Health Organization (WHO) guidelines (WHO, 1999) is fundamental at the primary care level to make a rational initial diagnosis and to select the appropriate clinical management. The collection and analysis of the semen must be undertaken by properly standardized procedures in appropriately qualified and accredited laboratories (De Jonge, 2000).

The "basic" semen evaluation should include: (1) assessment of physical semen characteristics (volume, liquefaction, appearance, consistency, pH and agglutination); (2) evaluation of sperm concentration, grading of motility and analysis of morphological characteristics (using strict criteria) (Kruger, *et al.*, 1986); (3) determination of sperm vitality (viability), testing for sperm auto-antibodies (using the mixed antiglobulin reaction and/or the direct immunobead tests), presence of leukospermia and immature sperm cells; and (4) bacteriological studies. The identification and separation of the motile sperm fraction is also an integral part of the initial semen evaluation (Oehninger, 1995: Mortimer, 2000).

Sperm function tests

Other categories of assays that are usually considered include: (1) tests that examine defective sperm functions indirectly through the use of biochemical tests (i.e., measurement of the generation of reactive oxygen species or evidence of peroxidative damage, measurement of enzyme activities such as creatine phosphokinase and others); (2) bioassays of gamete interaction (i.e., the heterologous zona-free hamster oocyte test and the homologous sperm-zona pellucida binding assays) and induced-acrosome reaction scoring; and (3) computer-aided sperm motion analysis (CASA) for the evaluation of sperm motion characteristics (Yanagimachi, *et al.*,1976; Cross, *et al.*,

1986:Burkman, *et al.*,1988; Liu *et al.*, 1988; Aitken, *et al.*, 1989a; Aitken, *et al.*, 1989b; Aitken, *et al.*, 1991; WHO, 1992; WHO, 1999; Huszar, *et al.*, 1992; Huszar, *et al.*, 1994; ESHRE, 1996).

We recently published the results of a meta-analysis that aimed to determine the diagnostic test accuracy and predictive value of various sperm function assays for IVF outcome (Oehninger, *et al.*, 1996). The scrutinized tests were CASA, acrosome reaction testing, the zona-free hamster egg penetration test or sperm-penetration assay (SPA) and sperm-zona pellucida binding assays.

The validity of such assays was objectively assessed through results obtained from 2,906 subjects evaluated in 34 prospectively designed, controlled studies. Results demonstrated a high predictive power of the sperm-zona pellucida binding and the induced-acrosome reaction assays for fertilization outcome. On the other hand, the findings indicated a poor clinical value of the SPA as predictor of fertilization and a real need for standardization and further investigation of the potential clinical utility of CASA systems.

The interaction between spermatozoa and the zona pellucida is a critical event leading to fertilization and reflects multiple sperm functions (i.e., completion of capacitation as manifested by the ability to bind to the zona pellucida and to undergo ligand-induced acrosome reaction) (Oehninger, *et al.*, 1992a; Oehninger, *et al.*, 1992b; Oehninger, *et al.*, 2000a). The two most common sperm-zona pellucida binding tests currently utilized are the hemizona assay (or HZA) (Burkman, *et al.*, 1988) and a competitive intact-zona binding assay (Liu, *et al.*, 1992). The high positive and negative predictive values, but more importantly, the low false negative rate (i.e., robust power to identify patients at high risk for fertilization failure) underscore the predictive ability of these tests.

The induced-acrosome reaction assays appear to be equally predictive of fertilization outcome and are simpler in their methodologies. The use of a calcium ionophore to induce acrosome reaction is at the present time the most widely used methodology (Tesarik, 1989; Cummins, *et al.*, 1991). Nevertheless, the implementation of assays using small volumes of human solubilized zonae pellucidae (Franken, *et al.*, 1996; Franken, *et al.*, 2000), biologically active recombinant human ZP3 (van Duin, *et al.*, 1994; Chapman, *et al.*, 1996; Dong, *et al.*, 2001) or active, synthetic ZP3 peptides (Hinsch, *et al.*, 1998) will probably allow for the design of improved, physiologically oriented acrosome reaction assays.

Franken et al (2000) devised a new micro assay that is easy and rapid to perform, and facilitates the use of minimal volumes of solubilized zona pellucida (even a single zona) for

assessment of human acrosome reaction. The micro assay has been validated as compared to standard macro assays and consequently offers a unique arena to test for the physiological induction of acrosomal exocytosis by the homologous zona pellucida. Moreover, preliminary clinical studies using the micro assay have demonstrated that the zona-induced acrosome reaction (ZIAR) can predict fertilization failure in the IVF setting. The micro assay ZIAR can therefore refine the therapeutic approach for male infertility prior to the onset of therapy (Esterhuizen, et al, 2001a and b).

Recently, Bastiaan et al (2001) prospectively evaluated the relationship between sperm morphology, acrosome resonsiveness to solubilized zona pellucida using the micro assay, sperm-zona biniding potential (HZA) and IVF outcome. Receiver operator characteristics (ROC) curve analyses indicated ZIAR to be a robust indicator for fertilization failure during IVF therapy with a sensitivity of 81% and specificity of 75%. In addition, a positive and significant correlation existed between ZIAR results and sperm morphology (r= 0.65) and sperm-zona binding (r= 0.57).

Genetic testing

It is now well established that a variety of genetic disorders are associated with male infertility, some of them potentially transmissible to the offspring (St John, 1999). Chromosomal anomalies (structural and numerical, involving autosomal and sexual chromosomes) can be diagnosed by peripheral karyotyping and should be studied in men with severe oligozoospermia and non-obstructive azoospermia (Chandley, 1998a; Chandley, 1998b; Martin, 1998). At the gene level, reproductive failure may be associated with cystic fibrosis mutations (in men presenting with obstructive azoospermia due to congenital absence of the vas deferens) and with Y-microdeletions (in men with severe oligozoospermia and non-obstructive azoospermia due to spermatogenic failure) (Wong, 1998: Roberts, 1998). Such abnormalities can also be detected by peripheral blood screening using PCR methodologies (conventional, nested, multiplex, fluorescent or quantitative PCR) (St. John, 1999).

Spermatozoa of infertile men have also been shown to contain various nuclear alterations. Some of them include an abnormal chromatin structure, aneuploidy, chromosomal microdeletions and DNA strand breaks (Gorczyca, *et al.*, 1993; In't Veld, *et al.*, 1993; Manicardi, *et al*, 1995; Hughes, *et al.*, 1996; Lopes, *et al.*, 1998;; Aitken, *et al.*, 1998; Evenson, *et al.*, 1999; Sakkas, *et al.*, 1999; Pfeffer, *et al.*, 1999). Different theories have been proposed to explain the origin of DNA damage in spermatozoa. Damage could occur at the time of or be the result of DNA packing during

the transition of histone to protamine complex during spermiogenesis. DNA fragmentation could also be the consequence of direct oxidative damage (free radical-induced DNA damage has been associated with antioxidant depletion, smoking, xenobiotics, heat exposure, leukocyte contamination of semen and presence of ions in sperm culture media). Alternatively, DNA damage could be the consequence of apoptosis.

Presently, various tests are available for detection of some of those anomalies, including the aniline blue staining (Hoffman and Holscher, 1991), acridine orange (Tejada, *et al.,* 1984), sperm chromatin structure assay (SCSA) (Evenson, *et al.*, 1999) and assessment of DNA damage or fragmentation (Aitken, *et al.*, 1998; Sakkas, *et al.*, 1999; Barroso, *et al.*, 2000).

Because of de novo aberrations occurring at the time of spermatogenesis, some infertile men may have chromosomal/genetic damage in their spermatozoa that are not found peripherally. Analysis of sperm by FISH has demonstrated a high incidence of chromosomal alterations in ejaculated spermatozoa in men with severe oligo-astheno-teratozoospermia as compared to fertile men (Pfeffer, *et al.*, 1999).

How to manage the infertile man

Clinicians and scientists are still searching for semen parameter thresholds in the so-called "normal fertile populations" in order to be able to more accurately define fertility, sub-fertility and infertility. Recent publications have appropriately re-addressed those issues as part as both European and American studies (Ombelet, *et al.*, 1997; Zinaman, *et al.* 2000).

Notwithstanding such lack of uniform criteria, if sperm abnormalities are observed in the "basic" semen analysis or if the couple is diagnosed as "unexplained" infertility, the work-up should proceed to the analysis of sperm function/biochemical tests. The diagnosis of sub-fertility or infertility, based upon the first-tier (initial "basic" evaluation) and the "expanded" (functional/biochemical) screenings, will direct management toward a variety of therapeutic options (Oehninger, *et al.*, 1991; Oehninger, *et al.*, 1997a; Oehninger, 2000a). We have previously proposed that laboratory evaluation of sperm quality/quantity for assisted reproduction should be approached using such sequential, multi-step diagnostic scheme (Oehninger, *et al.,* 1991; 1992 a and b; 1997a) (table1). This concept has also been proposed by others (Amann and Hammerstedt, 1993).

Before treating the infertile man, it is mandatory to examine the female counterpart. The simultaneous presence of female factors is frequently observed and such abnormalities may have an impact on the decision-making process. Pregnancy success varies among different therapies, but the presence of multi-factorial (male and female) infertility and/or a female age >35 years may direct the couple earlier to the more advanced and successful techniques (such as ICSI) rather than recommending less efficient (albeit less expensive) approaches.

Male infertility problems may be successfully treated with (a) urological interventions (surgical or non-surgical treatments, such as conventional, micro-surgical or laparoscopic surgery, including correction of varicocele, epididymo- and vaso-vasostomy and modern approaches for ejaculatory disorders); (b) medical therapies (such as specific treatment of hypogonadism, hyperprolactinemia and infection); while (c) a significant proportion will proceed to low and high complexity ARTs.

It is at this time that sperm function/biochemical tests may be of highest value in order to direct the couple to ART. Assisted reproduction can be indicated as a result of (a) failure of urological/medical treatments, (b) the diagnosis of "unexplained" infertility in the couple; (c) the presence of "basic" sperm abnormalities of moderate-high degree; or (d) abnormalities of sperm function as diagnosed by predictive bio-assays (such as the HZA or induced acrosome reaction test).

Currently recommended ART options include: "low complexity" intra-uterine insemination (IUI) therapy, "standard" IVF and embryo transfer, and IVF augmented with ICSI. If the female partner is <35 years, typically 4-6 cycles of intrauterine inseminations (IUI) using husband's sperm are recommended as a simple (low complexity) ART approach.

Although there are no established sperm cut-off levels, it is preferable to perform IUI if >5 million total motile spermatozoa can be used per insemination (particularly if sperm morphology is normal or only slightly abnormal). Pregnancy results for IUI in male infertility are higher with concomitant ovarian stimulation. Such regimens include use of clomiphene citrate, a combination of clomiphene and gonadotropins, or gonadotropins alone. Sperm preparation techniques involve a simple washing step (in a capacitating medium) or washing/separation of the motile fraction (typically using a gradient centrifugation method). The latter will result in a better selection of functional cells at the price of a decreased total number of spermatozoa.

Results of IUI therapy from our program (including couples with and without a male factor) are presented in table 2. As shown, the cumulative pregnancy rate at 6 months appears to be higher in IUI-cycles performed following gradient separation techniques. Gradient separation should also be recommended in the presence of round cells and/or anti-sperm antibodies. Female cycle monitoring is mandatory to avoid ovarian hyperstimulation and multiple pregnancies. Although an overall IUI clinical pregnancy rate per cycle of 12% to 14% is within expectation, those couples with abnormal sperm parameters (moderate oligoastheno-teratozoospermia) typically have a lower rate of about 8% to 12% per cycle, depending upon the severity of the anomalies. In such cases, the cumulative pregnancy rate (4 to 6 cycles of insemination) may vary from 30% (in 30-year-old women) to only 10% (in 39-year-old women) (Morshedi et al, 1999).

Patients with a motile sperm fraction (>1.5 million motile spermatozoa following swim-up or gradient centrifugation), but with mild to moderate teratozoospermia (in the range of 4-14% normal forms by strict criteria) should be offered "standard" IVF therapy. In those cases, good fertilization and pregnancy rates are achieved with an increase in the sperm insemination concentration (Oehninger, *et al.*, 1988; 1996).

In our program, patients are selected for IVF augmented with ICSI according to the following indications: [1] poor sperm parameters (i.e., $<1.5 \times 10^6$ total spermatozoa with adequate progressive motility after separation, severe teratozoospermia with <4% normal forms in the presence of a borderline to low total motile fraction, and/or poor sperm-zona pellucida binding capacity with a hemizona assay index <30%) (Oehninger, *et al.*, 1997a); [2] failure of IUI therapy in cases presenting with abnormal sperm parameters including presence of anti-sperm antibodies); [3] previous failed fertilization in IVF; and [4] presence of obstructive or non-obstructive azoospermia, where ICSI is combined with sperm extraction from the testes or the epididymis (Oehninger, 2000a).

In the presence of severe oligo-astheno-teratozoospermia or if the outcome of sperm function testing indicates a significant impairment of fertilizing capacity, couples should be immediately directed to ICSI. This approach is probably more cost-effective and will avoid loss of valuable time (particularly in women >35 years). The results of ICSI using ejaculated, epididymal (using MESA or microsurgical epididymal sperm aspiration) and testicular sperm (using TESA or testicular sperm extraction) performed in our program in the last five years are shown in (table 3) (Monzo, *et al.*, 2001).

As can be observed, good results can be achieved in terms of fertilization, implantation and pregnancy, irrespective of the origin of the spermatozoa (semen, epididymis or testicle). The only exception is the outcome of non-obstructive azoospermia where testicular sperm are aspirated from men with severe impairments of spermatogenesis, including Sertoli cell-only syndrome, partial maturation arrest, hypospermatogenesis and other pathological conditions. Such poorer results may be the consequence of a compromised male gamete (in terms of chromosomal content, DNA damage or other membrane-cytoplasmic abnormalities) (Oehninger, 2000a).

In addition, sperm cryopreservation continues to be a valuable clinical aid in the management of male infertility. Its current principal indications include: (1) donor sperm insemination; (2) freezing before cancer therapy to maintain reproductive capacity; and (3) because of the outstanding success with ICSI even cases with oligo-astheno-teratozoospermia or azoospermia with extracted testicular/epididymal sperm may be offered freezing for future use in assisted fertilization (Oehninger, *et al.*, 2000b).

Conclusions

On the basis of current evidence, the use of ICSI should be restricted to male-factor infertility, for which it seems to be cost-effective and relatively safe (Oehninger, 2001b). However, as in other areas of the ever-expanding specialty of ART, vigilance is required to identify any potential negative impact on the long-term health of children conceived after ICSI. Although ICSI constitutes a validated, formidable therapy that can help most cases of male infertility, the identification of specific sperm defects should allow the development of simpler, directed therapies. The basic semen analysis remains the cornerstone in the evaluation of the male partner. Validated sperm functional tests such as the HZA and ZIAR should expand the initial work up as indicated. The urologist and reproductive endocrinologist should work as a team to offer the best option to each couple suffering from male infertility.

References

1. Aitken RJ, Gordon E, Harkiss D, *et al.*, (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod*, **59**, 1037-1046.
2. Aitken RJ, Irvine DS, Wu FC. (1991) Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol,* **164**, 542-551.
3. Aitken RJ, Clarkson JS, Fishel S. (1989a) Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol Reprod*, **41**, 183-187.
4. Aitken RJ, Clarkson JS, Hargreave TB, *et al.*, (1989b) Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J. Androl,* **10**, 214-220.
5. Amann RP, Hammerstedt RH. (1993) In vitro evaluation of sperm quality: an opinion. *J. Androl,* **14**, 397-406.
6. Barroso G, Morshedi M, Oehninger S. (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod,* **15**, 1338-1345.
7. Bastiaan HS, Windt ML, Menkveld R, Kruger TF, Oehninger S, Franken DR. (2002) The relationship between zona pellucida induced acrosome reaction (ZIAR), sperm morphology, sperm-zona ellcuida binding and in vitro fertilization (*Fertil Steril*, submitted)
8. Bhasin S, De Kretser D, Baker HWG. (1994) Pathophysiology and natural history of male infertility. *J Clin Endocrinol Metab*, **79**, 1525-1529.
9. Bonduelle M, Camus M, De Vos A., *et al.*, (1999) Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. *Hum Reprod,*, 1999, **14**, 243-264.
10. Burkman LJ, Coddington CC, *et al.*, (1988) The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril,* **49**, 688-697.
11. Center for Disease Control and Prevention.Reprodution Technonlogy Success Rates.(1998) National summary and Fertility Clinic reports. U.S. Department of Health and Human Servises, 9-32.
12. Centers for Disease Control and Prevention. (1997) Fertility, Family Planning and

Women's Health: New Data from the 1995 National Survey of Family Growth. US Department of Health and Human Services, USA, 1-11.

13. Chandley AC. (1998a) Chromosome anomalies and Y chromosome microdeletions as causal factors in male infertility. *Hum Reprod,* **13**, 45-50.
14. Chandley AC. (1998 b) Genetic contribution to male infertility. *Hum Reprod,* **13**, 76-83.
15. Chapman NR, Barratt CLR. (1996) The role of carbohydrate in sperm-ZP3 adhesion. *Mol Hum Reprod,* **2**, 767-774.
16. Cross NL, Morales P, Overstreet JW, *et al.*, (1986) Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res*, **15**, 213-226.
17. Cummins JM, Pember SM, *et al.*, (1991) A test of the human sperm acrosome reaction following ionophore challenge. Relationship to fertility and other seminal parameters. *J. Androl,* **12**, 98-103.
18. De Jonge C. Commentary. (2000) Forging a partnership between total quality management and the andrology laboratory. *J. Androl,* **21**, 203-205.
19. Dong KW, Chi TF, Juan YW, *et al.*, (2001) Characterization of the biological activities of a recombinant human zona pellucida protein 3 (ZP3) expressed in human ovarian (PA-1) cells. *Am J Obstet Gynecol,* **184**, 835-844.
20. ESHRE Andrology Special Interest Group (1996) Consensus Workshop on Advanced Diagnostic Andrology Techniques. *Hum Reprod,* **11**, 1463-1479.
21. Esterhuizen, A.D., Franken, D.R., Lourens, J.G.H., *et al.*, (2001a) Clinical importance of a micro-assay for the evaluation of sperm acrosome reaction using homologus zona pellucida. *Andrologia*, **33**, 87-93.
22. Esterhuizen, A.D., Franken, D.R., Lourens, J.G.H., *et al.*, (2001b) Clinical importance of zona pellucida induced acrosome reaction (ZIAR test) in cases of failed human fertilization. *Hum Reprod,* **16,** 136-144
23. Evenson DP, Jost LK, Marshall D, *et al.*, (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod,* **14**, 1039-1049.
24. Franken DR, Bastiaan HS, Oehninger S. (2000) Physiological induction of the acrosome reaction in human sperm: Validation of a microassay using minimal volumes of solubilized, homologous zona pellucida. *J Assist Reprod Genet,* **17**, 156-160.
25. Franken DR, Morales PJ, *et al.*, (1996) Inhibition of G protein in human sperm and its

influence on acrosome reaction and zona pellucida binding. *Fertil Steril,* **66**,1009-1011.

26. Gorczyca W, Traganos F, *et al.*, (1993) Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: anology to apoptosis of somatic cells. *Exp Cell Res*, **207**, 202-205.
27. Hinsch E, Oehninger S, Hinsch K. (April, 1998) ZP3-6 peptide and acrosome reaction. Semen evaluation, testing and selection: a new look in the 2000s. *In Andrology in the Nineties Precongress Training Course. Genk, Belgium*, 25-30.
28. Hoffman N, Hilscher B. (1991) Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. *Hum Reprod*, **6**, 979-982.
29. Hughes CM, Lewis SEM, *et al.*, (1996) W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men using a modified comet assay. *Mol Hum Reprod,* **2**, 613-619.
30. Hull MGR, Glazener CMA, *et al.*, (1985) Population study of causes, treatment and outcome of infertility. *Br Med*, **291**, 1693-1697.
31. Huszar G, Vigue L. (1994) Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. *J. Androl,* **15**, 71-77.
32. Huszar G, Vigue L, Morshedi M. (1992) Sperm creatine phosphokinase M-isoform ratios and fertilizing potential of men: a blinded study of 84 couples treated with in vitro fertilization. *Fertil Steril,* **57**, 882-888.
33. In't Veld P, Brandenburg H, Verhoeff A, *et al.*, (1995) Sex chromosomal abnormalities and intracytoplasmic sperm injection. *Lancet*, **346**,773.
34. Irvine DS. (1997) Declining sperm quality: a review of facts and hypotheses. *Baillieres Clin Obstet Gynaecol*, **11**, 655-671.
35. Irvine DS. (1998) Epidemiology and etiology of male infertility. *Hum Reprod,* **13**, 33-44.
36. Kruger TF, Menkveld R, Stander FSH, *et al.*, (1986) Sperm morphologic features as a prognostic factor in in-vitro fertilization. *Fertil Steril,* **46**, 1118-1123.
37. Liu DY, Baker HWG. (1992) Tests of human sperm function and fertilization in vitro. *Fertil Steril,* **58**, 465-483.
38. Liu DY, Lopata A, *et al.*, (1988) A human sperm-zona pellucida binding test using oocytes that failed to fertilize in vitro. *Fertil Steril,* **50**, 782-788.
39. Lopes S, Jurisicova A, Sun J-G, *et al.*, (1998) Reactive oxygen species: potential cause

for DNA fragmentation in human spermatozoa. *Hum Reprod,* **13**, 896-900.

40. Manicardi GC, Bianchi PG, Pantano S, *et al.*, (1995) Underprotamination and nicking of DNA in ejaculated human spermatozoa are highly related phenomena. *Biol Reprod,* **52**, 864-867.
41. Martin RH. (1998) Genetics of human sperm. *J Assist Reprod Genet,* **15**, 240-245.
42. Monzó A, Kondylis F, Lynch D, *et al.*, (2001) Outcome of ICSI in azoospermic patients: stressing the liason between the urologist and reproductive medicine. *Urology,* 2001a, **58**, 69-75.
43. Morshedi M, Taylor S, *et al.*, (1999) A prospective randomized study comparing ISolate™-processed versus washed semen in intrauterine insemination (IUI) therapy using husband's sperm: Efficiency and pregnancy outcome. *Presented at the ASRM Annual Meeting and Canadian Fertility and Andrology Society, Toronto, Canada,* **396** (S217).
44. Mortimer D. (2000) Sperm preparation methods. *J. Androl,* **21**, 357-366.
45. Mosher WD, Pratt WF. (1990) Fecundity and Infertility in the United States, 1965-1988. Advance Data from Vital and Health Statistics. DHHA publication no. (PHS) 91-1250. Hyattsville, MD: Public Health Service.
46. Oehninger S. (2001) Place of intracytoplasmic sperm injection in clinical management of male infertility. *Lancet*, **357**, 2068-2069.
47. Oehninger S, Franken DR, *et al.* Sayed E, Barroso G (2000a) Sperm function assays and their predictive value for fertilization outcome in IVF therapy: a meta-analysis. *Hum Reprod Update,* **6**, 160-168.
48. Oehninger S, Duru NK, Srisombut C, Morshedi M (2000b) Assessment of sperm cryodamage and strategies to improve outcome. *Mol Cell Endocrinol*, **169**, 3-10.
49. Oehninger S. (2000) Clinical and laboratory management of male infertility: An opinion on its current status. *J Androl*, **21**, 814-821.
50. Oehninger S, Franken D, Kruger T. (1997a) Approaching the next millennium: how should we manage andrology diagnosis in the intracytoplasmic sperm injection era? *Fertil Steril,* **67**, 434-436.
51. Oehninger S, Mahony M, Ozgur K, Kolm P, *et al.*, (1997b) Clinical significance of human sperm-zona pellucida binding. *Fertil Steril,* **67**, 1121-1127.
52. Oehninger S, Kruger TF, Simon T. (1996) A comparative analysis of embryo implantation potential in patients with severe teratozoospermia undergoing in vitro fertilization with a high insemination concentration or intracytoplasmic sperm injection.

Hum Reprod, **11**, 1086-89.

53. Oehninger, S. (1995) An update on laboratory assesment of male fertility. *Hum Reprod,* **10**, 38-45.
54. Oehninger S, Toner JP, Muasher SJ, Coddington C, *et al.*, (1992a) Prediction of fertilization in vitro with human gametes: Is there a litmus test? *Am J Obstet Gynecol,* **167**, 1760-1767.
55. Oehninger S, Franken D, Alexander N, Hodgen GD (1992b) Hemizona assay and its impact on the identification and treatment of human sperm dysfunctions. *Andrologia,* **24**, 307-321.
56. Oehninger S, Acosta AA, Veeck L. (1991) Recurrent failure of in vitro fertilization: role of the hemizona assay (HZA) in the sequential diagnosis of specific sperm/oocyte defects. *Am J Obstet Gynecol,* **164**, 1210-1215.
57. Oehninger S, Acosta AA, Morshedi M, Veeck L, *et al.*, (1988) Corrective measures and pregnancy outcome in in-vitro fertilization patients with severe sperm morphology abnormalities. *Fertil Steril,* **50**, 283-387.
58. Ombelet W, Bosmans E, Janssen M, *et al.*, (1997) Semen parameters in a fertile versus subfertile population: a need for change in interpretation of semen testing. *Hum Reprod,* **12**, 987-993.
59. Pfeffer J, Pang M-G, Hoegerman SF. (1999) Aneuploidy frequencies in pellet and swim-up semen fractions from ten oligoasthenoteratozoospermic patients donating sperm for intracytoplasmic sperm injection (ICSI). *Fertil Steril,* **72**, 472-478.
60. Roberts KP. (1998) Y-chromosome deletions and male infertility: state of the art and clinical implications. *J. Androl,* **19**, 255-259.
61. Sakkas D, Mariechoz E, Manicardi G, *et al.*, (1999) Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod,* **4**, 31-37.
62. Society for Assisted Reproductive Technology/American Society for Reproductive Medicine. (1995) Assisted reproductive technology in the United States and Canada: 1993 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology Registry. *Fertil Steril,* **64**, 13-21.
63. St John JC. (1999) Incorporating molecular screening techniques into the modern andrology laboratory. *J. Androl,* **20**, 692-701.
64. Tejada RI, Mitchell JC, Norman A *et al.*, (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil Steril,* **42**, 87-91
65. Tesarik J. (1989) Appropriate timing of the acrosome reaction is a major requirement

for the fertilizing spermatozoon. *Hum Reprod,* **4**, 957-961.

66. van Duin M, Polman J, De Breet IT. (1994) Recombinant human zona pellucida protein ZP3 produced by Chinese Hamster Ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. *Biol Reprod*, **51**, 607-617
67. Vogt PH. (1998) Human chromosome deletions in Yq11 AZF candidate genes and male infertility: history and update. *Mol Hum Reprod,* **4**, 739-744.
68. Wilcox LS, Mosher WD. (1993) Use of infertility services in the United States. *Obstet Gynecol*, **82**,122-127.
69. Wong PY. (1998) CFTR gene and male fertility. *Mol Hum Reprod,* **4**,107-110.
70. World Health Organization. (1999) WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge, United Kingdom: Cambridge University Press, 4-33.
71. World Health Organization. (1992) WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 3rd ed. Cambridge, UK: Cambridge University Press, 23-26.
72. Yanagimachi R, Yanagimachi H, Rogers BJ. (1976) The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod*, **15**, 471-476.
73. Zinaman MJ, Brown CC, Selevan SG, Clegg ED. (2000) Semen quality and human fertility: A prospective study with healthy couples. *J. Androl,* **21**, 145-153

Table I. Laboratory tests for the sequential investigstion of the infertile man.

FIRST LEVEL	
Semen characteristics	• Volume, pH, viscosity and presence of agglutination • Identification of round cells (leukospermia or others), anti-sperm antibodies and micr organisms
Sperm parameters	• Vitality (vital dyes, hypo-osmotic swelling test, others) • Concentration (million/mL) • Progressive motility (%) • Morphology (strict criteria) (% normal forms) • Separation of the motile fraction (total motile fraction in millions)
EXPANDED LEVEL	
Sperm function tests	• Motion parameters (CASA) • Sperm-zona pellucida binding test (hemizona assay) • Induced acrosome reaction (ZIAR)
Sperm biochemical tests	• Generation of reactive oxygen species and measurement of lipid peroxidation • Measurement of enzymes (creatine kinase and others) • DNA/chromatin tests (aniline blue, acridine orange, SCSA, DNA fragmentation)
GENETIC TESTING	• Karyotype, microdeletions of Y chromosome, cystic fibrosis mutations

Table II. Insemination therapy: results of 2,131 consecutive cycles using husband's (with or without male infertility) and donor spermatozoa.

	Pregnancy rate/cycle (%)	Cumulative pregnancy rate (%)	
		6 months	12 months
Fresh			
Husband sperm (n=673)			
- **IUI Wash**	12	39	—
- **IUI Gradient Separation**	14	58 *	—
Cryopreserved-thawed			
Donor Sperm (n=1458)			
- **ICI (intra-cervical)**	17	44	55
- **IUI (intra-uterine)**	15	57	70

* $P<0.05$ compared to IUI wash.

Table III. ICSI therapy: results of 633 consecutive procedures.

	Ejaculated Sperm	MESA	TESA	
			Obstructive	Non-Obstructive
No. of Cycles	532	18	60	23
Fertilization rate (%)	72	71	73	64*
Pregnancy rate/transfer (%)	36	41	47	13*
Implantation rate (%)	13	11	12	5
Abortion rate (%)	19	16	15	13

$P<0.05$ compared to obstructive azoospermia.

Chapter 5.

Conclusions and future directions

Based on the results of our studies we conclude that:

1- The evaluation of the infertile man in the clinical setting includes a thorough history and physical examination followed by repeated semen analysis. An endocrine, urological and/or imaging workup should be performed as appropriate. Genetic testing (peripheral karyotype and/or mutations screening by PCR, and sperm aneuploidy by FISH and/or mutations screening by PCR) should be performed based upon defined indications. The analysis of gene expression in mature spermatozoa by novel micro array technology appears to be a promising technique ready to be introduced among the battery of genetic tests (Figure 1).

2- The examination of the semen should be performed following a sequential multi-step diagnostic scheme including the analysis of the seminal plasma and basic sperm parameters followed by sperm function testing (Figure 2). There are a variety of fundamental sperm functions acquired after completion of capacitation and essential for a successful sperm-oocyte interaction (Figure 3). The hemizona assay (HZA) and the zona pellucida-induced acrosome reaction (ZIAR) are validated and useful functional tests to predict the outcome of fertilization under *in vitro* conditions (Figure 4). CASA allows for the identification of sperm populations showing hyperactivation but the clinical value in the prediction of fertilization or pregnancy outcomes remains to be validated.

3- Consequently, the HZA and ZIAR tests should be immediately added to the diagnostic armamentarium in the clinical arena to assess male fertility potential. Used as part of the sequential diagnostic scheme presented herein, these bioassays offer the clinician useful means to direct therapy in assisted reproduction. More prospectively designed studies are needed to establish the value of these tests to predict pregnancy outcome in the IUI and natural reproduction settings.

4- Notwithstanding their present clinical value, it is estimated that new assays based on biotechnological advances (such as [i] the use of cloned and *in vitro* expressed zona pellucida proteins used as recombinant vectors in solid or liquid phase assays, or [ii] the production of agonistic molecules and identification of complementary receptors) may soon be developed to provide simpler and more universally applicable diagnostic tools.

5- The HZA and ZIAR examine critical sperm functions that are essential for fertilization to ensue. Zona pellucida binding and the zona-induced acrosome reaction reflect multiple sperm functions

including the acquisition and completion of the capacitated state. Consequently, these assays proved to be highly predictive of fertilization in IVF therapy. However, ICSI bypasses these physiological steps and other assays need to be developed to predict pregnancy potential following the use of such micromanipulation technique. The assessment of sperm DNA quality, the identification of the yet putative egg activating factor and of the molecular basis of events leading to sperm head decondensation and pronuclear formation, and the impact of the timing and dysfunctions of the activated male genome, will help us understand the roles of the male gamete during fertilization as well as the paternal contributions to embryogenesis in the normal and abnormal situations.

6- On the basis of current evidence, the use of ICSI should be restricted to male-factor infertility, for which it seems to be cost-effective and relatively safe. However, as in other areas of the evolving specialty of assisted reproduction, vigilance is required to identify any potential negative impact on the long-term health of children conceived after ICSI. Although ICSI constitutes a validated, formidable therapy that can help most cases of male infertility, the identification of specific sperm defects should allow the development of more directed and maybe less complex and invasive therapies. The basic semen analysis remains the cornerstone in the evaluation of the male partner. Validated sperm functional tests such as the HZA and ZIAR should expand the initial work up as indicated. The urologist and reproductive endocrinologist should work as a team to offer the best option to each couple suffering from male infertility. Consequently, Andrology remains an ever-growing subspecialty.

7- Finally, extended cellular-molecular investigations of the processes of human sperm capacitation and interaction with components of the cumulus oophorus and its homologous zona pellucida, including changes of phospholipid dynamics in preparation for receptor- ligand interactions, priming and synergistic affects of follicular fluid components, activation of second messengers and identification of intracellular cascades resulting in acrosomal exocytosis and egg fusion, are needed for translational research resulting in new diagnostic and therapeutic opportunities (Figures 5-8). As the human genome project and the area of proteomics advance, their results and those of studies performed in combination with more classic reproductive biology-endocrinology techniques, will unveil the basis of sperm-oocyte interaction dysfunctions to alleviate male infertility.

Figure 1

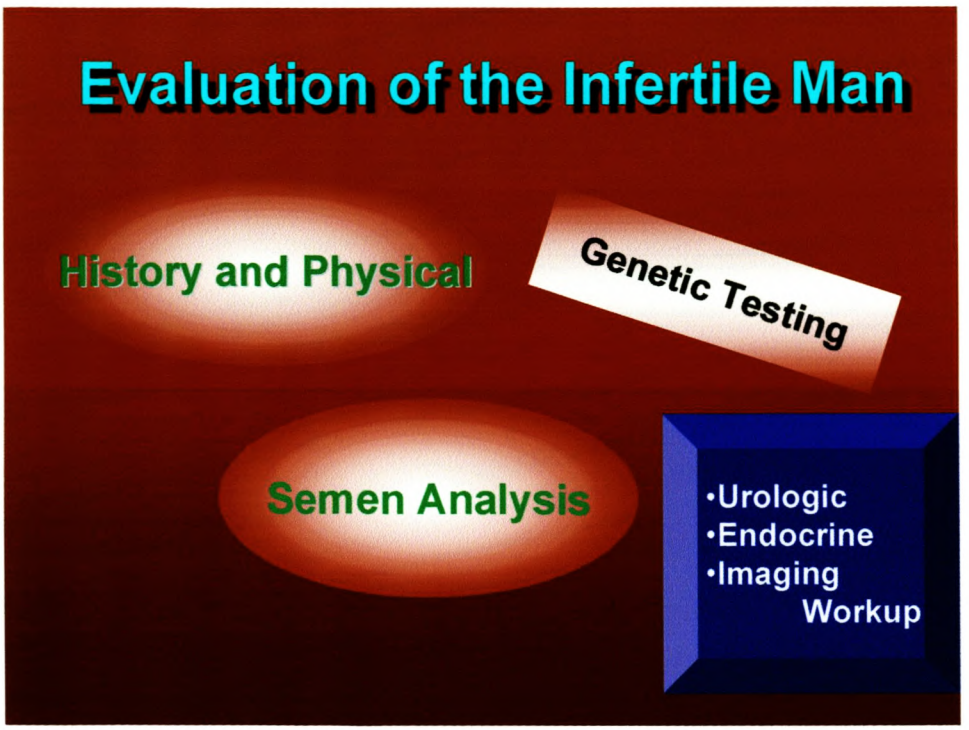


Figure 2

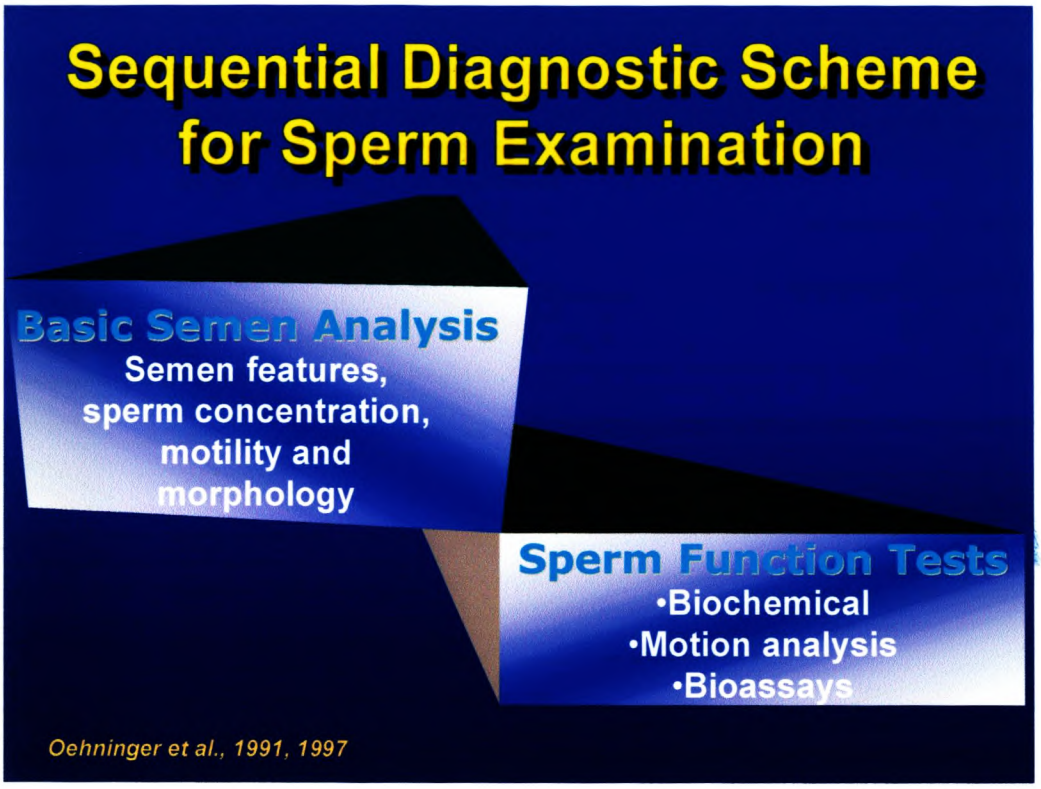


Figure 3

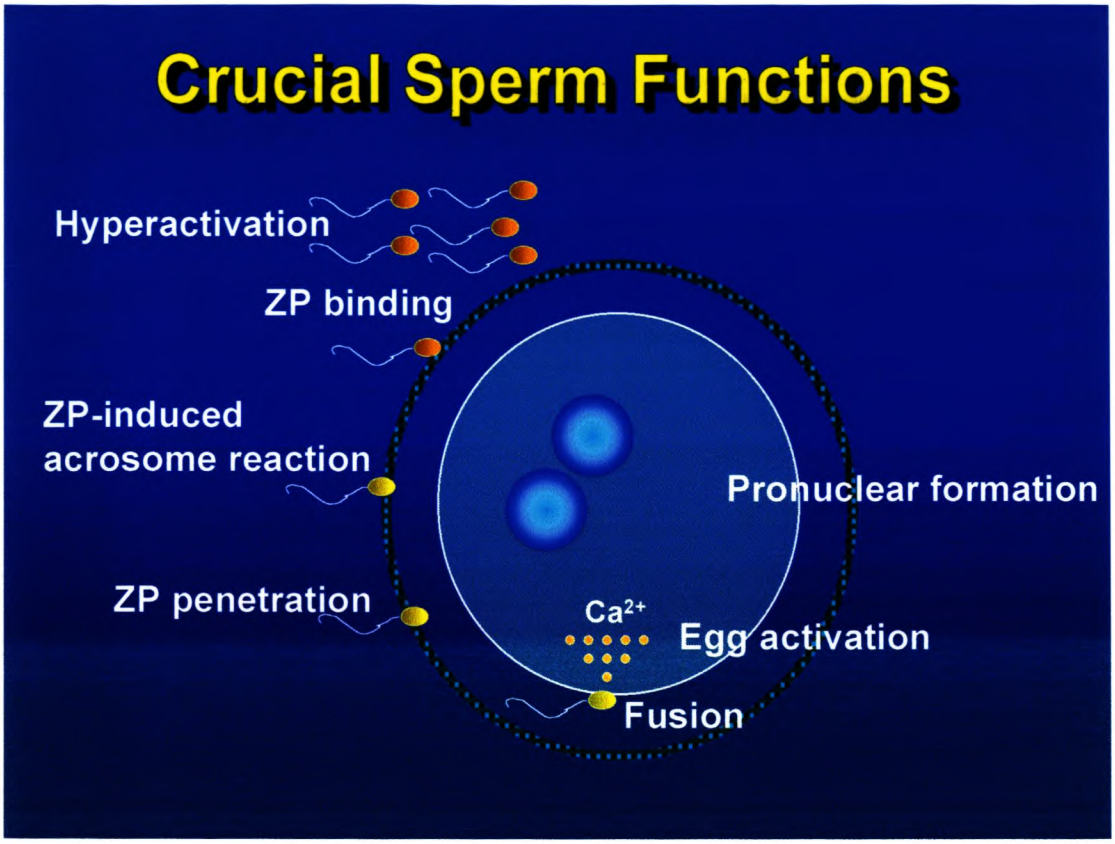


Figure 4

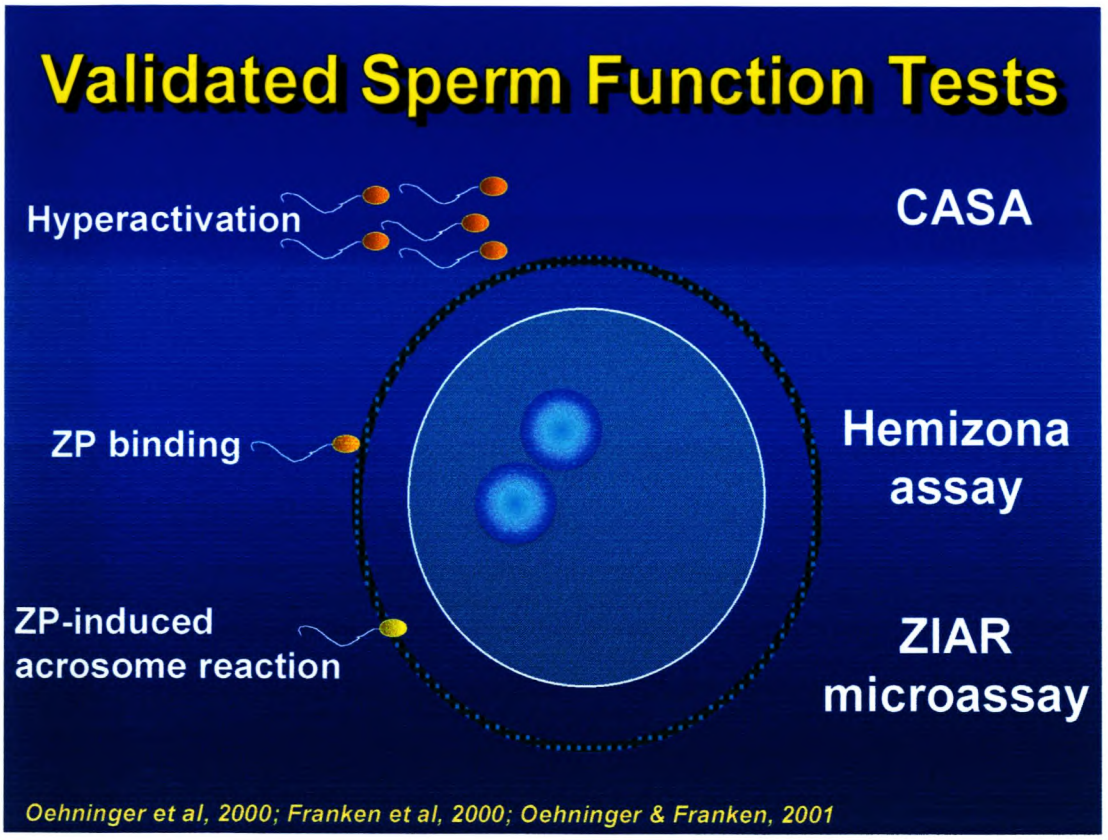


Figure 5

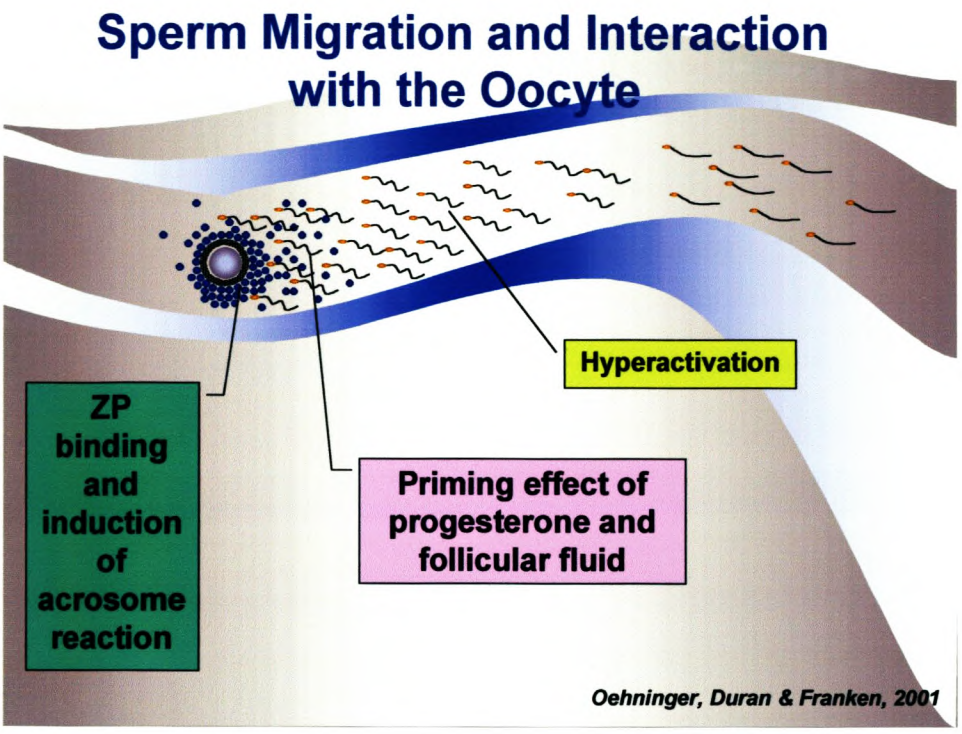


Figure 6

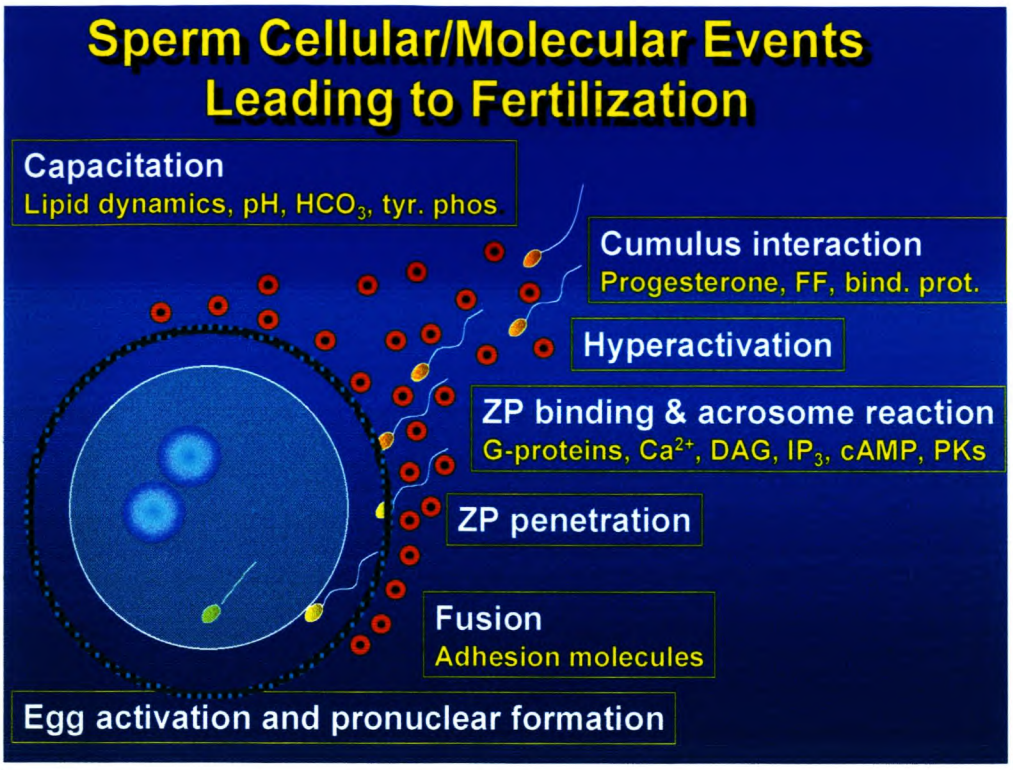


Figure 7

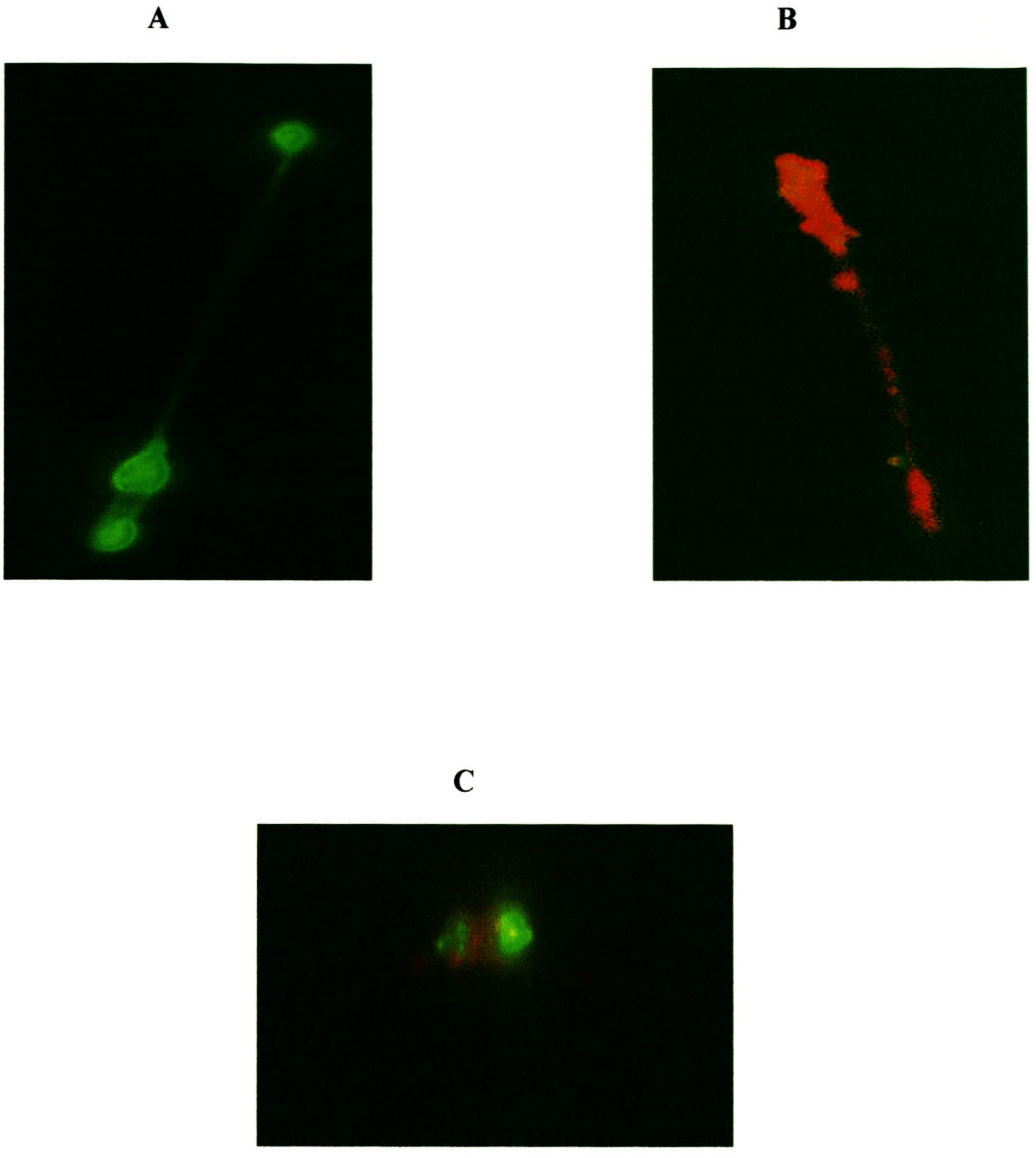
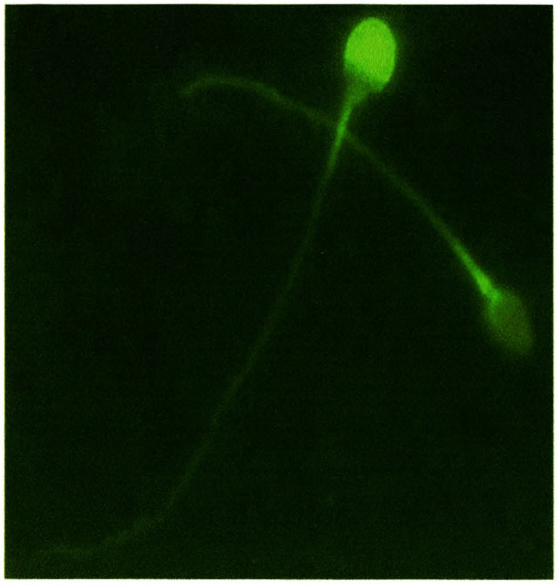
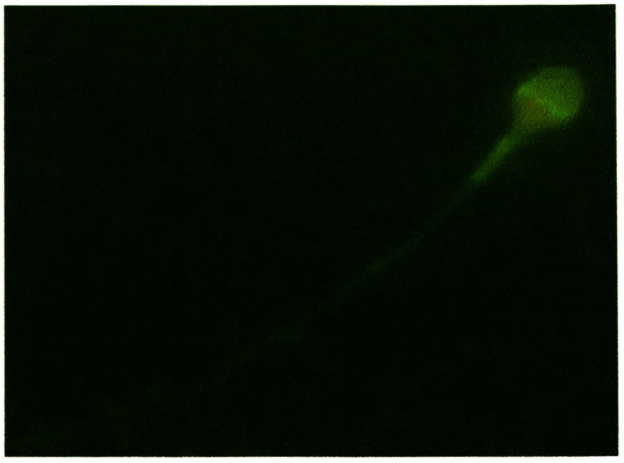


Figure 8

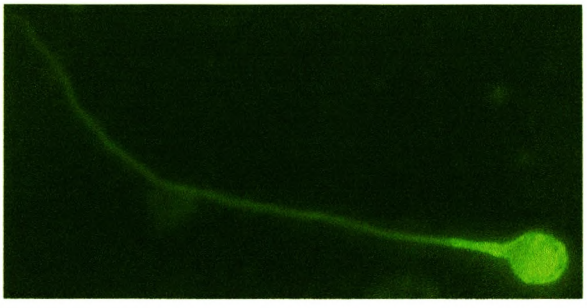
A



B



C



Legends to figures

Figure 1. Management of the infertile man in the clinical setting.

Figure 2. Sequential multi step diagnostic scheme for sperm examination.

Figure 3. Essential sperm functions that follow the acquisition of capacitation.

Figure 4. Validated sperm function tests.

Figure 5. Schematic representation of *in vivo* sperm-oocyte interaction.

Figure 6. Sequence of cellular-molecular events leading to capacitation, sperm-oocyte interaction and early embryogenesis.

Figure 7. Representative categories of spermatozoa as assessed by the annexin V binding assay: (A) live-normal spermatozoon (green), (B) dead spermatozoon (red) and (C) live cell with PS translocation (green-red).

Figure 8. Representative acrosome intact and reacted spermatozoa as observed under fluorescent microscopy with PSA staining. (A): Spermatozoon with intact acrosome (upper sperm cell); spermatozoon with no staining in acrosomal region (acrosome reacted) (lower sperm cell); (B): spermatozoon with no staining at acrosomal cap but staining at equatorial region (acrosome reacted, bar pattern); (C): spermatozoon with patchy staining at acrosomal cap (acrosome reacted).

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Sergio Oehninger, MD, MS	**Professor, Dept of Obstetrics and Gynecology & Dept of Urology; Director, Division of Reproductive Endocrinology and Infertility**

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of the Republic, Montevideo, Uruguay	MD	1980	Medicine
	MS	1981	Physiology
Rutgers University, New Brunswick, New Jersey	Specialty	1985	Ob/Gyn
	Fellow	1988	Reprod Endocrinol
University of the Republic, Montevideo, Uruguay			
Eastern Virginia Medical School, Norfolk, Virginia			

RESEARCH AND PROFESSIONAL EXPERIENCE:

Professional Experience

1978 – 1986 Assistant Professor, Dept of Physiology, University of the Republic, Uruguay
1988 – 1993 Assistant Professor, The Jones Institute, Dept of Ob/Gyn, Eastern Virginia Medical School, Norfolk, VA
1993 – 1999 Associate Professor, The Jones Institute, Dept of Ob/Gyn, Eastern Virginia Medical School, Norfolk, VA
1998 – present Visiting Professor, Dept of Ob/Gyn, University of Stellenbosch, Cape Town, South Africa
1999 – present Professor, The Jones Institute, Dept of Ob/Gyn, Eastern Virginia Medical School, Norfolk, VA
2000 – present Professor, Department of Urology, Eastern Virginia Medical School, Norfolk, VA
2000 present Director, Division of Reproductive Endocrinology and Infertility, The Jones Institute, Dept. Ob/Gyn.

Honors and Awards

Fulbright Scholar, Rutgers University, New Jersey, 1981; Poster Prize: American Fertility Society, 1990 and IV World Congress for IVF, 1991; Movie Award: American Fertility Society, 1992; Faculty of Biomedical Sciences, PhD Program, Old Dominion University/Eastern Virginia Medical School, 1992; Adjunct Professor, Hospital Militar, Uruguay, 1995; Member, Andrology Expert Advisory Committee, FIGO, 1998; First Prize Award, American Society for Reproductive Medicine, 2000; Membership to the American Gynecological Obstetrical Society, 2001.

Editor: Andrologia **Editorial Board:** Reproduction; Biology of Reproduction **Ad Hoc Reviewer:** Fertility and Sterility; Journal of Assisted Reproduction and Genetics; Obstetrics and Gynecology; Int. Journal of Andrology; Molecular Reproduction and Development; Contraception; Journal of Andrology; Andrologia; Int. Journal of Fertility; Human Reproduction.

Research Projects Ongoing or Completed during the last Years:
Oehninger S, Co-PI, Acosta AA, PI: Contraceptive potential of FSH suppression in male primates. Contraceptive Research and Development (CONRAD) Program, [$90,000/year for three years, 1988-1991].
Oehninger S, PI: Physiologic and biochemical studies of frozen/thawed human spermatozoa: an attempt to define the nature of cryodamage. Eastern Virginia Medical School [$5,000/1 year, 1990].
Oehninger S, PI: Influence of acrosomal status on sperm binding ability to the zona pellucida. Eastern Virginia Medical School [$6,000/1 year, 1991].
Clark G, Oehninger S (Co-PI): Investigation of the role of glycoconjugates in human sperm-egg binding using the hemizona assay system. Jeffress Memorial Trust Research Grant. [$45,000 per yr/3 years, 1991].
Oehninger S: LIF and murine implantation studies. Eastern Virginia Medical School [$9600/1 year, 1994].
Oehninger S, Dong K: Characterization of a human recombinant ZP3. Jeffress Trust [$30,000 per year/2 years, 1995-1997].
Oehninger S: Apoptosis in human sperm. Commonwealth of Virginia Health Board. [$60,000/1 year, 2000].
Oehninger S: Characterization of cellular pathways of apoptosis in human sperm. Jeffress Trust. [$65,000/3years, 2000-02].

Institutional Organizations
Chairman and Vice Chairman, Institutional Review Board, 1996-1999; Faculty Awards Committee, 2000-02, Faculty Appointments Committee, 2001-02, Eastern Virginia Medical School

Scientific Societies: American Society for Reproductive Medicine; American Society of Andrology; Society of Assisted Reproductive Technologies; Society for Gynecologic Investigation; New York Academy of Sciences; Society for the Study of Reproduction; European Society of Human Reproduction and Embryology

BIBLIOGRAPHY: Last five years (selected from more than 150 peer-reviewed publications since 1988)

1. Mitchell M, Swanson RJ, Hodgen GD, Oehninger S. Enhancement of in vitro murine embryo development by recombinant leukemia inhibitory factor (rLIF). J. Soc Gynecol Invest 1994; 1:215-219.
2. Kamada S, Blackmore PF, Kubota T, Oehninger S, Asada Y. The role of endothelin-1 in regulating human granulosa cell proliferation and steroidogenesis. J. Clin Endocrinol Metab 1995:80:3708-14, 1995.
3. Dell A, Morris H, Easton R, Panico M, Patankar M, Oehninger S et al. Structural analysis of the oligosaccharides derived from glycodelin, a human glycoprotein with potent immunosuppressive and contraceptive activities. J Biol Chem 1995; 270:24116-24126.
4. Oehninger S, Hinsh E, Pfisterer S, Kolm P, Shill W, Hodgen GD, Hinsh KD. Use of a specific ZP3 antiserum as a clinical marker for human-zona pellucida integrity and function. Fertil Steril 1996; 65:139-145.
5. Patankar M, Ozgur K, Oehninger S, et al. Expression of glycans linked to natural killer cell inhibition on the human pellucida. Mol Hum Reprod 1997; 3:501-505.
6. Ozgur K, Patankar MS, Oehninger S, Clark GF. Direct evidence for the involvement of carbohydrate sequences in human sperm-zona pellucida binding. Mol Hum Reprod 1998; 4:318-324.
7. Oehninger S. Will ovarian autotransplantation have a role in reproductive and gynecologic medicine? Fertil Steril 1998; 70: 20-21.
8. Hsu M-I, Kolm P, Leete J, Dong K-W, Muasher S, Oehninger S. Analysis of implantation in assisted reproduction through the use of serial human chronic gonadotropin (hCG) measurements. J Assist Reprod Genetics 1998; 15:495-502.
9. Develioglu O, Cox B, Toner J, Oehninger S, Muasher S. The values of basal serum follicle stimulation hormone, luteinizing hormone and estradiol concentrations following pituitary down-regulation in predicting ovarian response to stimulation with highly purified follicle stimulating hormone. Hum Reprod 1998; 14:1168-1174.
10. Huey S, Abuhamad A, Barroso G, Hsu M-I, Kolm P, Mayer J, Oehninger S. Perifollicular blood flow Doppler indices, but not follicular pO2, pCO2, predict oocyte developmental competence in IVF. Fertil Steril 1999; 72:707-712.

11. Hsu M-I, Mayer JF, Aronshon M, Lanzendorf S, Muasher S, Oehninger S. Embryo implantation in IVF and ICSI: impact of the number of embryos transferred, morphology grade and cleavage status. Fertil Steril 1999; 72:679-685.
12. Hinsch E, Oehninger S, Schill W-B, Hinsch KD. Species specificity of human and murine anti-ZP3 synthetic peptide antiserum and use of the antibodies for localization and identification of ZP3 or ZPC domains of functional significance. Hum Reprod 999; 14:419-428
13. Develioglu O, Hsiu J-G, Nikas G, Toner JP, Stacey MW, Mayer J, Oehninger S, Jones H. Endometrial estrogen and progesterone receptor and pinopode expression in stimulated cycles in oocyte donors. Fertil Steril 1999; 1040-1047.
14. Barroso G, Barrionuevo M, Rao P, Graham L, Danforth D, Huey S, Abuhamad A, Oehninger S. Vascular endothelial growth factor, nitric oxide and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil Steril 1999; 72:1024-6.
15. Oehninger S, Mayer J, Muasher S. Impact of different clinical variables on pregnancy outcome following embryo cryopreservation. Mol Cell Endocrinol 2000; 169:73-77.
16. Oehninger S, Duru N. et al. Assessment of sperm cryodamage and strategies to improve outcome. Mol Cell Endocrinol 2000: 169:3-10.
17. Hsu M-I, Barroso G, Mayer, Lanzendorf S, Gibbons W, Muasher S, and Oehninger S. Is the timing of implantation affected by zona pellucida micromanipulation? J Assist Reprod Genetics 2000; 17:34-38.
18. Brown S, Seppala M, Jones HW, Toner J, Mandelin E, Oehninger S. Increased endometrial glycodelin-A expression throughout the "implantation window" in controlled ovarian hyperstimulation cycles. Fertil Steril 2000; 74:130-33.
19. Brown S, Oehninger S, Schnorr J, Toner J, et al. Evaluation of outpatient hysteroscopy, saline enhanced hysterosonography and hysterosalpingography in infertile women: prospective, randomized study. Fertil Steril 2000: 74:1029-4034.
20. Brown S, Mandelin E, Oehninger S et al. Histochemical localization of endometrial insulin-like growth factor binding proteins during the luteal phase in controlled ovarian hyperstimulation cycles: a controlled study. Fertil Steril 2000; 74:338-42
21. Oehninger S. Molecular basis of human sperm-zona pellucida interaction. Cells Tissue Organs 2001; 168:58-64.

22. Duru K, Morshedi M, Schüffner A, Oehninger S. Cryopreservation thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. Fertil Steril 2001; 75:2, 263-268.

23. Dong K-W, Chi TF, Juan YM, Chen CW, Lin Z, Xiang XQ, Mahony M, Gibbons W, Oehninger S. Characterization of the biological activities of a recombinant human zona pellucida protein 3 (ZP3) expressed in human ovarian (PA-1) cells. Am J Obstet and Gynecol 2001; 184:835-844.

24. Lanzendorf S, Boyd C, Wright D, Muasher S, Oehninger S, Hodgen G. The use of human oocytes obtained from anonymous donors for the production of human embryonic stem cells lines. Fertil Steril, 2001;76:132-7.

25. Oehninger S. Place of intracytoplasmic sperm injection in management of male infertility. Lancet 2001, 357:2068-69 Mitchell M, Swanson RJ, Oehninger S. In vivo effect of LIF and anti-LIF polyclonal antibody on murine embryo/fetal development following exposure at the time of transcervical blastocyst transfer. Biol Reprod, 2002 (in press).

26. Wright D, Jones E, Mayer J, Oehninger S, Gibbons W, Lanzendorf S. Characterization of telomerase activity in the human oocyte and preimplantation embryo. Mol Hum Reprod 2001, 7: 947-955

27. Schnorr J, Oehninger S, Toner J, Hsiu J, Lanzendorf S, Williams R, Hodgen G. Functional studies of subcutaneous ovarian transplants in non-human primates: steroidogenesis, endometrial development, ovulation, menstrual patterns and gamete morphology. Hum Reprod 2002, 17: 612-19

28. Oehninger S. Place of intracytoplasmic sperm injection in management of male infertiliy. Lancet 2001, 357:20 68-69