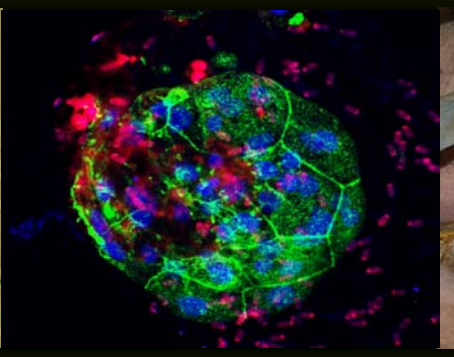
IN VITRO EMBRYO DEVELOPMENT IN THE PIG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KIDSON

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In vitro embryo development in the pig

impact of oocyte maturation milieu on blastocyst morphology and viability

In vitro embryo-ontwikkeling in het varken:

effect van het milieu van eicelmaturatie op de morfologie en levensvatbaarheid van de blastocyst

(met een samenvatting in het Nederlands)

Proefschrift

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"Life is full of cactus, but we don't have to sit on it."

Unknown

Vir my familie,

Vir Stephan,

En vir Mart,

Deo Gloria!

CHAPTER 1

General introduction

Assisted reproductive technologies (ART), such as in vitro embryo production, cryopreservation and transfer, have become an integral part of commercially applied breeding techniques in a variety of animal species [1]. Whereas progress of genetics through ART has played a core role in food-producing domestic species, such as the cow and sheep, during the past two decades it has not shown the same progress in the pig. Its importance as a food-producing animal, as well as a biological model in human biomedical applications, such as transgenesis directed at the pig as donor of cells, tissues and organs (xenotransplantation) [2], has prompted increased efforts to improve the efficiency of reproductive technologies in the pig. Despite the establishment of several systems to generate embryos in vitro, the efficiency is still poor and the quality of resultant blastocysts inferior compared with their in vivo counterparts. This limits the improvement of other reproductive techniques, such as embryo transfer and embryonic stem cell technology, which are dependent on the blastocyst as source of material. Advanced reproductive technologies rely more on the basic techniques of oocyte maturation and fertilization, and the development and identification of baseline requirements for these processes are essential and still much in need of further refinement in the pig.

In vitro embryo production (IVP) places major demands on oocytes and embryos with regard to developmental transitions, i.e. oocyte maturation, fertilization, compaction, blastocyst formation, all of which need to be traversed successfully in order for the embryo to develop to a new individual. These transitions are defined by exquisite remodelling of the gamete, zygote and embryo at intra- and intercellular levels, which sustains the continuation of its developmental program. Oocytes and embryos need to summon many signal transduction mechanisms, cytoskeletal constituents and genes in order to successfully negotiate and complete all the necessary developmental processes. Support of all these functions and processes is of cardinal importance when applying in vitro embryo production techniques to produce embryos capable of generating functional cell lines or live healthy offspring. Therefore, IVP systems are generally comprised of three stage-specific culture environments, specially designed to supply in the needs of each stage of oocyte, zygote and embryo development: 1) in vitro oocyte maturation (IVM), 2) in vitro fertilization (IVF), and 3) in vitro embryo culture (IVC). Although culture media have been adapted to suit the specific individual needs of the pig oocyte and embryo (i.e. North Carolina State University-23 (NCSU-23) medium [3]), not much progress in the improvement of embryo viability has been made since the adoption of its use a decade ago. Chronic and cumulative stresses caused by sub-optimal culture conditions or insufficiencies in the constituents of the culture medium, can lead to oocyte or embryo anomalies at a genetic or metabolic level and severely jeopardise the viability of the resultant embryo [4]. Markers for the best developmental potential of pig oocytes and embryos are scarce, thus confronting us with a dilemma, as we cannot estimate the developmental outcome for the in vitro period or the subsequent remaining in vivo period. As we become more sophisticated in the in vitro production of pig embryos, so

our battery of markers will increase and eventually improve our understanding and design of each separate phase of the IVP system for the pig.

This introductory chapter provides a general overview of in vitro embryo production in the pig, with particular reference to the difficulties and special requirements characteristic of this species.

In vitro maturation of oocytes

Oocyte maturation consists of two aspects, i.e. nuclear and cytoplasmic maturation (Figure 1). Nuclear maturation refers to the resumption of meiosis and progression to the metaphase II (MII) stage, whereas cytoplasmic maturation encompasses other, poorly understood, maturational events related to the cytoplasmic capacitation of the oocyte. These processes are believed to progress in parallel to one another, and synchronization of nuclear and cytoplasmic maturation are essential for establishing optimal oocyte developmental potential. Cumulus-oocyte-complexes (COCs) are typically harvested from the ovaries of slaughterhouse sows and prepubertal gilt, and specifically from follicles 3 to 6 mm in diameter. Selection of follicle size ensures the harvest of fully grown oocytes and hence the ability of the oocytes to resume and complete meiosis [5-7]. Hormonal supplements, such FSH, eCG or hCG, are added to the IVM medium in order to mimic the in vivo situation and stimulate nuclear maturation of the oocyte. Since its beneficial effects became known a decade ago [8, 9], it is now common practice in the pig to add these supplements only during the first half of maturation. Follicular fluid supplementation of the IVM medium has been proven to provide a beneficial microenvironment for the further development of the immature pig oocyte [8, 9]. Under these circumstances pig oocytes develop to MII without much difficulty, with a reasonable degree of cytoplasmic maturation. Nevertheless, the cytoplasmic component of oocyte maturation is not completely satisfactory and believed to be incomplete or delayed. Efficiency of cytoplasmic maturation includes the ability of the oocyte to block the penetration of more than one sperm [10], and also to support the decondensation of the sperm head within the ooplasm of the fertilized oocyte. Whereas nuclear maturation can be evaluated by simple nuclear staining methods, such as aceto-orcein or DAPI, cytoplasmic maturation can only be determined by indirect means such as the blastocyst yield and cell number, glutathione content of the oocyte and the percentage male pronucleus formation. Cytoplasmic maturation of pig oocytes can be improved by reducing oxidative stress caused by the COCs production of reactive oxygen species (ROS) due to the in vitro culture environment [11, 12]. Metabolic pathways, mediated by enzymes such as glutathione, control ROS cellular levels and protect the oocyte against the damaging effects of oxidative stress. Glutathione content of the oocyte can be increased by adding thiol compounds such as cysteine, cysteamine, glutamine, ß-mercaptoethanol and/or follicular fluid to the maturation medium [13, 14]. Apart from its protective action, glutathione also increases amino acid transport and stimulates DNA and protein synthesis [15, 16].

An important element for both nuclear and cytoplasmic maturation of the oocyte, are the layers of cumulus cells surrounding the oocyte. These cells act as a 'go-between' between the oocyte and the follicular or culture environment. Intracellular communication between the oocyte and the cumulus cells takes place via gap junctions which are facilitated by means of microfilamentar transzonal projections of the cumulus cells directly in contact with the zona pellucida, i.e. corona radiata cells [17]. These

processes transverse the zona pellucida and terminate upon the oocyte plasma membrane (oolemma), thereby establishing a route of communication by which direct transfer of substances important for oocyte growth and maintenance of meiotic arrest can take place [18]. Cumulus cells also play a protective and metabolic role in oocyte cytoplasmic maturation, by reducing cystine to cysteine and promoting the uptake of cysteine in the oocyte [12], thereby protecting the oocyte against oxidative stress caused by ROS. By evaluating the degree of expansion in response to gonadotrophin stimulation [19-22], cumulus cell function and viability can be estimated and serves as a gauge to approximate the COCs developmental potential. Hormonal stimulation also regulates remodelling of the actin cytoskeleton at the level of the cumulus cell transzonal processes, but also within the oocyte cortex. The actin cytoskeletal changes, mediated by the cumulus cells, are believed to stabilize the distribution of cortical granules in the cortex of the oocyte and aid in the migration of organelles such as mitochondria and the endoplasmic reticulum which also influences the ability of the oocyte to support normal fertilization [23, 24]. Other factors known to enhance nuclear and cytoplasmic maturation of the oocyte include somatic cell co-culture, or addition of growth factors to the IVM medium, which can have beneficial effects on embryo development and blastocyst cell number [25].

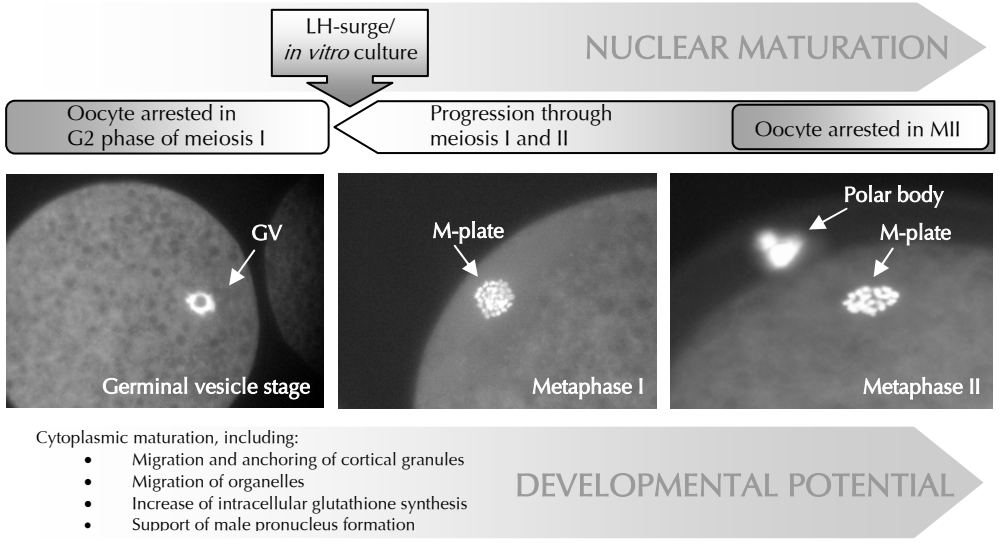

Figure 1. Follicular oocytes are arrested in the G2 phase (germinal vesicle stage) of the first meiotic division. The immature oocyte will resume meiosis and complete its final maturation upon the LH surge in vivo, or after being liberated from its follicular environment in vitro. Resumption of meiosis is characterized by condensation of the chromatin, breakdown of the germinal vesicle and organization of the chromosomes into the metaphase I (MI) plate and spindle. At this stage the oocyte enters into meiosis II and upon extrusion of the first polar body remains arrested in the metaphase II (MII) stage until activation of the oocyte takes place at fertilization. Parallel with nuclear maturation, cytoplasmic maturation/capacitation of the oocyte progresses and is characterized by migration of the cortical granules to the periphery of the ooplasm, migration of the mitochondria to the inner cytoplasm, and increased synthesis of glutathione (GV: germinal vesicle; M-plate: metaphase plate).

In vitro fertilization

The main feature, widely perceived to be a distinctive trait in porcine IVF, is the high prevalence of polyspermic fertilization. Polyspermy is an abnormality of the fertilization process (Figure 2), during which more than one sperm enters the oocyte due to failure of initiation or completion of the zona reaction to block the entry of multiple sperm (Figure 3). It is a potentially lethal condition, which in the majority of cases leads to the early demise of the conceptus. The exact cause of this phenomenon, which has an exceptionally high prevalence in the pig, has not yet been determined. Only recently has it been linked to the age of the oocyte donor. In the greater majority of IVP studies in the pig, oocytes are harvested from the ovaries of prepubertal gilts out of necessity, due to the relative unavailability of adult sow ovaries. In other species, such as the cow, sheep and goat [26-28], differences in oocyte developmental potential between prepubertal and adult donors have been reported and the prepubertal oocyte used as a negative model for studying oocyte developmental competence [26]. Similar studies in the pig have shown that age of the donor appeared to be the main cause of the polyspermy phenomenon and subsequent poor developmental of the porcine oocyte [29, 30]. Conversely, penetration rates exceeding 80% are typically achieved in prepubertal gilt oocytes, but polyspermy rates rarely measure less than 40%. When using sow oocytes, polyspermy rates in the range of 10% are routinely achieved, but penetration rates remain low (40-50%) [29, 30]. An ideal IVF system should support a high sperm penetration rate (>80%) and a low polyspermy rate (<10%).

In neither donor age group has the optimal fertilization result been achieved yet. Various strategies for the reduction of polyspermy have been developed, focussing on enhancement of either oocyte cytoplasmic maturation or modulation and adaption of sperm and IVF treatments. This has included co-culture of the male and/or female gamete with oviductal secretions or proteins, supplementation of fertilization media with glycosaminoglycan or thiol compounds, and IVF in the presence of cumulus cells [31]. Although these approaches have led to some degree of improvement, the level of polyspermy in prepubertal gilt oocytes remains far from satisfactory or comparison with that achieved in sow oocytes.

Akin to the calf and lamb, prepubertal gilt oocytes show intrinsic differences in both cytochemical and ultrastructural aspects [28, 29, 32]. Redistribution of organelles (cortical granules and mitochondria), also linked to cytoplasmic maturation, does not occur to the same extent in prepubertal gilt as in ovulated oocytes [33], although cortical granule (CG) exocytosis appears to be normal [34]. In sheep, the size of the mitochondria and CGs are smaller, and the glutamine metabolism lower in prepubertal than in adult oocytes. The size of the perivitelline space (PVS) has also been linked to the ability of the oocyte to prevent polyspermy, as ovulated oocytes have a much wider PVS than those matured in vitro [34]. Culture of oocytes in low NaCl medium results in a wider PVS and a also a reduction in polyspermy [9]. As pre-ovulatory oocytes have a narrower PVS and suffer a similar fate as in vitro maturated oocytes [35], post-ovulatory contact with the oviductal environment appears to supply the oocyte with the cytoplasmic machinery required to prevent polyspermy. Furthermore, after ovulation cumulus cells surround the oocyte at the time of sperm contact and thus also play an important role in promoting fertilization [18]. In the pig, though, due to the initial high polyspermy rates following IVF of cumulus-intact oocytes in early studies [36-39], it is now common practise to

denude COCs prior to fertilization, which may further contribute to the poor fertilization results commonly experienced.

Baseline values and characteristics, for sow vs. prepubertal gilt oocytes, yet need to be established, and will help to elucidate the intrinsic age-related differences between prepubertal and adult oocytes in order to identify specific means by which the developmental potential of prepubertal oocytes can be enhanced.

Embryo culture and development

Advanced technologies, such as embryo transfer or embryonic stem cell studies, rely on the blastocyst as its source of material. The production of large numbers of viable blastocysts is therefore one of the main goals in the majority of porcine IVP laboratories [4]. In the pig, the initially experienced developmental block at the 4-cell stage was overcome by adapting the then available culture media to suit the development and specific needs of the pig embryo. Current embryo culture systems are now able to support in vitro-derived zygotes up to the blastocyst stage. In fact, a number of porcine IVP groups routinely obtain a blastocyst formation rate of 30% or more, from in vitro matured oocytes [30, 40, 41] which is on par with that achieved in other farm animal species [42, 43]. The percentage of zygotes cleaved to the four to eight-cell stage 48 h after IVF, can be used as a gauge to estimate the potential of cleaved zygotes to develop to the blastocyst stage. Those embryos that consist only of evenly sized blastomeres are believed to have a better chance of further development than zygotes with cytoplasmic fragments, which frequently contain no nuclei. To which extent fragmentation is linked to polyspermy is not yet fully established, but cytoplasmic fragmentation has also been associated with oocyte aging and early developmental arrest [44].

Using the blastocyst yield as the ultimate parameter for evaluating the success of a given porcine IVP system is also not without its pitfalls. The resultant blastocyst should also be of optimal quality and viability. The term embryo quality is a fuzzy concept in any species, but even more so in the pig where non-invasive means or criteria for scoring blastocyst quality have not yet been developed. When taking into account the abovementioned problems with cytoplasmic maturation, and subsequent polyspermy, appearances can be deceptive as far as blastocyst quality are concerned in the pig. Polyspermic zygotes readily develop to blastocysts [45], but their viability after transfer is severely restricted: of blastocysts developing from polyspermic zygotes, 78% have been reported to suffer from abnormal ploidy [45] and also lag behind in development compared with their normospermic counterparts [46, 47]. In vitro produced blastocyst differ distinctly from their in vivo counters parts, with the former containing much lower numbers of nuclei. This is thought to be due to deficiencies in actin filament content and distribution within the cytoplasm [44], most likely related to poor cytoplasmic maturation of the original oocyte. Parameters used in other species to define embryo quality include blastocyst morphology, total and inner cell mass cell number, kinetics of development, post-cryopreservation survival, chromosomal abnormalities, metabolism, gene expression and apoptosis [48]. Of these parameters only blastocyst morphology and kinetics of development are non-invasive techniques, and neither has been developed for use in pig embryos yet. The remaining factors have indeed proven differences between in vivo and

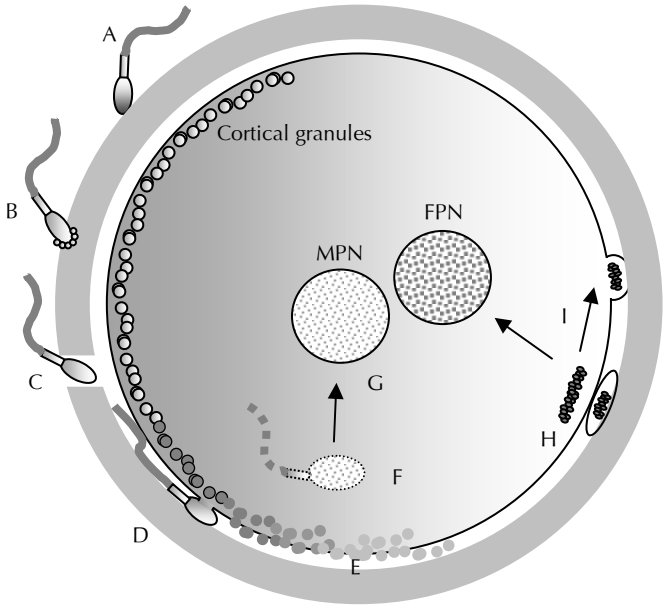

Figure 2. A schematic overview of the event sequence occurring during fertilization. (A) Acrosome intact, capacitated sperm bind to the zona pellucida, thereby (B) triggering the acrosome reaction. The enzymatic acrosomal contents lyse the zona pellucida (C), thus enabling the now hypermotile sperm cell to enter the perivitelline space, fuse with the oolemma (D) and activate the oocyte. Upon activation, release of cortical granule content takes place (E) which prevents the penetration of supplementary sperm. The sperm head, now incorporated within the ooplasm, begins to swell and decondense (F) eventually forming the male pronucleus (G). The oocyte, until now arrested in metaphase II (H), commences progression through meiosis II to extrude the second polar body (I), and form the female pronucleus (I). The male and female pronuclei are now in apposition, as a final prelude to syngamy.

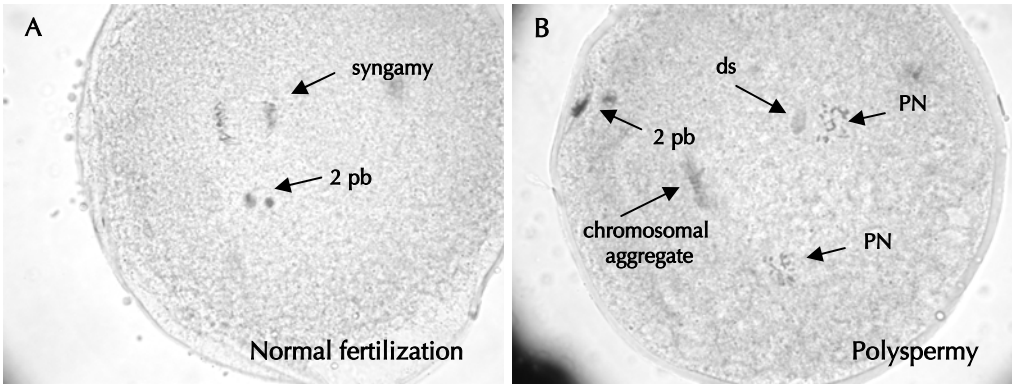

Figure 3. Micro-images of orcein stained porcine oocytes 20 h after the addition of sperm to the oocytes for IVF. In A) a normally fertilized oocyte is seen, with two polar bodies and the male and female chromosomal complement involved in the process of syngamy. In B), polyspermic fertilization has taken place and although two pronuclei can be seen, they are accompanied by a decondensed sperm head, and an extra set of aggregated chromosomal material. (2 pb: 2 polar bodies; ds: decondensed sperm head; PN: pronucleus)

in vitro derived porcine blastocysts, but none have been utilised to distinguish viable from non-viable embryos.

One parameter of particular interest in the pig is apoptosis, as in vivo-produced blastocysts present few or no apoptotic cells, whereas in vitro produced embryos show a much higher incidence. Apoptosis, or programmed cell death, serves a variety of purposes such as elimination of inappropriately differentiated inner cell mass cells [49], removal of cells containing chromosomal abnormalities such as haploidy [50], or of cells failing to activate the embryonic genome [51, 52]. It is therefore a naturally occurring cellular process in which the cell literally commits suicide without the neighbouring cell noticing. Apoptosis differs from necrosis in that coordinated gene-directed energy-dependent processes are required for its execution [53]. A cell undergoing apoptosis presents characteristic morphological features in which the cell shrinks, accompanied by organelle and cytoplasmic condensation, chromatic condensation, nuclear and DNA fragmentation, blebbing of the plasma membrane, cell fragmentation and finally phagocytosis by neighbouring cells [54]. The cascade of apoptotic events (Figure 4) is initiated by two pathways: an intrinsic and an extrinsic pathway [55, 56]. The intrinsic pathway, mediated via the mitochondria, may be activated by culture stress conditions including deprivation of growth factors, presence of toxic substances, excess free radicals or metabolic stress. These stimuli act directly on the pro-apoptotic Bcl-2 family, by phosphorylating Bad/Bax which in turn acts on the mitochondria causing the leakage of cytochrome c, which activates the apoptosis protease activating factor (apaf) [55, 56]. The initiator caspases are then cleaved leading to activation of downstream caspases which in turn cause nuclear and/or DNA fragmentation. The extrinsic, or receptor dependent pathway, is initiated by ligand-receptor binding which acts directly on the initiator caspases. This family of caspases are responsible for proteolytic events e.g. cleavage of cytoskeletal components such as lamin/actin which are thought to be responsible for plasma membrane blebbing. The nuclear apoptotic features are telltale signs of apoptosis which can be visualized using relatively simple fluorescent DNA-labelling techniques [52].

Embryo transfer

The acid test for determining the developmental potential of in vitro produced embryos is their survival after transfer to the uterus of a recipient female. In the pig, embryo transfer is still in its development stages which further complicate the progression of the associated ART-forms. Determining blastocyst viability is only one of many potential applications of ET, and the combination of IVP and ET holds much promise for both the agricultural and biomedical application of porcine embryo-related technologies. At the top of the pig breeding pyramid, the combination of ET and IVP could introduce complete genomes of a superior combination of animals (instead of 50% through sperm of a superior boar), minimize the risk of pathogen transfer associated with the introduction of

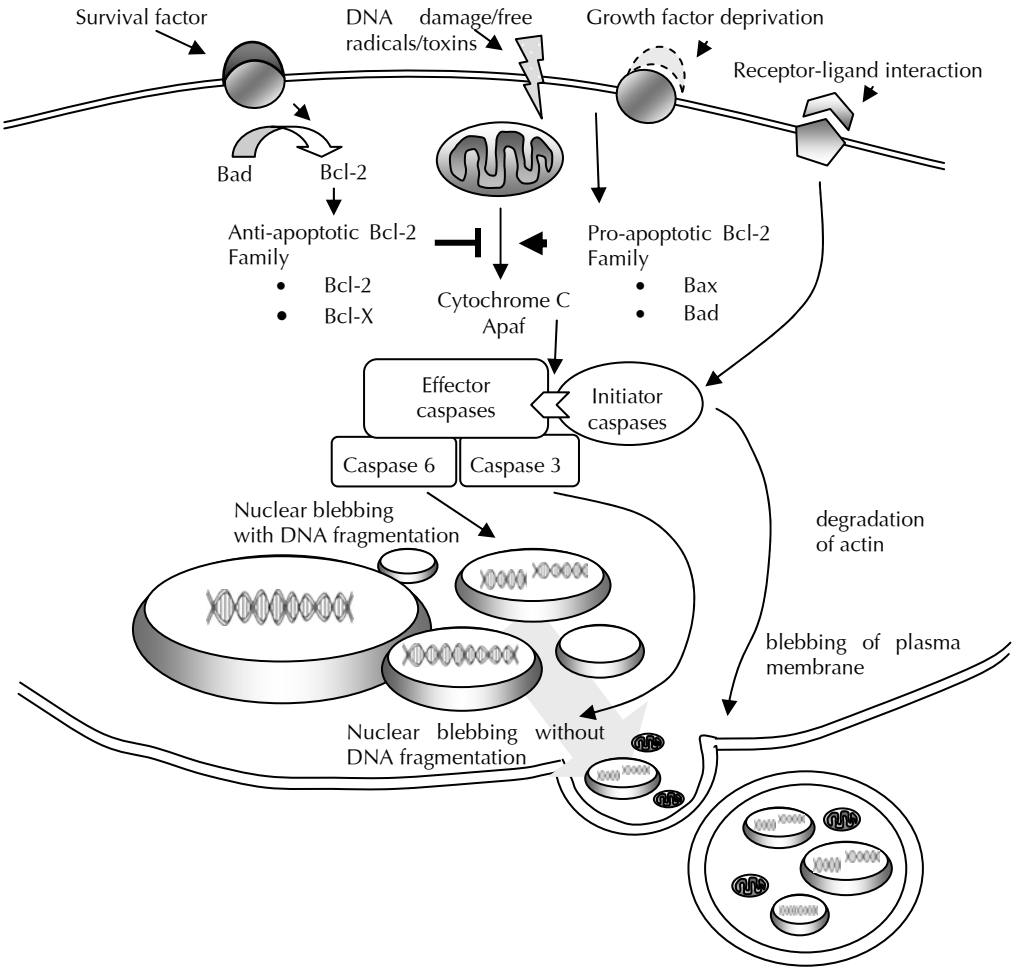

Figure 4. Schematic overview of the proposed major apoptotic pathways relevant in the embryo. The mitochondria-mediated 'intrinsic' pathway can be triggered by a wide range of stimuli, including deprivation of growth factors, presence of toxic substances or excess free radicals or metabolic stress. In contrast, apoptosis can be inhibited by presence of survival factors, which include growth factors and hormones. The system is regulated by the interplay of proapoptotic and antiapoptotic proteins of the Bcl-2 family. The proapoptotic proteins, such as Bad, contain a domain which forms a heterodimer with Bcl-2, blocking its survival promoting activity. The abundance of these pro- and antiapoptotic proteins determines the susceptibility of the cell to apoptosis. The proapoptotic proteins act on the mitochondrial membrane by decreasing the transmembrane potential and promoting leakage of cytochrome c. In the presence of dATP, cytochrome c activates Apaf, which in turn activates downstream caspases which results in apoptosis. The "extrinsic" receptor-dependent pathway, is initiated by ligand-receptor binding which acts directly on the initiator caspases. This family of caspases are responsible for proteolytic events e.g. cleavage of cytoskeletal components such as actin which are thought to be responsible for plasma membrane blebbing.

breeding animals into a herd, and also reduce the costs and welfare issues of live animal transport. The immediate impetus of ET/IVP, though, lies in the potential progression of developing technologies such as transgenesis and nuclear transfer [57, 58], which of course relies on IVP for the production of blastocysts suitable for transfer.

Transfer of in vivo produced pig blastocysts has mainly been performed surgically, with reasonable success rates [59]. At present, 60% of transfers lead to pregnancies, with 60% of transferred embryos surviving to farrowing. Recently, it was reported that the transfer of IVP blastocysts could lead to pregnancies and live births [30, 60], albeit at a low degree of efficiency. In these surgical transfer studies, vast numbers (50 to 100) of randomly chosen IVP blastocysts were transferred to uterus of recipient animals resulting in less than 20 % survival to farrowing. Explanations for these poor survival rates are most likely found in the inherent poor quality of in vitro produced blastocysts, worsened by the lack of selection criteria. It is therefore not yet clear whether an accurate picture of the survivability of IVP blastocysts is created by the recent transfer results. Data to correlate embryo morphological selection criteria and post-transfer survival rates therefore still need development to serve the needs of the actively developing field of in vitro pig embryo production.

Surgical transfer of porcine embryos is not allowed in the Netherlands due to the invasive nature of the procedure, which has necessitated the development of non-surgical ET (nsET). Being a less invasive procedure, requiring neither surgery nor sedation [61], nsET promises to be a valuable tool for both research and commercial purposes, due to its on-farm applicability and reduced need for special facilities. In the past, nsET was thought to be impossible in the pig due to the convoluted cervical canal, long coiled uterine horns and the location of the embryos in the tip of the uterine horns. However, the design of a pig-specific transfer instrument allowed the transcervical deposition of embryos into the uterine body of the recipient sow [59, 61, 62]. Pregnancy rates comparable to that of surgical ET have been achieved after the non-surgical transfer of in vivo produced blastocysts [61, 63], but embryo survival rates are slightly lower necessitating the transfer of larger numbers of embryos per recipient. One of the problems affecting embryonic survival is the synchronicity of the donor embryos and the recipient uterus. In general, careful timing and synchronization of donor and recipient are essential, but the most optimal transfer-time has not yet been established for pig nsET purposes. Nevertheless, nsET holds much promise as a tool to evaluate the viability of in vitro produced blastocysts.

Design and scope of thesis

The approach adopted at the design of this dissertation was directed at obtaining insight into the fundamental aspects regulating oocyte developmental potential by modification of the basic in vitro embryo production system to mimic certain aspects prevalent in in vivo conditions. The supplementation of culture media with follicular fluids, somatic cells and growth factors was selected to improve our understanding of the mechanisms influencing critical aspects of in vitro oocyte maturation and fertilization and their consequences on blastocyst morphology and viability.

The first specific aim of the research presented in this thesis (**Chapter 2**), was to examine the influence of the supplementation of follicular fluids, collected from mature sows or prepubertal gilts, to the IVM medium on the developmental capacity of prepubertal gilt

oocytes. Following maturation in the presence of either follicular fluid source, oocytes were then either denuded or left with an intact cumulus mass for in vitro fertilization, in order to establish the more desirable combination leading to the most optimal fertilization and embryo development results. Special attention was given to the effects of the different IVM and IVF combinations on the quality of subsequent developing blastocysts, as evaluated using gross morphology, cell number and the incidence and level of apoptosis as parameters. The follicular fluid was also characterized for steroid and pituitary hormones, selected growth factors and glycosaminoglycan content, in order to explain specific developmental effects caused by either type of follicular fluid. In **Chapter 3**, the effects of sperm dose and presence of cumulus cells during IVF on the prevalence and pattern of polyspermy was studied, after maturation of the oocytes in sow or gilt follicular fluid. In addition, the consequences of these fertilization parameters on blastocyst development and quality were evaluated. In **Chapters 4 to 7**, slaughterhouse sow ovaries were used as the source of oocyte supply. The baseline effects of step-wise or continuous hormonal stimulation on the nuclear and cytoplasmic maturation of sow oocytes was studied in **Chapter 4**, including its consequences on blastocyst yield and cell number. **Chapter 5** was devoted to improving cytoplasmic maturation of the sow oocyte, by co-culture with homologous oviductal epithelial cells (pOEC) during the final phase of IVM. Its effects on sperm penetration and blastocyst cell number were used as evaluation parameters. In the following study, described in **Chapter 6**, an alternative approach was adopted by supplementing the embryo culture medium with growth hormone (GH), which in other species has been associated with increases in blastocyst cell number and reduction of apoptosis. In this study, for the first time, the non-surgical transfer of control or GH-treated blastocysts was attempted and embryonic survival until Day 11 of gestation was evaluated. In **Chapter 7**, blastocyst morphological scoring criteria were developed and tested using a novel non-invasive culture-based assay. The selection criteria were subsequently put to the test by non-surgically transferring morphologically classified blastocysts, and following their survival to Day 20 of pregnancy and subsequent farrowing. In conclusion, **Chapter 8** summarizes the findings of these studies, and discusses its implications for porcine IVP in general, but also its possible impact and future application within the field of advanced techniques such as embryonic stem cell technology.

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CHAPTER 2

The influence of sow or gilt follicular fluid in vitro maturation microenvironment, on fertilization, blastocyst morphology and apoptosis in prepubertal gilt oocytes

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Abstract

Current porcine in vitro embryo production systems do not fully support oocyte cytoplasmic maturation, leading to a high prevalence of polyspermy. Sow oocytes matured and fertilized in vitro are not as susceptible to polyspermy, indicating the importance of the follicular environment from which the oocyte is harvested. Sow follicular fluid (FF) could provide an improved culture microenvironment, more supportive of cytoplasmic maturation. This study evaluated the effect of sow or gilt FF during IVM, on the developmental potential of prepubertal gilt oocytes and the contribution of cumulus cells during IVF on subsequent embryo development. In vivo matured ovulated oocytes were used as a control. Nuclear maturation was not affected by the source of FF, but sow FF enhanced cumulus expansion and zona pellucida dissolution times. Sow FF also improved normal fertilization, and in combination with cumulus intact IVF, reduced polyspermic fertilization. Embryo development was not influenced by the FF type, but blastocyst morphology was improved by sow FF. Neither cell number, nor apoptosis was affected by the IVM treatment, but blastocysts classified as Normal contained higher total cell numbers. Sow FF contained higher concentrations of estradiol, progesterone, LH, growth hormone and prolactin, but a lower concentration of hyaluronic acid and no detectable levels of interleukin-1ß. In conclusion, the positive effects of sow FF occurred at the level of oocyte cytoplasmic maturation, with a cumulative effect on cumulus cells creating a combination in which fertilization and embryo quality similar to that of in vivo matured oocytes was obtained.

Submitted for publication

Introduction

Current porcine in vitro oocyte maturation systems lack the in vivo dominant and preovulatory follicular development, which may contribute to the poor embryo development results obtained in this species. It is believed that cytoplasmic capacitation and final maturation of the oocyte takes place during the later phases of follicular development, i.e. prematuration in the dominant follicle and final maturation in the preovulatory follicle [1]. The developmental potential of oocytes recovered from prepubertal animals is documented to be lower than that of adult animals in a number of species [2-4], including the pig [5-7], and is possibly related to the lack of oocyte prematuration. What distinguishes in vitro embryo production (IVP) using prepubertal gilt oocytes, is the inability of current culture systems to prevent the high prevalence of supernumerary entry of sperm (polyspermy) during IVF. Concerted cytoplasmic and nuclear maturation in vitro is essential for establishing the optimum developmental potential of the oocyte. Whereas nuclear maturation proceeds without much difficulty in prepubertal gilt oocytes, cytoplasmic differentiation is believed to be delayed or incomplete and thus contributes to the poor ability of the oocyte to block polyspermy. Sow oocytes fertilized in vitro are not as susceptible to polyspermy as prepubertal gilt oocytes [5, 7], indicating that the follicular environment from which the oocyte is harvested plays an elemental role in cytoplasmic maturation of the oocyte. Although chemically-defined media for IVM have been shown to be successful for the production of viable embryos [8], the supplementation of IVM media with follicular fluid (FF) provides an improved maturation environment for subsequent blastocyst production [9, 10]. Sow FF could thus provide an improved culture microenvironment, more supportive of cytoplasmic maturation and comparable with in vivo follicular prematuration.

In human IVP, correlations between the concentration of various substances in FF, and oocyte quality have been established, and steroid and pituitary hormone, cytokine, growth factor and glycosaminoglycan content are used as markers for oocyte developmental competence [11, 12]. Comprehensive characterization of porcine FF is lacking in literature, and could provide a useful means for the identification of other substances that influence the developmental competence of prepubertal gilt oocytes. A recent study [6], comparing FF from prepubertal gilts or sows for their ability to promote or support cytoplasmic maturation of prepubertal gilt oocytes, reported that the developmental competence of prepubertal gilt oocytes was not affected by the FF donor age. As the in vitro matured oocytes in this study were parthenogenetically activated, and not fertilized in vitro, the effects of the two FF sources on polyspermy and subsequent blastocyst development yet needs to be determined.

Despite many attempts to modify the IVM or IVF systems in order to reduce polyspermy, the only moderately successful technique is leaving the cumulus intact for IVF, as opposed to the widely used procedure of denudation prior to IVF. The exact role of cumulus cells during IVF yet needs to be determined, but cumulus-associated factors, such as hyaluronic acid (HA) content of the mucified extracellular matrix of the cumulus-oocyte-complex (COC), have been proposed to play a role in the prevention of polyspermy [13-15]. The extent to which the IVM environment influences the cumulus contribution during fertilization has not yet been addressed, but may play a role in the oocyte's ability to block polyspermy under IVF conditions.

The objective of this study was to evaluate the effect of the microenvironment provided by sow or gilt FF during IVM of prepubertal gilt oocytes on in vitro fertilization and blastocyst formation rate and quality. In addition, we investigated the contribution of cumulus cells during IVF on the afore-mentioned characteristics. For comparison, oocytes matured in vivo were collected from the oviduct shortly after ovulation and subjected to IVF and further culture in vitro. Nuclear maturation, cumulus expansion and zona pellucida dissolution time were used as indicators for oocyte maturational capacity. Fertilization was assessed by determining the rate of sperm penetration, and polyspermy. Blastocyst quality was evaluated by cell number, morphology and level of apoptosis. Finally, the sow and gilt FF were analyzed for steroid and pituitary hormone, cytokine, growth factor and glycosaminoglycan content to identify any potential substances contributing to differential effects on oocyte developmental potential.

Materials and Methods

Collection of follicular fluid

Ovaries from sows and prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory in insulated containers. Only morphologically healthy 3 to 6 mm follicles were aspirated. Follicular health was established according to the criteria for the bovine of Kruip and Dieleman [16] (Non-atretic: uniform bright appearance, extensive and very fine vascularization and no free-floating particles in the follicular fluid. Atretic: loss of translucency, slightly or dull greyish and/or opaque appearance and free-floating particles in follicular fluid). Recently, the same criteria have been validated for the porcine [17]. The pooled fluids (sow or gilt FF) were centrifuged at 1900 *g* for 30 min at 4°C, the supernatant collected and filtered through a 0.8 µm syringe filter (Sterivex, Millipore, MA) and stored at -20ºC until use. One batch of FFs was used throughout the experiments since this procedure provides a highly constant degree of quality [9].

In vivo oocyte maturation

All experiments were conducted according to USDA ethical committee guidelines. A total of 18 crossbred gilts, 6 months of age or older weighing at least 100 kg, were used as oocyte donors. Ovulation of the donors was synchronized as follows. Between day 11 and 15 following an observed estrus, the gilts were fed 20 mg altrenogest (Regu-Mate®, Hoechst Roussel Pharmaceuticals, Somerville, NJ) once daily for 4 days and 20 mg altrenogest twice on the 5th day. On the 6th day these gilts were administered 1750 IU of eCG (Folligonan; Intervet International BV, Boxmeer, The Netherlands) and 750 IU hCG (Chorulon; Intervet International BV) 83 h later. Donors were slaughtered 48 h after hCG for collection of ovulated oocytes. Immediately after exsanguinations, the reproductive tracts were removed and prepared for flushing by trimming the oviducts and a small portion of uteri away from the connective tissue while maintaining tissue temperature at 39^{o}C. Oocytes were flushed from the oviducts with warm (39^{o}C), sterile, pH-balanced BECM-3 [18] supplemented with 2 mM sodium bicarbonate, 10 mM HEPES and 0.3% BSA-V (A-7906, Sigma, St. Louis, MO). A total of 434 oocytes were recovered, which equals an average of 24 oocytes/donor (28 corpora lutea per donor) with a recovery rate of 87%. Of the oocytes recovered, 17% (74/434) were still cumulus-intact, and were denuded by carefully prizing the oocyte loose from the cumulus mass using a blunt 21 g needle. The oocytes were then directly processed for IVF.

In vitro oocyte maturation

Oocyte recovery, in vitro maturation, fertilization and embryo culture proceeded as previously described [19]. Briefly, ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in insulated containers. The ovaries were then washed in pre-warmed saline placed in beakers for aspiration. For oocyte and embryo searching and selection, 10 mM HEPES-buffered modified North Carolina State University 23 medium (NCSU-23) [20] containing 0.4% BSA (mNCSU-23H) was used. Cumulus-oocyte complexes (COCs) were aspirated from 3 to 6 mm non-atretic follicles with an 18-gauge needle fixed to a vacuum pump via 50 ml conical tube. Follicles were regarded to be atretic when they were highly vascularized, or opaque and milky in appearance. Contents were collected into the tube and allowed to settle for 10 minutes at room temperature. The supernatant was removed and sediment was resuspended in mNCSU-23H at room temperature and allowed to settle. COCs were washed in mNCSU-23H. This treatment was repeated once more and the content of the tube was observed under a stereomicroscope on a heated stage (38.5 ^{o}C). COCs surrounded by two or more layers of compact cumulus investment and containing oocytes of equal size were selected, washed twice in mNCSU-23H which had been pre-warmed to 38.5ºC prior to use, and transferred in groups of 40 to 50 to a four-well dish containing 500 µl of equilibrated IVM-I medium in each well. The IVM-I medium used for the first 20 to 22 h of oocyte in vitro maturation was BSA-free NCSU-23 supplemented with 10% (v/v) sow or gilt FF, 1mM db-cAMP, 0.8 mM cysteine, 25 µM ß-mercaptoethanol, 10 IU/ml eCG and hCG. The four-well dish was then incubated for 20 to 22 h at 38.5 ºC in 5% CO_2 in humidified air. After 20 to 22 h all the oocytes were washed twice in IVM-wash medium and placed in 500 µl IVM-II medium for an additional 18 to 20h of culture. The second IVM (IVM-II) culture period (20 to 22 h) occurred without db-cAMP, eCG and hCG added to the medium.

In vitro fertilization and embryo culture

After maturation the oocytes were placed in a wash dish containing pre-warmed equilibrated IVF medium. Modified Tris-buffered medium (mTBM) was used as fertilization medium. This medium consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate. Caffeine (1 mM) and 0.1 % BSA (w/v) (A-6003, Sigma) were supplemented to mTBM for use as IVF medium. Using a micropipette, half of the contents of each dish were vigorously pipetted for 10 to 30 sec to remove the expanded cumulus cells. The denuded oocytes were washed once more in IVF medium before being placed in 500 µl wells of equilibrated IVF medium, in groups of 40 to 50, and incubated at 38.5 ºC in 5% CO_2 for 30 min until the addition of the sperm. The remaining oocytes with intact cumulus were washed twice and also placed in 500 µl wells of equilibrated IVF medium, in groups of 40 to 50, and incubated at 38.5 ºC in 5% CO_2 for 30 min until the addition of the sperm.

Semen from the sperm rich fraction of the ejaculate was collected from two mature fertility-proven boars and immediately transported to the laboratory in an insulated container. Eight milliliters of saline containing 0.1% (w/v) BSA and 100 mg/l kanamycin sulfate was added to two ml sperm. The sperm suspension was centrifuged for 3 minutes at 900 *g*. The supernatant was removed and sperm resuspended in saline and centrifuged again. Following one more wash, the sperm was resuspended in 3 ml IVF medium. The

sperm concentration was determined using a hematocytometer (single technician) and adjusted to achieve a final concentration 50 motile cells/oocyte when adding 10 µl of sperm-suspension to each well containing oocytes. After warming to 38.5 °C for 30 min, 10 µl of the sperm suspension was added to the oocytes and co-incubated with the oocytes for 6 h at 38.5ºC at 5% CO_2 in air. After 6 h, the presumptive zygotes, from oocytes denuded prior to IVF, were washed three times in mNCSU-23H before being transferred in groups of 40 to 50 into 500 µl of IVC medium. Oocytes fertilized with an intact cumulus were gently pipetted to remove the remaining cumulus cells, and then washed three times before being placed in IVC medium. The in vitro embryo culture medium was NCSU-23 containing 0.4 % BSA (w/v).

At 48 h after the addition of sperm for IVF, the cleavage rate was determined (structures judged to be degenerated or uncleaved were not removed). At 96 h of culture, embryos were transferred to BSA-free NSCU-23 supplemented with 10% Fetal Calf Serum until 150 h after fertilization when blastocyst formation and morphology was evaluated.

Microscopy of oocytes

At 0, 24 and 42 h of IVM, images of COCs in each treatment group were recorded at 60x to 80x magnification (stereo microscope) in order to evaluate the degree of cumulus expansion. After digitalization, the diameter of each COC was measured twice, perpendicularly, using Adobe Photoshop 5.0 software (Adobe Systems, San Jose, CA) in order to obtain the mean diameter. Values are presented as the mean surface area occupied by the COC, and was calculated as the product of the two diameter measurements, i.e. diameter A x diameter B = diameter^2.

To determine the stage of nuclear maturation after termination of IVM at 42 h, a sample of COCs from each treatment group was removed, vigorously pipetted to remove the cumulus cells, washed twice in NCSU-23H and fixed in 2% paraformaldehyde until further processing. The fixed oocytes were then washed three times in NCSU-23H and stained with 4,6-diamino-2-phylindole (DAPI; Sigma) to visualize the chromosomal material. The stained oocytes were then mounted on glass slides, and viewed under epifluorescence at 200x magnification. Nuclear maturation stages were classified as metaphase I (MI) or metaphase II (MII). When aberrations were observed in the alignment of the chromosomes on the metaphase plate, during MII, they were sub-classified as abnormal.

The zona pellucida (ZP) dissolution time was assessed according to the method of Kim et al. [21]. From each treatment group, 8 oocytes (denuded by pipetting) per replicate (4 replicates/treatment group), were washed three times in NCSU-23H and transferred in 10 µl to a 0.1% pronase solution (w/v NCSU-23H) at 25°C. The ZPs were continually observed under a stereomicroscope at 200x magnification to accurately observe the dissolution of the zonae. The time from placing the oocytes into the pronase-solution, until complete dissolution of 4 of the 8 ZP, was designated as the ZP dissolution time.

Twenty hours after insemination, a sample of the oocytes were removed from the culture wells and evaluated for fertilization. The denuded oocytes were washed twice in NCSU-23H, fixed in 2% paraformaldehyde and stained with DAPI to visualize the chromosomal material. Penetration parameters were classified as follows - Penetration: oocytes

containing one or more decondensed sperm heads, one or more male pronuclei, syngamy, or two blastomeres each containing a normal nucleus in the presence of two polar bodies; Normal fertilized: monospermic oocytes with two pronuclei and two polar bodies, or a decondensed sperm head, a female pronucleus and two polar bodies, or syngamy with two polar bodies, or two blastomeres each containing a normal nucleus in the presence of two polar bodies; Polyspermy: oocytes containing more than one decondensed sperm head, or one sperm head in the presence of more than one pronucleus, or more than two pronuclei; Abnormal: oocytes containing aberrant chromosomal arrangements, or fragmented cytoplasm.

Embryo Development

To determine the efficiency of the culture systems, embryos were scored morphologically at 48 and 150 h after the addition of sperm for IVF (0 h). At 48 h, the percentage of cleaved embryos displaying 2 to 8 evenly-sized blastomeres was recorded and categorized as Even Cleaved; embryos with fragmented or uneven-sized blastomeres were categorized as Fragmented. The percentage blastocysts, expressed on the basis of the number of Even Cleaved embryos, was evaluated at 150 h post-insemination and all blastocysts were then individually evaluated according to their morphological appearance. Hereafter the blastocysts were fixed individually in 2% paraformaldehyde and stored at 4°C until further processing.

Blastocyst morphology

Blastocysts were strictly evaluated according to their morphological appearance using a stereo microscope at 160-200X magnification. When the blastocyst was symmetrical and spherical with a clear inner cell mass, intact trophoblast, containing no extruded blastomeres it was categorized as Normal. Abnormal blastocysts included embryos with: 1. one or more extruded blastomeres present under the zona pellucida, 2. one or more blastomeres extruded within the blastocoel cavity, 3. disruptions (vacuoles) in the trophoblast layer and 4. no visible inner cell mass. For correlation analysis purposes, blastocyst size was estimated according to Kidson et al. [19]. Briefly, when the perivitelline space was visible blastocyst were categorized as small, and when the perivitelline space was no longer visible they were classified as expanded. Early or small blastocysts included those in which the blastocoel was not fully formed, and an inner cell mass not yet clearly distinguishable.

Apoptosis by TUNEL assay

Blastocyst apoptosis was evaluated as previously described by Kidson et al. [19]. Biochemical detection of DNA strand breaks was performed on each individual blastocyst using TUNEL (fluorescein-conjugated dUTP and TdT, Roche, Mannheim, Germany) according to manufacturer's instructions, with 0.05 µg/ml DAPI as a counterstain. Fixed blastocysts were washed twice in mNCSU-23H and then permeabilized for 15 min on ice in 0.1% Triton X-100 (0.1% sodium citrate in PBS). After rinsing twice more in mNCSU-23H blastocysts were incubated in microdrops (25 µl per 1 to 12 embryos) of the TUNEL reaction mixture, for 1 h under oil in a humidified atmosphere in the dark. After TUNEL culture the embryos were washed twice in mNCSU-23H and then stained with DAPI (5 µl/ml) for 5 min at room temperature in the dark. The embryos were then mounted in a

minimal amount of DAPI fluid, flattened completely by applying firm pressure to the cover slip, and examined using a fluorescence microscope (200x magnification, BH2-RFCA Olympus, Tokyo, Japan) to assess the total number of nuclei and the proportion showing DNA fragmentation. Overlap of nuclei was negligible in all groups. As a positive control for TUNEL labeling, 5 embryos from each treatment group were treated with DNAse before TUNEL staining and for a negative control the terminal transferase enzyme was omitted during TUNEL labeling. To rule out necrosis, all embryos were stained with 4 μM EthD-I (Molecular Probes Europe BV, Leiden, The Netherlands) before fixing and subsequent TUNEL and DAPI staining.

Nuclei were classified according to three nuclear morphologies: 1) 'healthy' interphase nuclei with a uniform DAPI staining and a clear outline but without TUNEL staining, also including mitotic nuclei; 2) fragmented nuclei with no TUNEL labeling; 3) TUNEL labeled nuclei which were condensed and/or fragmented. Embryos were classified according to the morphology of the nuclei contained within: TUNEL & Morphology – blastocysts containing one or more TUNEL labeled condensed or fragmented nucleus, also in combination with fragmented non-TUNEL labeled nuclei; Morphology only – blastocysts containing only one or more fragmented non-TUNEL stained nuclei, and no other apoptotic morphologies; EthD-I – blastocyst containing one or more EthD-I labeled nuclei in the absence of any other apoptotic labeling or morphology. The total nuclei count consisted of all nuclei, whether they displayed apoptosis or not. The apoptotic index was calculated for each embryo as follows:

Total apoptotic index = (TUNEL-positive nuclei, either fragmented or condensed) + (no. of TUNEL-negative fragmented nuclei)/ (total no. of nuclei) X 100

Assays for hormones and factors in follicular fluid

Concentrations of LH and prolactin (PRL) were determined in three duplicate aliquots of 50, 100 and 200 μl pFF by homologous radio immuno assays (RIA) as validated for pig plasma [22, 23]. Porcine LH-LER 778.4 (kindly supplied by Dr. L.E. Reichert, Tucker Endocrine Research Institute LLC, Atlanta, GA) and pPRL-A7 were used for iodination and standards, and rabbit anti-pLH (UCB A528; Campro Scientific, Veenendaal, The Netherlands) and rabbit anti-pPRL (7703L; [23]) as antiserum, respectively. Specificity of the RIAs was high as indicated by low cross-reactivity for other pituitary hormones [22, 23] and by the observed parallelism. The limit of quantitation was 0.2 ng/ml for LH and PRL.

Concentrations of progesterone (P_4) and estradiol-17β (E_2) were determined by solid-phase ^{125}I RIA methods (Coat-A-Count TKPG and TKE, respectively; Diagnostic Products Corporation, Los Angeles, CA) as reported for bFF [24, 25] and validated for cow plasma [25]. Briefly, duplicate aliquots of 1 to 2 μl pFF were diluted into 50 μl 0.02 M borate buffer (pH 8.5) in 0.9% (w/v) NaCl and extracted with 2 ml diethylether. After evaporation of the diethylether the residues were dissolved in 250 μl zero calibrator plasma (Diagnostic Products Corporation, Breda, The Netherlands) or borate buffer for the RIA of progesterone and estradiol, respectively. Duplicate aliquots of 100 μl were used in the respective RIAs. Extraction efficiency was determined in parallel samples with tritiated steroid. Specificity of the RIAs was high as indicated by low cross-reactivity for other steroid hormones [25] and

by the observed parallelism. The limits of quantitation, using 1 µl of pFF, were 3 and 2 ng/ml for progesterone and estradiol, respectively.

Concentrations of GH and interleukin-1β (IL-1β) were estimated by sandwich enzyme immuno assays (hGH and h-Interleukin-1β ELISA, respectively; Roche Diagnostics Nederland B.V., Almere, The Netherlands) according to the manufacturer. Triplicate aliquots of undiluted pFF and of a series of 5 different dilutions were used. Specificity of the hGH assay for pGH was limited as a moderate parallelism was observed. For the h-Interleukin-1β assay parallelism could not be established since all observed sample values were below detection. The limits of quantitation, using undiluted aliquots of 200 and 20 µl of pFF, were 0.02 and 0.03 ng/ml for GH and IL-1β, respectively.

Concentrations of hyaluronic acid as a measure for glycosaminoglycans were estimated by enzyme-linked binding protein assay as validated for human serum or plasma (Hyaluronic acid (HA) test kit; Corgenix Medical Corporation, Denver, CO) according to the manufacturer. Four triplicate aliquots of 30, 40, 50 and 75 µl of pFF were used. Since parallelism was not present the values obtained with the standard, 30 µl sample volume were used for further analysis. The limit of quantitation was 50 ng/ml. In general, the intra- and inter-assay coefficients of variation were <10 and <15% for all assays, respectively.

Statistical analyses

All experiments consisted of a minimum of 3 replicates for follicular fluid analyses and 6-8 replicates for oocyte maturation, fertilization and embryo development. Follicular fluid components in sow or gilt FF and cumulus expansion were compared by t-test, and zona pellucida dissolution time by SPSS GLM. Statistical analysis of oocyte maturation and blastocyst abnormalities was carried out using Chi Square or Fisher's Exact where appropriate. After testing for normality (Levene's test) and testing for equal variances (F-test for two groups and Bartlett's test for multiple groups) fertilization and embryo development data, apoptotic indices and blastocyst cell numbers were analyzed using SPSS GLM, with Origin of oocyte or follicular fluid and Cumulus during IVF, as well as their interaction, taken into account as factor variables; these data were subjected to ArcSine transformation for normalization. Data are presented as the mean ± SEM. Correlation analysis was calculated using Pearson's correlation coefficient. Differences of $P \leq 0.05$ were considered significant. Analyses were completed using the statistical analysis program SPSS for Windows 10.0.0, or Graphpad Prism®.

Results

Oocyte Maturation

At the termination of in vitro maturation at 42 h, 90% (125/139) of sow FF matured oocytes and 84% (131/156) of gilt FF treated oocytes had developed to MII. A small percentage of oocytes in both treatment groups had not yet progressed to MII and were still in the MI stage; 9% (13/139) and 14% (22/156) for sow FF and gilt FF, respectively ($P>0.05$). Of oocytes in MII at 42 h of in vitro maturation, a substantial proportion showed abnormalities in chromosomal arrangements which consisted mainly of spindle

aberrations, judged by scattered or malaligned chromosomes in the metaphase plate; 26% (33/125) vs. 32% (42/131) for sow FF and gilt FF, respectively (P>0.05).

In vitro maturation of prepubertal gilt oocytes in the presence of sow FF had a positive effect on cumulus expansion. Although no differences in the mean surface area of the COCs were seen from 0 to 24 h, the cumulus mass of sow FF matured oocytes (5 348 ± 312 $\mu m^2 x10^2$) was significantly (P<0.01) more expanded than gilt FF matured COCs (4 266 ± 206 $\mu m^2 x10^2$), which constitutes a 25% increase in cumulus expansion induced by the sow FF.

Denuded oocytes derived from COCs matured in vitro for 42 h in the presence of sow or gilt FF, and denuded oocytes flushed from the oviducts of donor gilts within 3 h of ovulation, were exposed to 0.1% pronase for up to 30 minutes. The zona pellucida of sow FF oocytes was more resistant to pronase digestion than gilt FF zona pellucida (P<0.01) and on average remained intact 1 minute and 7 seconds (15%) longer than gilt FF zona pellucida. In general, the zona of ovulated oocytes was much more resistant to pronase digestion, and by 30 minutes of incubation with 0.1% pronase was still intact.

Table 1. Effect of follicular fluid origin, and presence or absence of cumulus during IVF, on fertilization parameters at 22 h after addition of sperm for in vitro fertilization of prepubertal gitl oocytes.

	N	% Penetrated	% Normal Fertilized (of Penetrated)	% Cleaved/ normal	% Polyspermy (of Penetrated)
In vivo Control	214	62 ± 4[a]	76 ± 3[a]	62 ± 6[a]	15 ± 4[a]
SowFF Nude	233	73 ± 4[a]	55 ± 4[b,d]	9 ± 5[b]	41 ± 4[b]
Sow FF Intact	202	30 ± 5[b]	89 ± 4[c]	10 ± 5[b]	6 ± 4[a]
GiltFF Nude	228	75 ± 7[a]	38 ± 5[e]	10 ± 9[b]	53 ± 6[b]
Gilt FF Intact	228	43 ± 4[b]	69 ± 4[a,d]	4 ± 3[b]	22 ± 5[c]
Statistical factor interaction:					
Origin oocyte or FF:		P = 0.125	P < 0.001	P < 0.001	P < 0.001
Cumulus during IVF:		P < 0.001	P < 0.001	P = 0.937	P < 0.001
Origin oocyte or FFxCumulus:		P = 0.382	P = 0.314	P = 0.284	P = 0.778

[a,b,c,d,e]Within columns values with superscripts differ significantly: P<0.05

Fertilization Parameters

As presented in Table 1, the penetration rate of oocytes was mainly determined by the presence or absence of cumulus cells during fertilization (P<0.01). Irrespective of oocyte or follicular fluid origin, the penetration rates for cumulus intact oocytes was significantly lower (P<0.05) compared with denuded oocytes. The ability of the penetrated oocytes to support normal fertilization was influenced by both the maturation microenvironment of the oocyte, as well as the presence or absence of cumulus cells during IVF (P<0.001). Cumulus intact oocytes matured with sow FF (Sow FF intact) had the highest incidence of normal fertilization (89 ± 4%; P<0.05), followed by ovulated oocytes (In vivo Control; 76 ± 3%; P<0.05) and cumulus intact gilt oocytes (Gilt FF intact; 69 ± 4; P<0.05) which had similar percentages of normal fertilization (P>0.05). Oocytes matured with sow FF, and

denuded for IVF (Sow FF nude), had a higher incidence of normal fertilization (55 ± 4; P<0.05) than denuded gilt FF matured oocytes (Gilt FF nude) which had the lowest incidence (38 ± 5; P<0.05) of normal fertilization of all groups. Fewer than 10% of penetrated oocytes in each treatment group presented abnormalities in fertilization (data not shown). Ovulated oocytes showed advanced progression of development with more than 60% of oocytes already having undergone the first cleavage division by 22 h, after the addition of sperm during IVF, compared with 10% or less of in vitro matured oocytes. Similar to normal fertilization, polyspermic penetration of oocytes was determined by both the origin of the oocyte or FF, as well as the presence or absence of cumulus cells during IVF. Sow FF matured cumulus intact fertilized oocytes and in vivo matured oocytes were the least prone to polyspermy (6 ± 4% vs. 15 ± 4% for Sow FF intact and In vivo Control, respectively; P>0.05), followed by Gilt FF intact oocytes (22 ± 5; P<0.05). Gilt FF nude oocytes had a greater percentage of polyspermy (53 ± 6%) than Sow FF nude oocytes (41 ± 4%)(P>0.05).

Four different types of pronucleus distribution were seen in polyspermic oocytes and are depicted in Table 2. Type I: all pronuclei progressed to the center of the oocyte and participated in syngamy; Type II: two pronuclei were apposed with one or more supplementary pronucleus or decondensed spermheads located eccentrically; Type III: polyspermy consisted of three or more non-apposed pronuclei with or without one or more eccentrically arranged decondensed sperm heads; Early PPN: a single pronucleus with two or more decondensed sperm heads.

In all groups, polyspermy consisted mainly of Type II pronuclear location, indicating successful apposition of pronuclei, and to a lesser extent Type III (except in Sow FF intact) containing only non-apposed pronuclei. In the three groups generally suffering from a greater degree of polyspermy, i.e. Sow FF nude, and both Gilt FF groups, a small incidence of Type I pronuclear arrangements occurred in addition to Type II. In these oocytes polyspermy was caused by either di- or trispermic fertilization, and to a lesser extent tetraspermy or an even higher degree of supernumerary sperm penetration, except for Gilt FF nude oocytes of which almost half had been penetrated by four or more sperm. All oocytes denuded for IVF presented a small percentage of Early PPN, which was not seen in cumulus intact fertilized oocytes.

Table 2. Distribution of pattern of polyspermy after IVF of oocytes matured in different microenvironment.

Treatment	n	Type I (%)	Type II (%)	Type III (%)	Early PPN (%)
					
Vivo	21	0 (0)	18 (86)	2 (10)	2 (5)
Sow FF	67	1 (1)	50 (75)	10 (15)	6 (9)
Gilt FF	122	10 (8)	78 (64)	25 (21)	9 (7)

Embryo Development

Embryo development at 48 h and 150 h after the initiation of IVF is presented in Table 3. Embryo cleavage rates were categorized into two groups, i.e. Even Cleaved and Fragmented. The percentage of oocytes which had undergone even cleavage was determined by both the maturation microenvironment of the oocyte, and followed a trend

similar to the normal fertilization rate. Ovulated oocytes were the most likely to be Even cleaved (P<0.05), followed by sow and gilt FF in vitro matured oocytes denuded for IVF (P<0.05). In vitro matured oocytes fertilized with the cumulus intact presented the lowest percentage of even cleavage (P<0.05). Ovulated and sow FF matured, cumulus intact fertilized, oocytes showed a smaller percentage of fragmentation compared with embryos derived from denuded sow FF oocytes, and all gilt FF derived embryos (P<0.05). The percentage of Even Cleaved embryos developing to blastocysts did not differ between any of the treatment groups.

Table 3. Percentage embryo development (Mean ± SEM) on Days 2 and 6 after IVF, of prepubertal gilt oocytes matured in vitro in the presence of sow (Sow FF) or gilt follicular fluid (Gilt FF), and fertilized in vitro with intact cumulus (intact) or after cumulus removal (nude) in comparison to ovulated oocytes.

	n	% Even Cleaved	% Fragmented	% Total cleaved	% Day 6 Blast/ even cleaved	% Blasts with Normal Morphology
In vivo Control	219	54 ± 2^{a}	10 ± 4^{a}	64 ± 5^{a}	86 ± 10^{a}	87 ± 6^{a}
Sow FF Nude	349	32 ± 4^{b}	$37 \pm 3^{b,c}$	69 ± 2^{a}	87 ± 8^{a}	93 ± 5^{a}
Sow FF Intact	304	18 ± 2^{c}	20 ± 4^{a}	38 ± 4^{b}	68 ± 16^{a}	86 ± 8^{a}
Gilt FF Nude	353	37 ± 3^{b}	$36 \pm 5^{b,c}$	72 ± 4^{a}	78 ± 8^{a}	70 ± 5^{b}
Gilt FF Intact	267	23 ± 3^{c}	$25 \pm 6^{b,c}$	49 ± 7^{b}	73 ± 6^{a}	71 ± 7^{b}
Statistical factor interaction:						
Origin oocyte or FF:		P < 0.001	P < 0.001	P = 0.116	P = 0.923	P < 0.001
Cumulus during IVF:		P < 0.001	P = 0.004	P < 0.001	P = 0.236	P = 0.624
Origin oocyte or FFxCumulus:		P = 0.846	P = 0.450	P = 0.343	P = 0.495	P = 0.844

[a,b,c]Within columns values with superscripts differ significantly: P<0.05; n=number of oocytes for IVF and IVC.

The major difference between treatment groups was found in the quality of the blastocysts at 150 h after IVF, and was mainly determined by the oocyte maturation microenvironment. Ovulated oocytes and oocytes matured in vitro in the presence of sow FF had a greater potential to develop to blastocysts with normal morphology, than those derived from oocytes matured with gilt FF. Blastocysts exhibited four distinct abnormalities: 1. disruptions/vacuoles in the trophoblast layer, 2. extruded blastomeres present under the zona pellucida, 3. extruded blastomeres present within the blastocoel cavity, and 4. no visible inner cell mass. As seen in Figure 1, the pattern of morphological abnormalities differed according to the treatment regimen. Blastocysts derived from In vivo Control or Sow FF intact oocytes, invariably contained a distinct inner cell mass. The only visible abnormalities seen in blastocysts from sow FF cumulus intact oocytes, were disruptions in the trophoblast layer. All other blastocysts in the remaining treatment groups with abnormal morphology contained extruded cells as well as trophoblast layer disruptions. Blastocysts of normal or abnormal morphology consisted of all stages of development, i.e. early blastocyst, expanded blastocyst or hatched/hatching blastocyst stages, and there were no differences (P>0.05) in the distribution thereof in any of the treatment groups (data not shown).

To evaluate any relationships between different embryo quality parameters (i.e. blastocyst size, cell number, morphology and apoptosis) data from all blastocysts were subjected to correlation analyses. Normal blastocyst morphology was positively correlated with greater cell numbers in the In vivo Control (0.33; P<0.05), Sow FF intact (0.44; P<0.05) and Gilt FF intact (0.47; P<0.05) groups. Interestingly, there was no association between

the incidence of apoptosis and blastocyst morphology. In all treatment groups blastocyst size was significantly ($P<0.05$) positively correlated with cell number (In vivo Control: 0.29; Sow FF nude: 0.23; Sow FF intact: 0.35; Gilt FF nude: 0.40; Gilt FF intact: 0.44).

We subsequently analyzed the mean number of nuclei contained in the blastocysts in general, as well as those for Normal and Abnormal blastocysts in each treatment group. The overall mean number of nuclei did not differ between treatment groups (intertreatment range: 40 ± 2 to 45 ± 2 nuclei per blastocyst), nor did that of Normal (intertreatment range: 43 ± 2 to 48 ± 2 nuclei per blastocyst) or Abnormal blastocysts (intertreatment range: 23 ± 7 to 35 ± 3 nuclei per blastocyst). In agreement with the correlation analysis, we found that Normal blastocysts tended to contain greater cell numbers than Abnormal blastocysts ($P<0.05$).

As depicted in Figure 2, of all blastocysts containing one or more nuclei showing signs of apoptosis, more than 90% contained TUNEL-labeled nuclei, indicating DNA fragmentation, in combination with apoptotic morphology, irrespective of treatment group. A small percentage of Normal blastocysts also presented signs of apoptosis or cell death in the absence of TUNEL labeling (EthD-I only), whereas the greater majority of Abnormal blastocysts contained only biochemical signs of apoptosis, i.e. TUNEL labeling. The overall incidence of apoptosis did not differ between Normal and Abnormal blastocysts, nor between the different treatment groups (X; Fig. 2) ($P>0.05$), which confirms the correlation data which showed no association between apoptosis and blastocyst morphology.

The apoptotic index (ApI) of the embryos is shown in Table 4. In general, a greater percentage of nuclei in Abnormal blastocysts presented one or more signs of apoptosis, with the exception of Sow FF blastocysts for which the apoptotic index of Normal and Abnormal did not differ. The percentages of apoptotic nuclei in general did not differ between the blastocysts derived from in vitro matured oocytes, but was slightly, although significantly, lower for blastocyst derived from ovulated oocytes. When the ApI was expressed as the number of whole apoptotic nuclei per blastocyst, no differences in ApI were found between treatment groups, nor between Normal or Abnormal blastocysts.

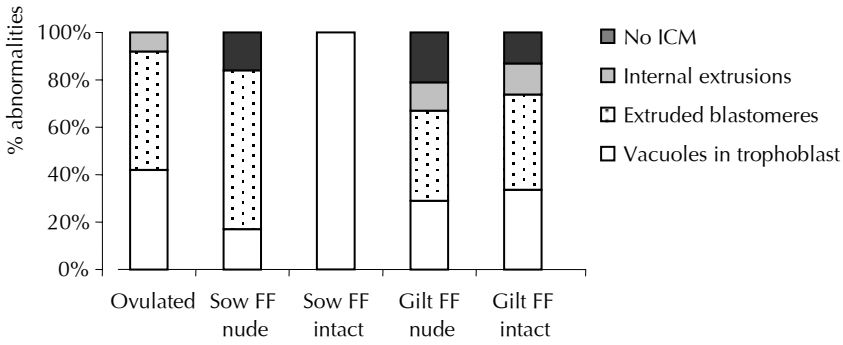

Figure 1. Distribution of pattern of morphological abnormalities in 150 h post IVF cultured blastocysts according to follicular fluid origin and presence or absence of cumulus during IVF, as compared with ovulated oocytes.

Follicular fluid analysis

The partial characterization of follicular fluid, aspirated from 3 to 6 mm diameter sow or prepubertal gilt follicles, is presented in Table 5. The mean concentrations of E_2, P_4, LH, GH and PRL were significantly ($P<0.05$) higher in sow FF than in prepubertal gilt FF. The ratio of P4:E2 was 4.15 in sow FF and 2.8 in gilt FF. Interestingly, the hyaluronic acid concentration was significantly ($P<0.05$) higher in gilt than in sow FF. There were no detectable levels of IL-1β in either sow or gilt FF.

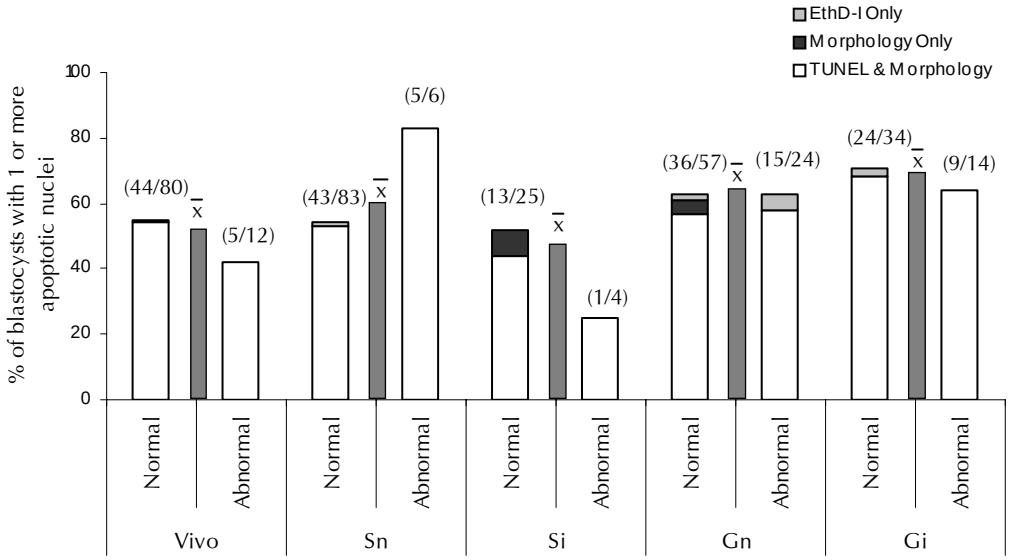

Figure 2. Apoptosis in 150 h post IVF cultured porcine blastocysts according to follicular fluid origin and presence or absence of cumulus during IVF, as compared with ovulated oocytes.

Discussion

This study demonstrates that the microenvironment created by sow or gilt follicular fluid during IVM, influences various aspects of prepubertal gilt oocyte maturation, and subsequent development through the zygote to blastocyst stages. As follicular environment plays a critical role in the establishment of developmental potential of the immature oocyte, it is therefore not surprising that in vitro oocyte maturation, and further embryonic development, may be influenced by the microenvironment provided by the type of FF supplemented to the IVM medium. The results presented in this report clearly indicate that sow FF has a more positive influence on prepubertal gilt oocyte developmental potential than gilt FF.

In the first instance, the differential effect of the two types of FF was evident in the enhanced cumulus expansion seen in COCs matured with sow FF. Cumulus expansion, in response to gonadotrophin stimulation, is facilitated by the secretion of its intercellular matrix of which hyaluronic acid (HA) forms the main structural macromolecule. In this study, though, we intriguingly found a higher concentration of HA in prepubertal gilt FF than in sow FF. Hyaluronic acid is mostly known for its role in peri-ovulatory follicles where it is involved in the detachment of the COC from the follicular wall, and transport

Table 4. The effect of follicular fluid maturation microevironment and presence or absence of cumulus cells during IVF on the extent of apoptosis within prepubertal gilt blastocysts in each treatment group as indicated the Apoptotic Index (ApI).

	In Vivo Control		Sow FF Nude		Sow FF Intact		Gilt FF Nude		Gilt FF Intact	
	ApI % nuclei	ApI # nuclei	ApI % nuclei	ApI # nuclei	ApI % nuclei	ApI # nuclei	ApI % nuclei	ApI # nuclei	ApI % nuclei	ApI # nuclei
Overall	4.1±0.4^{a}	2±0^{a}	6.0±1.0^{a,b}	2± 0^{a}	4.8±0.9^{a,b}	2±0^{a}	6.7±0.7^{b}	3±0^{a}	6.6±0.7^{b}	3±0^{a}
Normal	3.6±0.4^{a}	2±0^{a}	6.0±1.1^{b}	2± 0^{a}	4.8±1.0^{a,b}	2±0^{a}	5.5±0.6^{a,b}	2±0^{a}	5.5±0.6^{a,b}	2±0^{a}
Abnormal	8.1±1.5^{a}*	2±0^{a}	6.5±1.5^{a}	2± 0^{a}	4.4±0.0^{a}	2±0^{a}	9.4±1.9^{a}*	3±1^{a}	9.9±1.9^{a}*	3±0^{a}

$^{a,b/x,y}$Within rows values with superscripts differ significantly: P<0.05; *Within columns Normal differs from Abnormal: P<0.05.

Table 5. Mean concentrations of selected hormones and compounds of follicular fluid collected from slaughterhouse sows or prepubertal gilts used to supplement in vitro maturation medium.

	Concentration in ng/ml						
	Estradiol-17β	Progesterone	LH	Growth Hormone	Prolactin	Hyaluronic Acid	Interleukin -1β
Sow FF	17.09	70.98	0.49	0.04	1.83	87	<0.03
Gilt FF	6.29	17.70	0.36	0.00	1.47	114	<0.03

All significantly different: P<0.05 except Interleukin-1β

to and within the oviduct following ovulation. Importantly though, HA also plays a role in follicular fluid formation [27], and the greater HA concentration in the gilt FF may instead be related to follicular dynamics inherent in the prepubertal ovary. High concentrations of glycosaminoglycans in the culture medium can inhibit gonadotrophin binding to granulosa cells [28, 29] which may also explain the lesser degree of cumulus expansion seen in the gilt COCs this study. In the human, the concentration of HA in FF is negatively correlated with fertilization [30] and is regarded to be due to over-maturation/atresia of the oocytes [31]. In the pig and mammals in general, over-maturation or aging of the oocyte in vivo and in vitro [32] decreases its ability to block the supernumerary entry of sperm and the inappropriately high HA environment created during IVM with prepubertal gilt FF may therefore contribute to the inability of some prepubertal gilt oocytes to block polyspermy.

The main effect of sow FF appears to have occurred via the cumulus cells in the intact COC. Sow follicular fluid enhanced the percentage of normal fertilization, and IVF in combination with intact cumulus during IVF, greatly reduced the incidence of polyspermy thereby matching the results achieved when using in vivo matured ovulated oocytes. Normal fertilization was not solely determined by the IVM microenvironment, but in gilt FF matured oocytes it was improved to the level of denuded sow FF matured oocytes when the oocytes were fertilized with an intact cumulus, even though penetration rates were reduced as also reported in other studies [13, 33-35]. These results therefore indicate that oocyte and cumulus maturation are cumulatively influenced and enhanced by sow FF microenvironment in vitro, and that both of these aspects require concerted and improved maturational capacitation in order to achieve fertilization rates comparable with in vivo matured oocytes. In the pig, as in most mammals cumulus cells surrounding

the oocyte shortly after ovulation play an important role in promoting fertilization [36]. A number of authors have reported beneficial effects of the presence of cumulus cells during IVF of pig oocytes [13, 33-35], but efficiency of fertilization, i.e. sperm penetration and blastocyst formation, is not improved. The combination of cumulus cells and HA plays an important role in the preparation of the oocyte for fertilization. Hyaluronic acid is also known for its modulatory effect on sperm physiological status within the mature mucified cumulus mass in a variety of mammalian species [14, 15, 30, 37]. In pigs, addition of exogenous HA to the IVF medium reduces polyspermic penetration in denuded oocytes, but in combination with an intact cumulus polyspermy is increased [15] with a resultant drop in the developmental potential of the oocyte. The combination of hormonal content and lower HA concentration in sow FF could thus contribute the optimal effect achieved with cumulus intact fertilization of oocytes matured with sow FF, possibly by improving HA retention in the cumulus.

Other factors believed to influence supernumerary sperm entry into the oocyte in vitro, include the resistance of the ZP to pronase digestion and the presence of cumulus during IVF. In agreement with previous reports [38] our study also showed that the ZP of in vivo matured ovulated oocytes were much more resistant to enzyme digestion than those of in vitro matured oocytes. Although the sow FF matured ZP stayed intact significantly longer than gilt FF matured ZP, the difference was minor compared with the difference between the in vivo and in vitro matured ZP dissolution time. Taking into account that denuded in vitro matured oocytes were equally as likely to be polyspermic, this small increase in digestion resistance of sow FF ZP appears not to be of any physiological value.

The composition of porcine FF, with regard to steroid hormone content, is known to differ according to the developmental stage of the follicle [39, 40], the age [6, 41] and genetic disposition [42] of the donor animal. Consistent with other porcine studies [6, 41], we found greatly elevated levels of E_2 and P_4 in gilt FF, but the positive effects of enriched sow FF may not specifically be correlated with the steroid hormone content, as hypothesized in other reports [6]. Follicular fluid steroid hormone concentrations in the highly prolific Meishan breed do not differ from that of the Large-White hybrid, but large differences in developmental competence of the oocytes, i.e. higher sperm penetration and male pronucleus formation rates, prevails following IVM with Meishan FF [42]. This is supported by the reported lack of direct influence of steroids on in vitro maturation of porcine oocytes, and thus suggests an indirect role in oocyte development for these factors [43]. Indeed, the higher E_2 and P_4 content may rather be the consequence of the elevated GH and LH concentrations in the sow FF which act via the follicular somatic cells by increasing steroidogenesis in granulosa cells [44-46]. LH is further known to be involved in the initiation of cumulus expansion in the porcine oocyte by downregulation and discoupling of gap junctions between cumulus cells [47], and may also contribute to the greater degree of cumulus expansion in sow FF matured oocytes. The differences in the ratio of P_4:E_2 found in this current study are in agreement with a recent report [6] where lower P_4:E_2 ratios were evident for gilt FF. These differences were attributed to the differential ability of adult and prepubertal theca and granulosa cells to synthesize various steroid hormones, and in their report no effect of steroid hormone concentration, nor ratio was seen in the development potential of the oocyte following parthenogenetic activation. In addition, studies examining the effect of exogenous steroid hormones on IVM of prepubertal gilt oocytes have described either negative [48] or no effect [43] of E_2 or progesterone on oocyte cytoplasmic maturation. Negative effects of E_2 supplementation of IVM media have also been reported in the bovine where it appears to

cause meiotic spindle aberrations [49]. The high percentage of abnormalities found in MII of in vitro matured oocytes in this study, could thus be related to the yet inappropriate non-cyclic hormonal environment provided by the current IVM culture systems in general.

Extensive analysis of the composition of porcine follicular fluid is lacking in current literature, but various follicular fluid markers have the potential to influence oocyte development competence, as explored in human IVF [11, 12]. The results obtained in this study indeed indicated a number of parallelisms with the human FF reports. Clear correlations exist between certain compounds present in human FF and the subsequent in vitro developmental capability of the oocyte [11, 12], where follicular P_4, GH, PRL and IL-1β are positively correlated with the rate of normal fertilization, LH and IL-1β with morphologically normal cleavage and GH with embryo viability [11]. In the current study, the concentration of GH and PRL were statistically significantly higher in sow FF, but consisted of only a marginal increase in magnitude which, although of debatable direct physiological significance, may synergistically or cumulatively contribute to the positive effects seen in this study. In the human [11, 12], rabbit [50] and bovine [51-53], PRL is indicated in the final phase of oocyte maturation and is believed to enhance cytoplasmic maturation. In these reports, exogenous application of intrafollicular concentrations of PRL has proven to be correlated with the ability of the oocyte to undergo normal fertilization and embryonic development. The concentration of IL-1β is also thought to be instrumental in stimulating oocyte cytoplasmic maturation as it is positively correlated with morphologically normal and rapid cleavage in human embryos [12]. It reaches a peak in human [11, 54, 55] and porcine [56] peri-ovulatory follicles and has been shown to stimulate gonadotrophin-supported steroidogenesis [57]. It is not yet clear whether IL-1β plays a similar role in the pig as it does in the human. The complete absence of IL-1β in porcine follicular fluid collected from medium-sized follicles, as analyzed in this study, may be a contributing factor in the retardation in the first cleavage division as seen in this report and commonly experienced in porcine IVP [58, 59].

The percentage of blastocyst development was influenced by neither oocyte maturation treatment, nor cumulus presence during IVF. This is not surprising as blastocyst development readily takes place in both normal fertilized and polyspermic zygotes, but with inherent differences in cell number and viability [26, 58]. Surprisingly, in porcine IVP literature, the only reported means of assessing blastocyst quality has been the determination of mean cell number of a cohort of blastocysts within a specific treatment, which offers no indication towards the quality of individual blastocysts. As no differences were found in the mean blastocyst cell number in each treatment group within our data, cell number by itself did not provide a strong enough tool for evaluating blastocyst quality. To our knowledge, this study presents the first morphological characterization of in vitro produced blastocysts in order to estimate their quality in combination with, and support of, cell number. Our evaluation criteria used in this study thus, provides the means for non-invasively estimating blastocyst quality in the pig. These data show a clear negative correlation between morphological abnormalities and the number of nuclei contained in such blastocysts. Furthermore, the pattern of abnormalities differed according to the maturation treatment of the oocyte, and appeared to be related to the fertilization status of the zygotes. In the treatment groups which had higher percentages of polyspermy a number of blastocysts appeared to contain no visible inner cell mass.

It has been hypothesized that blastocysts developing from Type II polyspermic zygotes may have an improved likelihood of developing to diploid and normal blastocysts, than those developing from Type I polyspermy [26] which could lead to either diploid or triploid embryos. Furthermore, it is tempting to relate extruded cells at the blastocyst stage with Type II polyspermy in which two pronuclei participate in syngamy, in the presence of one or more eccentrically located pronuclei. If the two apposed pronuclei should participate in cleavage, the remaining surplus pronuclei could divide separately giving rise to two diploid and one or more haploid blastomeres. The latter could thus be extruded during further cleavage, explaining the presence of extruded cells at the blastocysts stage. Such specific associations need to be addressed in a carefully controlled manner, and could be of great value to provide insight into the ploidy- and survival-related fate of in vitro produced blastocysts displaying abnormalities in morphology.

The percentage of normal morphology blastocysts in each treatment group was determined by the maturation treatment of the oocyte, and not influenced by the presence or absence of cumulus during IVF. This further substantiates the positive effects of in vivo maturation, or sow FF in vitro maturation, seen on the cytoplasmic maturation and hence developmental potential the oocytes. Differential staining for inner cell mass (ICM) and trophoblast cell number was not performed in this study, as it cannot be combined with TUNEL. The effect of oocyte maturation environment on the number of ICM cells could thus not be ascertained, but deserves further attention as blastocyst derived from in vitro matured oocytes, or polyspermic zygotes, are known to contain fewer ICM cells [58]. Recently, apoptosis as evaluated using TUNEL to detect DNA strand breaks, has also been used as an invasive tool to evaluated blastocyst quality [19, 60-62], but no clear correlation between the incidence of apoptosis and viability of such blastocysts has been provided in literature. In a recent study [19], we highlighted the importance of using both TUNEL and nuclear morphology to assess apoptosis in porcine blastocysts, as DNA strand breaks are not always associated with morphological signs of apoptosis such as nuclear blebbing and fragmentation [19, 63, 64]. The value of apoptosis as a tool to assess blastocysts quality in porcine embryos is becoming increasingly doubtful, as the apoptotic index (percentage of apoptotic cells in a blastocyst), as seen in this and other studies [19, 65], is extremely low compared with other species such as the bovine [66-70]. Whereas a threshold index for the number of apoptotic nuclei in bovine blastocysts is valuable for indicating the overall health status of such an embryo, very few apoptotic nuclei are found in IVP porcine blastocysts, as indicated in this study as well. In these data, the apoptotic index at first sight appeared to be elevated in abnormal blastocysts, but percentage data, even when expressed as an index, can be misleading. When one translates the apoptotic index in this study to actual cell numbers, even the greatest percentage of apoptosis found in gilt FF abnormal blastocyst translates to a total of only two apoptotic nuclei. Furthermore, the fact that neither the incidence, nor indices, of apoptosis differed between any of the treatment groups, despite the prevailing differences in cell number and morphology, additionally points to the poor embryo quality diagnostic value of apoptosis in the porcine blastocyst. Morphological evaluation of blastocysts therefore provides a stronger tool for estimating embryo quality than apoptosis, as it is also associated with the number of nuclei contained on the blastocyst.

In conclusion, the results of this study indicated that cytoplasmic maturation of prepubertal gilt oocytes was improved by the use of the enriched sow FF, and reflected in the greater ability of sow FF matured oocytes to support normal fertilization, with a larger percentage subsequently developing to blastocysts with normal morphology. It therefore appears that the positive effects of the microenvironment created by the sow FF occurs at the level of oocyte cytoplasmic maturation, but that the synergystic effect thereof on the cumulus cells creates a combination in which fertilization and embryo development comparable with that of in vivo matured oocytes can be obtained. These positive effects of sow FF could be related to a number of compounds explored, and provides a base for further studies to evaluate the effect of each individual component. In addition, it provides the potential to improve chemically-defined in vitro maturation media by supplementation thereof with one or more of these components in order to achieve a culture environment more supportive of oocyte pre- and final maturation. In vitro maturation with sow FF, followed by cumulus intact IVF, appears to represent the most suitable combination for attaining fertilization similar to that achieved when using in vivo matured oocytes.

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CHAPTER 3

Effects of sperm dose and cumulus cells on the incidence and pattern of polyspermy in prepubertal gilt oocytes, as modulated by sow or gilt follicular fluid during in vitro oocytes maturation.

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Abstract

We have previously shown that the presence of sow follicular fluid (FF), in contrast to gilt FF, during IVM of prepubertal gilt oocytes improves blastocyst quality. Sow FF during IVM enhances normal fertilization and reduces polyspermy to the level of ovulated oocytes when combined with cumulus intact IVF. Sperm penetration is greatly decreased in cumulus intact IVF, which reduces fertilization efficiency and blastocyst yield. We therefore investigated the effects of sperm dose on penetration and pattern of polyspermy, and the contribution of the cumulus on these parameters. The role of oocyte maturation microenvironment, that is sow vs. gilt FF, in nuclear maturation kinetics and fertilization parameters was also studied. Finally, the consequences of different combinations of sperm dose, oocyte maturation microenvironment and cumulus cells during IVF for subsequent blastocyst quality were evaluated. In vivo matured ovulated oocytes were used as a control. Results indicated that increasing the sperm concentration to obtain higher sperm penetration rates improved penetration only in sow FF or in vivo matured oocytes, but reduced normal fertilization and increased polyspermy. Cumulus presence during IVF of oocyte matured in sow FF could not prevent polyspermy increase upon challenge with a higher sperm dose. The pattern of polyspermy was modulated by the sperm dose and the maturation microenvironment, leading to differential effects on blastocyst cell number and morphology. The percentage normal blastocyst development was not reduced when an increase in polyspermy had occurred, and was determined only by the maturation microenvironment.

Submitted for publication

Introduction

In vitro embryo production (IVP) in the prepubertal gilt is generally characterized by the inability of current culture systems to prevent the high incidence of supernumerary entry of sperm (polyspermy; PPN) during IVF. As oocytes recovered from sows are not as susceptible to polyspermy when fertilized under identical circumstances [1-3], the origin of this persistent problem is most likely related to factors concerning the overall maturation status of the oocyte. Concerted nuclear and cytoplasmic maturation are essential for establishing optimal oocyte developmental potential, which includes the ability of the oocyte to block the penetration of more than one sperm [4]. We have previously shown that the presence of sow follicular fluid (FF), in contrast to gilt FF, during IVM improves blastocyst quality [5]. This positive effect of sow FF on blastocyst morphology is due to the richer hormonal and growth factor composition of sow FF than gilt FF, supposedly effecting an enhancement of oocyte cytoplasmic maturation. In particular, sow FF supplementation during IVM enhances normal fertilization, reduces polyspermy and modulates the pattern of pronuclear arrangements in prepubertal gilt oocytes [5]. Polyspermy can be classified into different types according to the location of the pronuclei before the first cell division, which may determine the developmental fate of such zygotes [5, 6]. It is possible that certain morphological abnormalities in blastocysts, i.e. extruded blastomeres or absence of an inner cell mass, may be correlated with the location of the pronuclei [5].

A further factor affecting the degree of polyspermy is the presence or absence of cumulus cells during IVF. Under in vivo circumstances the sperm immediately encounter the oocyte in its newly ovulated state in which the cumulus is still intact [7], indicating an integral role for the cumulus during fertilization. In fact, polyspermy rates are reduced to a level similar to that of ovulated oocytes when IVM takes place in the presence of sow FF in combination with cumulus intact IVF. In oocytes matured with gilt FF the cumulus also offers an effective block to polyspermy, but not to the level of sow FF matured oocytes. Unfortunately, the cumulus also appears to hamper sperm penetration, which inevitably leads to reduced fertilization efficiency and lower blastocyst yields [5, 8, 9]. In order to increase the efficiency of fertilization, substantial improvement in the sperm penetration rate is needed when exposing cumulus intact oocytes to the IVF environment. Denuding the oocyte from its cumulus before IVF increases fertilization, but also polyspermy rates [5]. It should be noted, however, that the effect of polyspermy on blastocyst quality is not clear. On the one hand, the increase in polyspermy arising from the denudation of oocytes, matured in sow FF before IVF, does not reduce blastocyst quality, while on the other hand the decrease in polyspermy seen in oocytes matured in gilt FF followed by cumulus intact IVF, does not improve blastocyst quality. Although maturation microenvironment, that is sow vs. gilt FF, clearly plays a determining role in blastocyst quality, it is not yet clear whether a threshold-value for polyspermy exists or has any effect.

In this report we studied the effects of sperm dose on penetration and pattern of polyspermy, and the contribution of the cumulus on these parameters. In addition, we investigated whether the oocyte maturation microenvironment, that is sow vs. gilt FF, plays a role in these fertilization parameters but also kinetics of oocyte maturation. Finally, the consequences of the different combinations of sperm dose, oocyte maturation microenvironment and cumulus cells during IVF for subsequent blastocyst quality were evaluated. In vivo matured ovulated oocytes were used as a control.

Materials and Methods

In general, new experiments were carried out with procedures that were largely the same as reported in a previous study [5] since the current study aimed to unravel the problems encountered concerning fertilization.

In vivo oocyte maturation

All experiments were conducted according to USDA ethical committee guidelines. A total of 16 crossbred gilts, 6 months of age or older weighing at least 100 kg, were used as oocyte donors. Ovulation of the donors was synchronized as follows. Between day 11 and 15 following an observed estrus, the gilts were fed 20 mg altrenogest (Regu-Mate®, Hoechst Roussel Pharmaceuticals, Somerville, NJ) once daily for 4 days and 20 mg altrenogest twice on the 5th day. On the 6th day these gilts were administered 1750 IU of eCG (Folligonan; Intervet International BV, Boxmeer, The Netherlands) and 750 IU hCG (Chorulon; Intervet International BV) 83 h later. Donors were slaughtered 48 h after hCG for collection of ovulated oocytes. Immediately after exsanguinations, the reproductive tracts were removed and prepared for flushing by trimming the oviducts and a small portion of uteri away from the connective tissue while maintaining tissue temperature at 39^{o}C. Oocytes were flushed from the oviducts with warm (39^{o}C), sterile, pH-balanced BECM-3 [10] supplemented with 2 mM sodium bicarbonate, 10 mM HEPES and 0.3% BSA-V (A-7906, Sigma, St. Louis, MO). Only those oocytes recovered and graded excellent or good for developmental stage and morphological appearance were used. A total of 363 oocytes were recovered, which equals an average of 23 oocytes/donor (28 corpora lutea per donor) with a recovery rate of 82%. Since at the time of collection a few hours after ovulation the majority of the oocytes have lost their cumulus, the cumulus was gently prized loose in the few that still had the cumulus [5]. The oocytes were then directly processed for IVF.

In vitro oocyte maturation

Collection of FF for IVM, oocyte recovery, and in vitro maturation proceeded as previously described [11]. Briefly, ovaries from sows and prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory in insulated containers. Only morphologically healthy 3 to 6 mm follicles were aspirated for FF. Follicular health was established according to the criteria for the bovine [12] as validated for the porcine [13]. The pooled fluids (sow or gilt FF) were centrifuged at 1900 *g* for 30 min at 4 °C, the supernatant collected and filtered through a 0.8 µm syringe filter (Sterivex, Millipore, MA) and stored at -20ºC until use. For culture, the ovaries of prepubertal gilts were washed in pre-warmed saline placed in beakers for aspiration. Modified 10 mM HEPES-buffered North Carolina State University 23 medium (NCSU-23) [14] containing 0.4% BSA (mNCSU-23H) was used for oocyte searching and selection as well as for embryo searching later. Cumulus-oocyte complexes (COCs) were aspirated from 3 to 6 mm non-atretic follicles with an 18-gauge needle fixed to a vacuum pump via 50 ml conical tube. Follicles were regarded to be atretic when they were highly vascularized, or opaque and milky in appearance. Contents were collected into the tube and allowed to settle for 10 minutes at room temperature. The supernatant was removed and sediment was resuspended in mNCSU-23H at room temperature and allowed to settle. (COCs) were washed in mNCSU-23H. This treatment was repeated once more and the content of the tube was observed under a stereomicroscope on a heated stage (38.5 ^{o}C). COCs

surrounded by two or more layers of compact cumulus investment and containing oocytes of equal size were selected, washed twice in mNCSU-23H which had been pre-warmed to 38.5ºC prior to use, and transferred in groups of 40 to 50 to a four-well dish containing 500 µl of equilibrated IVM-I medium in each well. The IVM-I medium used for the first 20 to 22 h of oocyte in vitro maturation was BSA-free NCSU-23 supplemented with 10% (v/v) sow or gilt FF (n=765 and 807 oocytes, respectively), 1mM db-cAMP, 0.8 mM cysteine, 25 µM ß-mercaptoethanol, 10 IU/ml eCG and hCG. The four-well dish was then incubated for 20 to 22 h at 38.5 ºC in 5% CO_2 in humidified air. After 20 to 22 h all the oocytes were washed twice in IVM-wash medium and placed in 500 µl IVM-II medium for an additional 18 to 20h of culture. The second IVM (IVM-II) culture period (20 to 22 h) occurred without db-cAMP, eCG and hCG added to the medium. Samples of oocytes were processed to determine progress of nuclear maturation at different times from 18 to 42 h after start of maturation (IVM-I).

In vitro fertilization and embryo culture

Fertilization and embryo culture methods were performed as described before [5, 11]. After maturation the oocytes were placed in a wash dish containing pre-warmed equilibrated IVF medium. Modified Tris-buffered medium (mTBM) was used as fertilization medium. This medium consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate. Caffeine (1 mM) and 0.1 % BSA (w/v) (A-6003, Sigma)) were supplemented to mTBM for use as IVF medium. Using a micropipette, half of the contents of each dish were vigorously pipetted for 10 to 30 sec to remove the expanded cumulus cells. The denuded oocytes were washed once more in IVF medium before being placed in 500 µl of equilibrated IVF medium, in groups of 40 to 50, and incubated at 38.5 ºC in 5% CO_2 for 30 min until the addition of the sperm. The remaining oocytes with intact cumulus and the in vivo matured oocytes were washed twice and also placed in 500 µl wells of equilibrated IVF medium, in groups of 40 to 50, and incubated at 38.5 ºC in 5% CO_2 for 30 min until the addition of the sperm.

Semen from the sperm rich fraction of the ejaculate was collected from two mature fertility proven boars and immediately transported to the laboratory in an insulated container. Eight milliliters of saline containing 0.1% (w/v) BSA and 100 mg/l kanamycin sulfate was added to two ml sperm. The sperm suspension was centrifuged for 3 minutes at 900 *g*. The supernatant was removed and sperm resuspended in saline centrifuged again. Following one more wash the sperm was resuspended in 3 ml IVF medium. In order to identify the highest sperm:oocyte ratio at which sperm penetration could be increased without jeopardizing normal fertilization different sperm concentrations were tested. Batches of sow or gilt FF matured oocytes (n=333 and 325 oocytes with sow and gilt FF, respectively), with an intact cumulus investment, were exposed to 50, 100, 150, 500 or 1000 sperm/oocyte during the 6 h of IVF. Results indicated that the standard number of sperm added to the oocytes, i.e. 50 sperm/oocyte, could be increased by threefold without a concurrent decrease in normal fertilization rate or increase of penetration rate (Figure 1 A and B, respectively). Two sperm concentrations, 50 vs. 150 sperm/oocyte, were consequently selected to evaluate fertilization parameters and also further development of subsequent embryos in all treatment groups.

The sperm concentration was determined using a hematocytometer (single technician) and adjusted to achieve a final concentration 50 or 150 motile cells/oocyte when adding 10 µl of sperm-suspension to each well containing oocytes. After warming to 38.5 °C for 30 min, 10 µl of the sperm suspension was added to the oocytes and co-incubated with the oocytes for 6 h at 38.5ºC at 5% CO_2 in air. After 6 h, the presumptive zygotes, from oocytes denuded prior to IVF, were washed three times in mNCSU-23H before being transferred in groups of 40 to 50 into 500 µl of IVC medium. Oocytes fertilized with intact cumulus were gently pipetted to remove the remaining cumulus cells, and then washed three times before being placed in IVC medium. The in vitro embryo culture medium was NCSU-23 containing 0.4 % BSA (w/v). At 20 h after insemination, a sample of the oocytes was processed for the analysis of fertilization, and the remaining oocytes were further cultured until the blastocyst stage.

During embryo culture, the cleavage rate was determined (structures judged to be degenerated or uncleaved were not removed) at 48 h after the addition of sperm for IVF. At 96 h of culture, embryos were transferred to BSA-free NSCU-23 supplemented with 10% Fetal Calf Serum until 150 h after fertilization when blastocyst formation and morphology was evaluated.

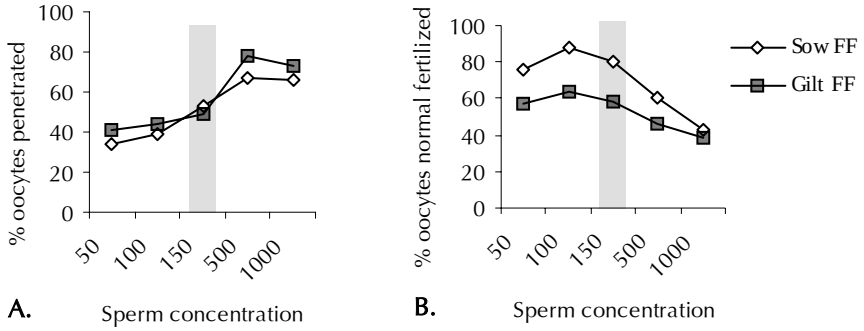

Figure 1. Sperm dose dependent fertilization of cumulus intact prepubertal gilt oocytes, matured in vitro with sow or gilt follicular fluid. Panel A: percentage of oocytes penetrated by sperm, and panel B: normal fertilized of penetrated oocytes; at each sperm dose the average number of oocytes in IVF was n=67.

Microscopy of oocytes before and after fertilization

To determine the progress of nuclear maturation COCs collected during maturation were vigorously pipetted to remove the cumulus cells, washed twice in NCSU-23H and fixed in 2% paraformaldehyde until further processing. The fixed oocytes were then washed three times in NCSU-23H and stained with 4,6-diamino-2-phylindole (DAPI; Sigma) to visualize the chromosomal material. The stained oocytes were then mounted on glass slides, and viewed under epifluorescence at 200x magnification. For the analysis of fertilization, the COCs were vigorously pipetted to remove the cumulus cells. The denuded oocytes were then washed twice in NCSU-23H, fixed in 2% paraformaldehyde and stained with DAPI to visualize the chromosomal material. Penetration parameters were classified as follows - Penetration: oocytes containing one or more decondensed sperm heads, one or more pronuclei, syngamy, or two blastomeres each containing a normal nucleus in the presence of two polar bodies; Normal fertilized: monospermic

oocytes with two pronuclei and two polar bodies, or a decondensed sperm head, a female pronucleus and two polar bodies, or syngamy with two polar bodies, or two blastomeres each containing a normal nucleus in the presence of two polar bodies; Polyspermy: oocytes containing more than one decondensed sperm head, or one sperm head in the presence of more than one pronucleus, or more than two pronuclei; Abnormal: oocytes containing aberrant chromosomal arrangements, or fragmented cytoplasm.

As described previously [5, 6], polyspermy could be classified into four categories according to the arrangement of the pronuclei: Type I in which all pronuclei progressed to the center of the oocyte and participated in syngamy [6]; Type II in which two pronuclei were located centrally with one or more supplementary pronuclei located eccentrically [6]; Type III polyspermy consisted of 3 or more eccentrically arranged pronuclei, in the presence of one or more decondensed sperm heads [5]; Early PPN consisted of an MII plate or single pronucleus with 2 or more decondensed sperm heads [5].

Assessment of Embryo Development

To determine the efficiency of the culture systems, embryos were scored morphologically at 48 and 150 h after the addition of sperm for IVF (0 h). At 48 h, the number of cleaved embryos displaying 2 to 8 evenly-sized blastomeres was recorded and categorized as Even Cleaved; embryos with fragmented or uneven-sized blastomeres were categorized as Fragmented. The percentage blastocysts was evaluated at 150 h post-insemination, expressed on the basis of the number of Even Cleaved embryos, and all blastocysts were then individually evaluated according to their morphological appearance. Hereafter representative samples of the blastocysts were fixed individually in 2% paraformaldehyde and stored at 4^{o}C until further processing.

Blastocyst morphology and cell number

Blastocysts were strictly evaluated according to their morphological appearance using a stereo microscope at 160-200X magnification, as previously described [5]. When the blastocyst was symmetrical and spherical with a clear inner cell mass, intact trophoblast, containing no extruded blastomeres it was categorized as Normal. Abnormal blastocysts included embryos with: 1. one or more extruded blastomeres present under the zona pellucida, 2. one or more blastomeres extruded within the blastocoel cavity, 3. disruptions (vacuoles) in the trophoblast layer and 4. no visible inner cell mass. To determine the cell number, fixed blastocysts were washed twice in mNCSU-23H and then stained with DAPI (5 µl/ml) for 5 min at room temperature in the dark. The embryos were then mounted in a minimal amount of DAPI fluid, flattened completely by applying firm pressure to the cover slip, and examined using a fluorescence microscope (x200 magnification, BH2-RFCA Olympus, Tokyo, Japan) to assess the total number of nuclei. Overlap of nuclei was negligible in all groups.

Statistical analyses

All experiments consisted of a minimum of 3 replicates for oocyte maturation, fertilization and embryo development. Statistical analysis of fertilization parameters, 48 h cleavage rates and blastocyst development was carried out using Chi Square or Fisher's Exact where appropriate. After testing for normality (Levene's test) and testing for equal variances (F-test for two groups and Bartlett's test for multiple groups) blastocyst cell numbers were analyzed using SPSS GLM. Data are presented as the mean percentage or mean ± SEM. Differences of $P \leq 0.05$ were considered significant. Analyses were done using the statistical analysis program SPSS for Windows 10.0.0 and Graphpad Prism®.

Results

Nuclear maturation kinetics

As shown in Figure 2, progress of nuclear maturation of prepubertal gilt COCs in the presence of sow or gilt FF was similar from 18 to 24 h of culture during GVBD and development to the MI stage. Between 24 and 30 h, though, both GVBD and MI development was accelerated in sow FF matured oocytes ($P<0.05$) compared with gilt FF matured oocytes. By 36 h of maturation this difference had been compensated for in gilt FF matured oocytes, and similar proportions of oocytes had developed to MI. Sow FF did not improve the overall synchronicity of nuclear maturation, as 24% of the oocytes had already progressed to MII by 36 h of IVM vs. 28% in gilt FF. At the termination of in vitro maturation (42 h), the majority of oocytes were in MII in both the sow and gilt FF treated groups. At 36 h of IVM, a substantial proportion of the oocytes in MII showed abnormalities in chromosomal arrangements (5% vs. 16% for sow and gilt FF, respectively; $P<0.05$), which was increased at 42 h of IVM (26% and 32% for sow and gilt FF, respectively; $P>0.05$).

Fertilization

Increasing the sperm concentration from 50 to 150 sperm/oocyte showed significantly higher penetration rates when oocytes had been matured in vivo (65 vs. 83%) or in vitro in the presence of sow FF (77 vs. 96 % for Sow FF nude; 31 vs. 55% for Sow FF intact) but not when matured with gilt FF (Table 1). This increase, however, was accompanied by a significant increase in polyspermic penetration, whereas the corresponding fertilization rates were neither reduced nor improved. Other comparisons of the fertilization parameters of corresponding groups (Table 1) did not show significant differences between the two sperm doses. When fertilization was carried out in the presence of the cumulus a higher proportion of the penetrated oocytes showed normal fertilization than when oocytes were denuded before IVF. This difference was significant at both sperm doses when oocytes had been matured in sow FF but not in gilt FF. Further, after IVF with intact cumulus, the proportions of normally fertilized penetrated oocytes remained similar at both sperm doses between oocytes matured with sow or gilt FF and the in vivo control. When applying a sperm dose of 50 sperm/oocyte, similar low rates of polyspermy were observed for in vivo control and in vitro cumulus intact fertilized oocytes which were significantly less than for their denuded counterparts. At the higher dose of 150 sperm/oocyte, however, the rate of polyspermy increased for the cumulus intact oocytes remaining significantly less than for denuded oocytes. Nevertheless the cumulus intact

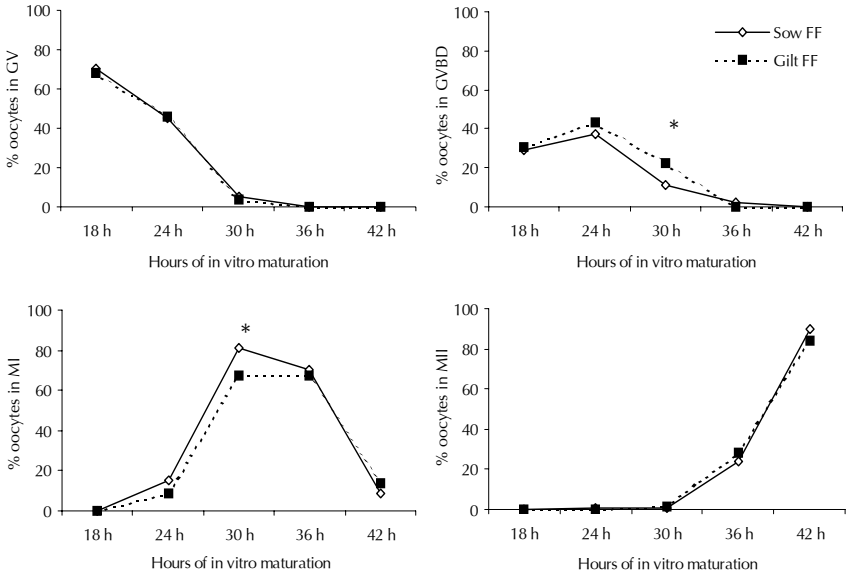

Figure 2. Kinetics of meiotic progression in pre-pubertal gilt oocytes from 18h to 42 h of in vitro culture. Nuclear stages of oocytes were scored as A) GV – germinal vesicle stage, B) GVBD – germinal vesicle breakdown, C) MI - metaphase I and D) MII – metaphase II. Prepubertal gilt oocytes were matured in vitro in the presence of sow (diamonds) or gilt (squares) follicular fluid. (Significant differences $P<0.05$ are indicated with an *); at each time point the average number of oocytes recorded was n=150.

fertilized oocytes were still able to maintain polyspermy at a level similar to that for in vivo matured oocytes. Overall low rates of abnormalities of chromosomal arrangements were seen. Only in denuded gilt FF matured oocytes fertilized with 150 sperm/oocyte a significant increase was found compared with other oocytes fertilized at this sperm dose. The increase in sperm concentration had no effect on the rate at which first cleavage took place in fertilized oocytes.

In general, polyspermy comprised the four categories. In addition, Type I polyspermy consisted of dispermic fertilization, Type II polyspermy of both di- and trispermy (with a very small incidence of tetraspermic or greater penetration), and in Type III and Early PPN pronuclear arrangements four or more sperm had typically penetrated the oocyte (data not shown). The pattern of polyspermy (Figure 3) appeared to be determined by the maturation treatment or the percentage polyspermy in the treatment group. The presence of cumulus cells during IVF also appeared to influence the pattern of polyspermy. When oocytes were denuded before IVF, irrespective of maturation treatment, a greater incidence of Type III polyspermy (Figure 3) was seen indicating the continuous supernumerary entry of sperm in to the oocyte surpassing that occurring in cumulus intact oocytes. The cumulus, therefore, appeared to provide an earlier block to 'new' sperm entry into the oocyte. Overall, in all treatment groups the largest proportion of polyspermy was represented by Type II in the presence of one or more decondensed sperm heads. The degree of polyspermy appeared to cause modulations in the type of pronuclear arrangements occurring, and was generally associated with the appearance of

one or two additional types of pronuclear arrangements. In the group presenting the lowest percentage of polyspermy, i.e. sow FF intact, fertilized at 50 sperm/oocyte, only Type II polyspermy was observed. Ovulated oocytes fertilized with 50 sperm/oocyte and sow FF intact oocytes in the 150 sperm/oocytes group, both suffering from a slightly higher incidence of polyspermy, mainly contained Type II with a small percentage of Type III prevailing as well. Gilt FF matured, cumulus intact fertilized, oocytes contained Type II with a small percentage of Type I. Sow FF matured oocytes, denuded for IVF, presented all four Types in similar ratios irrespective of sperm dose and polyspermy percentage. Gilt FF matured oocytes, denuded for IVF, presented Types I to III in similar percentages, irrespective of sperm dose. When the cumulus mass was present during IVF of gilt FF matured oocytes, the increase in sperm concentration to 150 sperm/oocyte caused the appearance of Type III polyspermy in addition to Types I and II. Early polyspermic penetration was detected only in sow FF matured oocytes that had been denuded for IVF.

Table 1. Effect of sperm dose and cumulus presence on penetration and polyspermy at 20 h after in vitro insemination of prepubertal gilt oocytes matured in sow and gilt FF.

	Penetrated (n)	Normal/ Penetrated (n)	‡Fertilization rate (n)	Cleaved/ Normal (n)	Total Abnormal (n)	PPN/ Penetrated (n)
50 sperm/oocyte						
In vivo Control	65^{a} (62/95)	76^{a} (47/62)	50^{a} (47/95)	57^{a} (27/47)	6^{a} (6/95)	16^{a} (10/62)
Sow FF Nude	77^{a} (59/77)	53^{b} (31/59)	$40^{a,b}$ (31/77)	13^{b} (4/31)	3^{a} (2/77)	44^{b} (26/59)
Sow FF Intact	31^{b} (27/88)	89^{a} (24/27)	27^{b} (24/88)	8^{b} (2/24)	3^{a} (3/88)	4^{a} (1/27)
Gilt FF Nude	76^{a} (32/42)	38^{c} (12/32)	29^{b} (12/42)	8^{b} (1/12)	12^{a} (5/42)	50^{b} (16/32)
Gilt FF Intact	47^{b} (27/58)	70^{a} (19/27)	33^{b} (19/58)	0^{b} (0/19)	9^{a} (5/58)	19^{a} (5/27)
150 sperm/oocyte						
In vivo Control	$83^{x,z}$* (57/69)	61^{x} (35/57)	51^{a} (35/69)	63^{x} (22/35)	6^{x} (4/69)	32^{x}* (18/57)
SowFF Nude	96^{x}* (44/46)	$34^{y,z}$ (15/44)	33^{a} (15/46)	20^{y} (3/15)	0^{x} (0/46)	66^{y}* (29/44)
Sow FF Intact	55^{y}* (22/40)	59^{x}* (13/22)	33^{a} (13/40)	8^{y} (1/13)	5^{x} (2/40)	32^{x}* (7/22)
GiltFF Nude	$69^{y,z}$ (29/42)	41^{z} (12/29)	29^{a} (12/42)	17^{y} (2/12)	10^{y} (4/42)	52^{y} (15/29)
Gilt FF Intact	64^{y} (25/39)	$60^{x,z}$ (15/25)	39^{a} (15/39)	7^{y} (1/15)	5^{x} (2/39)	32^{x} (8/25)

[a,b,c/x,y]Within columns values with different superscripts differ significantly: P<0.05; *Within column 50 sperm/oocyte group value differs from corresponding 150 sperm/oocyte group value: P<0.01; ‡Percentage normal fertilized oocytes developing from all oocytes subjected to IVF.

Embryo development

Increasing the sperm concentration from 50 to 150 sperm/oocyte affected the embryo development observed at Days 2 and 6 of in vitro culture (Table 2). In general, differences between treatment groups, which were apparent after IVF with the low sperm dose, were concealed or even reversed at the high dose. At 48 h of development, the proportion of even cleaved zygotes from intact-fertilized oocytes matured in gilt FF was significantly increased after IVF with the high sperm dose up to a level similar to that from their denuded counterparts, whereas with the low dose it was significantly less. In this respect, for sow FF matured oocytes the difference in result between denuded and intact oocytes remained significant at the high sperm dose. The higher sperm dose substantially reduced the proportion of blastocysts forming from even cleaved zygotes. This was significant for the sow FF matured intact-fertilized, the gilt FF denuded and in the in vivo control groups. At the high sperm dose the blastocyst formation rate of the in vivo control group was no longer significantly higher than of the other groups except for the rate of the sow FF matured intact fertilized oocytes. With regards to the developmental efficiency, i.e. the percentage blastocysts developing from all oocytes placed into IVF, it remained higher for the in vivo control group than for the other groups after IVF with the high sperm dose. The significant difference between the groups with intact or denuded oocytes at IVF with 50 sperm/oocyte disappeared at the high sperm dose for oocytes matured with gilt FF but not with sow FF. The poorest developmental efficiency was found in the groups with intact cumulus at IVF, where only 12% (sow FF) and 19% (gilt FF) of oocytes subsequently developed to blastocyst. The increase in sperm concentration did not affect the proportions of blastocysts with normal morphology although the percentage of normal blastocysts appeared to be lower at the high sperm dose. Blastocysts derived from gilt FF matured oocytes, irrespective of fertilization treatment, were of significantly poorer quality than either in vivo or sow FF origin blastocysts.

Blastocyst morphology and cell number

Increasing the sperm dose had a modulating effect on the pattern of morphological abnormalities of the blastocysts. The effect was in particular significant in the blastocysts developing from in vivo and sow FF matured oocytes. The in vivo control group presented a higher proportion of extruded blastomeres after IVF with the high sperm dose compared to the low dose (100 vs. 40% for 150 vs. 50 sperm/oocyte, respectively). Blastocysts derived from the sow FF matured denuded group demonstrated a larger percentage of trophoblast layer disruptions after IVF with the high sperm dose (100 vs. 25% for 150 and 50 sperm/oocyte, respectively). In blastocysts from the sow FF matured intact group the morphological abnormalities included externally extruded blastomeres after IVF with the high sperm dose, which was not evident their counterparts after IVF with the low sperm dose. In blastocysts derived from gilt FF matured oocytes the increase in sperm concentration did not alter the pattern of morphological abnormalities, except for the absence of blastocysts containing no visible ICM.

With regards to the cell number some effects of increasing the sperm dose were seen, in particular for blastocysts with abnormal morphology and for blastocysts derived from sow FF matured, intact-fertilized oocytes. The high sperm dose did not significantly affect the degree of similarity between treatment groups concerning the range in cell number over all blastocysts regardless their morphological appearance (inter-treatment range of the group means: 38 to 48 vs. 33 to 44 nuclei per blastocyst derived after IVF with 50 vs. 150

sperm/oocyte, respectively). The same was found for blastocysts with Normal appearance (inter-treatment range of the group means: 39 to 51 vs. 38 to 44 nuclei per blastocyst derived after IVF with 50 vs. 150 sperm/oocytes, respectively). For blastocysts with Abnormal appearance no significant differences were observed at the low sperm dose between the groups (inter-treatment range of the group means: 19 to 42 nuclei per blastocyst). But with the high sperm dose the cell number in Abnormal blastocysts from the in vivo control group was significantly higher than in the abnormal blastocysts derived from in vitro matured oocytes (44 vs. 20 to 24 nuclei per blastocyst for in vivo vs. in vitro matured groups). In general, though, for each treatment group the cell number in blastocysts containing morphological abnormalities was not affected by the sperm concentration.

A further significant, negative effect of the increase in sperm dose during IVF was evident for the blastocysts derived from sow FF matured intact-fertilized oocytes (48 vs. 33 and 51 vs. 38 nuclei per blastocyst with overall and with Normal morphological appearance, derived after IVF with 50 vs. 150 sperm/oocytes, respectively).

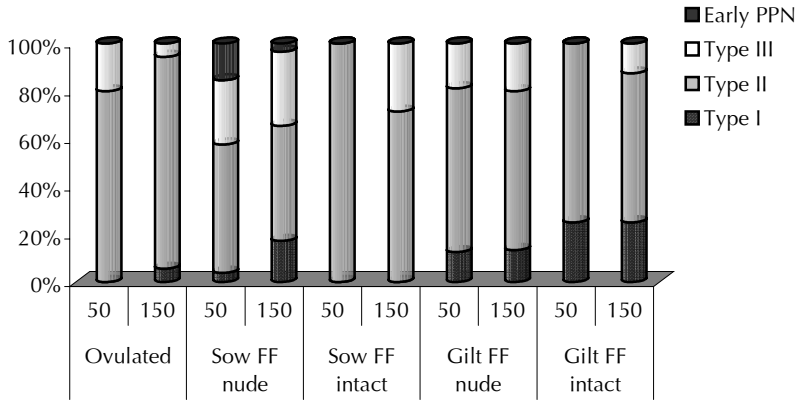

Figure 3. Sperm dose dependent polyspermy distribution patterns following IVF at 50 or 150 sperm/oocyte, of cumulus intact or denuded, prepubertal gilt oocytes after maturation in vitro in the presence of sow or gilt follicular fluid.

Discussion

In this study we demonstrated that the in vitro maturation microenvironment, established by sow or gilt follicular fluid, in combination with an intact cumulus mass for IVF could modulate the extent to which prepubertal gilt oocytes block polyspermic penetration when challenged with different sperm doses. Although the rate of fertilization was not affected by increasing the sperm dose nor by the presence or absence of cumulus cells during IVF or the conditions during IVM, the incidence and pattern of polyspermy was altered by both the type of FF added during IVM as well as the presence of cumulus cells during IVF. Blastocyst quality, on the other hand, was determined only by the in vitro maturation microenvironment, most likely due to its effects on oocyte cytoplasmic maturation as the incidence of polyspermy had no effect on embryo quality.

Cumulus-oocyte-complexes matured in vitro with sow FF undergo a greater degree of expansion than those matured with gilt FF [5], indicating that cumulus function in these COCs are possibly enhanced. The resultant improvement of blastocyst quality in sow FF can therefore be regarded as an indicator for the augmentation of cytoplasmic maturation under the influence and regulation of the cumulus cells. The positive effects of the cumulus thus supports the oocyte in its capacity to block the supernumerary entry of sperm and support male pronucleus formation to ensure normal and optimal embryo development [15, 16]. Cumulus cell presence during IVF, regardless of the preceding maturation microenvironment, also contributed to normal fertilization and polyspermy reduction, but the intact cumulus mass during IVF appeared to hinder sperm penetration. As result, a concomitant decline in developmental efficiency, as estimated by the decrease in the total blastocyst yield took place. To improve sperm penetration, the sperm dose for IVF was increased by threefold, which indeed lead to higher sperm penetration rates in cumulus intact oocytes but was persistently accompanied by the supernumerary entry of sperm into oocyte.

In the pig, the exact role of the cumulus oophorus during in vitro fertilization has not yet been fully elucidated, and studies concerning the role of the cumulus during IVF have been met with conflicting outcomes. In earlier studies, cumulus intact fertilization seemed to cause sperm penetration which consisted almost wholly of polyspermy [17-19], whereas more recent studies have found reduced penetration and fertilization rates [8, 9] as also seen in this present report. These conflicting outcomes might be linked to the type of IVF medium, as media now known to cause higher penetration rates, but also more polyspermy [17-19], were used in earlier studies, and not the modern pig-specific IVF medium mTBM [20].

Table 2. Effect of sperm dose and cumulus presence during IVF on embryo development at Days 2 and 6 of in vitro culture of prepubertal gilt oocytes matured in sow and gilt FF.

	% Even cleaved/ Total oocytes (n)		% Blastocyst/ Even cleaved (n)		†Developmental efficiency (n)		% Normal Blastocysts (n)	
	50	150	50	150	50	150	50	150
In vivo Control	55^{a} (54/98)	56^{a} (57/101)	89^{a} (48/54)	68^{a}* (39/57)	49^{a} (48/98)	39^{a} (39/101)	75^{a} (36/48)	82^{a} (32/39)
Sow FF Nude	46^{a} (71/154)	50^{a} (81/161)	62^{b} (44/71)	58^{a,c} (47/81)	29^{b} (44/154)	29^{a,c} (47/161)	84^{a} (37/44)	81^{a} (38/47)
Sow FF Intact	20^{b} (30/161)	29^{b} (45/157)	63^{b} (19/30)	40^{b,c}* (18/45)	12^{c} (19/161)	12^{b} (18/157)	95^{a} (18/19)	72^{a} (13/18)
Gilt FF Nude	41^{a} (65/158)	41^{b} (65/160)	72^{b} (47/65)	48^{a,c}* (31/65)	30^{b} (47/158)	19^{b,c}* (31/160)	51^{b} (24/47)	57^{b} (19/31)
Gilt FF Intact	24^{b} (37/153)	37^{b}* (62/168)	62^{b} (23/37)	52^{a} (32/62)	15^{c} (23/153)	19^{b,c} (32/168)	57^{b} (13/23)	63^{b} (20/32)

^{a,b,c}Within columns values with different superscripts differ significantly: $P<0.05$; * Value of 50 sperm/oocyte group differs from corresponding 150 sperm/oocyte group, †Percentage blastocysts developing from all oocytes subjected to IVF.

The types of sperm applied for IVF, i.e. fresh or frozen-thawed, are also known to have different requirements and effects during IVF leading to altered patterns of sperm penetration and fertilization [8, 9, 17, 19, 21, 22]. Whether fresh or frozen-thawed sperm are used also dictates the dose at which the sperm is added to the oocytes, and can vary from 50 or 150 sperm/oocyte (the present study), to 12 500 sperm/oocyte in other studies [8, 9, 23]. As similar penetration and polyspermy rates are often encountered using sperm at both extremes of the dose-range (50 or 12 500 sperm/oocyte) it seems feasible that not only the sperm concentration determines the fertilization fate, but that the functional status of the sperm also needs consideration. Very few have examined the capacitation status of spermatozoa at the time of addition to the fertilization wells or drops [9, 19, 24]. In vivo, sperm are sufficiently prepared, acrosome intact and functionally capacitated for fertilization by the time they reach the oviduct [25-27]. Upon encountering the newly ovulated COC, the sperm can readily penetrate the mucified cumulus mass surrounding the oocyte. Due to the lack of capacitative treatments or pre-incubations before IVF, the large differences in sperm penetration between denuded and cumulus intact fertilized oocytes, could thus possibly be attributed to the inappropriate capacitation status of the sperm.

In this study, sperm penetration and polyspermy rates were determined mainly by the sperm dose. For in vivo and sow FF oocytes the penetration and polyspermy rates increased with higher sperm dose, which upon increase masked the dose-dependent differences in fertilization parameters caused by the oocyte maturation regimen. Overall, the type of pronuclear arrangement during polyspermic fertilization appeared to have a specific order of first appearance which was related to the degree of polyspermy. At lower degrees of polyspermy Type II was either the only phenotype observed, with only a small incidence of Type I also occurring. These oocytes represent a group which presumably has the ability to correct ploidy at a later stage [6]. In the treatment groups predominantly low in polyspermy at the standard low sperm dose, i.e. in vivo or sow FF matured oocytes, subsequent development of embryos was affected by both the sperm concentration and oocyte maturation treatment and could therefore be related to the polyspermy rate and pattern. With the increase in polyspermy, following 150 sperm/oocyte fertilization, Type III arrangements in addition to Type II were also observed. In these groups, the percentage blastocyst development was typically also reduced, and might be related to the poorer developmental prognosis accompanying the Type III zygotes. We previously reported that Type III polyspermy was mainly caused by the penetration of 4 or more sperm [5], which in turn has also been described to disturb the interaction of microtubules and microfilaments, causing non-apposition of the pronuclei, and jeopardizes further development of the zygote [22].

Polyspermic zygotes readily develop to blastocysts, but their survival after surgical transfer is severely restricted [6]. Blastocysts developing from polyspermic zygotes, of which 78% have been reported to suffer from abnormal ploidy [6], also lag behind in development and are lower in cell number than their normospermic counterparts [28, 29]. The blastocyst cell number was also reduced in the sow FF intact 150 sperm/oocytes group which had a higher degree of polyspermy, and thus could possibly be related to chromosomal anomalies. In fact, blastocysts containing abnormalities, such as poly- or mixoploidy, have also been reported to contain fewer cells than their normal counterparts [29]. The pronuclear location before the first cell division appeared to play a role in the ploidy and hence the fate of the developing embryo [6]. Zygotes presenting Type II polyspermy, where one male and one female pronucleus were centrally located in

apposition and supplementary pronuclei eccentrically, have been hypothesized to have the ability to correct ploidy during further development, subsequently developing to term [6]. The correction of ploidy was hypothesized to occur when any haploid cell died later during embryo development, leaving only diploid cells in the embryo. Blastocysts with extruded cells, in this study, may have originated from polyspermic zygotes, having eventually 'corrected' their original ploidy by discarding the unwanted supplementary pronucleus. Moreover, such extruded blastomeres in many cases appeared to contain pronucleus-like structured, and not interphase or condensed nuclei (data not shown). As blastocysts developing from sow FF matured or ovulated oocytes which suffered from a higher incidence of polyspermy due to the higher sperm dose, but nevertheless contained fewer abnormalities, the improved maturation of these oocytes may have contributed to their ability to correct ploidy to a greater degree, and at an earlier stage than in gilt FF produced blastocysts. In the treatment groups which had higher percentages of polyspermy, i.e. sow FF nude, and the gilt FF groups, a number of blastocysts appeared to contain no visible inner cell mass. Interestingly, the groups containing this specific abnormality also were the only groups in which Type I polyspermy, but also a larger proportion of Type II polyspermy, was observed.

If one evaluates each maturation and fertilization treatment combination as an individual IVP system for prospective blastocyst production, the end result of developmental efficiency differed only between the denuded or cumulus intact fertilized groups and could not be improved regardless of microenvironment during IVM or the penetration rate after increasing the sperm concentration for IVF. Furthermore, contrary to the encouraging positive influence of cumulus intact IVF on subsequent normal fertilization, the developmental efficiency was greatly reduced in such oocytes. This phenomenon could possibly be related to the events taking place during nuclear maturation of the oocytes. One the one hand, this study demonstrated a positive effect of sow follicular fluid on synchrony of oocyte nuclear maturation up to 30 h of IVM, in addition to the apparent positive effects on cytoplasmic maturation. On the other hand, when taking into account the oocyte nuclear maturation kinetics data, an important concern relating to the age of the MII oocyte at the time of IVF comes to light. Almost a third of all oocytes had already reached MII by 36 h of maturation, irrespective of IVM microenvironment, leaving them 'overmatured' by 4 to 6 h and thus quality-jeopardized at the time sperm were added for IVF. Under ideal in vivo conditions the sperm are awaiting the oocyte at the site of fertilization prior to ovulation in order to maintain the stability of activation competence in the MII oocyte after ovulation [4]. If cortical granule exocytosis was to be initiated prematurely prior to sperm penetration of the ZP fertilizibility of the oocyte would be compromised, whereas premature progression to anaphase II (signifying parthenogenesis) followed by fertilization could jeopardize normal development of the zygote. Delayed fertilization in vivo causes a decrease in normal fertilization, and an increase in abnormal fertilization resulting in lower pregnancy rates and smaller litter sizes [30]. The temporal window for normal oocyte activation and fertilization is crucial for subsequent developmental potential, and may thus be a severely limiting obstacle during in vitro embryo production. Rapid changes in the oocyte cytoplasm take place during the pre- and post-ovulatory period, which narrows the window for optimal fertilization and subsequent normal development. Oocytes are deficient in activation competence prior to ovulation, but during the peri-ovulatory period activation competence is optimized [4]. Rather than suffering a loss of activation competence after ovulation, MII oocytes are hypothezised to rather become 'hypercompetent' for activation and thus more sensitive to aberrations in activation following late sperm entry. The fact

that fertilization efficiency, calculated as the percentage blastocysts developing from all oocytes subjected to IVF, was poorer when cumulus was present during could thus be due to the further delay in sperm entry brought about by the physical barrier created by the expanded mucified cumulus mass present during IVF. As the sperm penetration time for each treatment group was not assessed in this study, it is not a given that sperm penetration was delayed in the cumulus intact fertilized groups, and yet needs elucidation.

In conclusion, sow FF provides an improved microenvironment for establishing enhanced developmental potential for prepubertal gilt oocytes. This study indicates that both nuclear and cytoplasmic maturation of prepubertal gilt oocytes are influenced by the microenvironment established by sow or gilt follicular fluid supplementation to the IVM medium, as well as the presence of an intact cumulus mass for IVF. As a consequence, the incidence and pattern of polyspermy are modulated leading to differential effects on blastocyst quality. Simply increasing the sperm concentration to obtain higher sperm penetration rates does not suffice to increase normal fertilization, without incurring a higher degree of polyspermy and a reduction in the resultant developmental efficiency.

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CHAPTER 4

Effect of follicle-stimulating hormone on nuclear and cytoplasmic maturation of sow oocytes in vitro

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Abstract

A series of experiments were conducted to evaluate the effects of FSH supplementation during IVM on porcine oocyte nuclear maturation, and subsequent fertilization, cleavage and embryo development. Cumulus-oocyte-complexes (COCs) were cultured 40 h without FSH (control), 40 h with FSH (FSH 0-40 h), or 20 h with FSH followed by a 20 h culture period without FSH (FSH 0- 20 h). Nuclear stage of oocytes was assessed at intervals from 12 to 40 h of IVM. Furthermore, oocytes were in vitro fertilized, fixed and stained to determine normally fertilized and polyspermic oocytes. Additionally, COCs were matured with FSH, fertilized and zygotes cultured in NCSU-23. The percentage of cleaved embryos and blastocysts were determined and the number of nuclei was counted. The presence of FSH during the first 20 h of IVM retarded germinal vesicle breakdown. After 40 h of culture 84, 67 and 58% MII oocytes were observed in the FSH 0-20 h, FSH 0-40 h and control groups, respectively. After IVF, penetration rates were similar at 27, 26 and 29%, while the proportion of polyspermic oocytes was 7, 19 and 11% of penetrated oocytes for control, FSH 0-40 and FSH 0-20 h groups, respectively. Cleavage and blastocyst rates differed among treatments (21, 29 and 38%, and 7, 15 and 20% for control, FSH 0-40 and FSH 0-20 h groups, respectively). No differences in blastocyst cell number were found among groups. Blastocyst rates, based on number of cleaved embryos, were 51 and 52% for the FSH 0-40 and FSH 0-20 h groups, which differed significantly from the control group (31%). The results indicate that FSH has a stimulatory effect on nuclear and cytoplasmic maturation of sow oocytes. Addition of FSH for the first 20 h of culture was most beneficial, based on cleavage and blastocyst development rates.

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Introduction

Oocytes are arrested in the diplotene stage of the first meiotic division and resume meiosis immediately before ovulation. During this period of maturation, the oocyte changes from a developmentally incompetent cell to one with the capacity to direct and support the events of fertilization and early embryonic development. In the same period, cumulus cells lose contact with the oocyte and intercellular communication between these cells undergoes a progressive reduction. When oocytes from small and medium sized follicles are removed from their follicular environment and matured in vitro, spontaneous nuclear maturation does occur; only a low proportion of oocytes develop to blastocysts following IVF and early culture in vitro. These deficiencies may be attributed to abnormalities in cytoplasmic maturation, even though apparently normal nuclear maturation is observed. To increase the developmental ability of in vitro matured oocytes, gonadotropic hormones are added to the maturation medium. Although most in vitro maturation protocols currently utilize LH, FSH, or a combination of both, the effect of gonadotrophins on IVM and subsequent fertilization and early embryo development is still controversial [1].

Exposure of porcine cumulus-oocyte-complexes (COCs) to a combination of eCG, hCG and porcine follicular fluid (pFF) [2] or to eCG and pFF [3] during the first half of the maturation period increases male pronuclear formation following IVF. Addition of FSH during in vitro maturation increases the proportion of metaphase II (MII) pig oocytes [4-7]. Rath et al. [8] observed an increase of MII-stage oocytes only when FSH together with pFF was added to the maturation medium, while Bing et al. [7] reported that FSH affects nuclear progression only when cysteamine is added to the maturation medium. Maturation in the presence of FSH has no effect on male pronuclei formation following fertilization of in vitro matured pig oocytes [4,8,9]. However, the positive effect of pFF in the maturation medium on male pronuclei formation [9] or cleavage rate [8] is further enhanced in the presence of FSH. Singh et al. [5] observed a significant increase in the proportion of polyspermic oocytes when maturation medium was supplemented with FSH. It remains to be investigated whether FSH is required for only the first half or the whole maturation period. Moreover, results from the studies mentioned above [4-8] were obtained by using an IVM system where fetal calf serum (FCS) was added to the maturation medium. Naito et al. [9] reported that FCS at concentrations higher than 1% interferes with the effect of FSH with the progression of meiosis. Hence, the present study was undertaken to investigate the effect of duration of a physiological concentration of recombinant FSH, supplemented to a chemically defined maturation medium, both on the progression of meiosis of porcine oocytes and on the developmental competence of the matured oocytes following IVF.

Material and methods

Culture media

All chemicals for the preparation of culture media were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. The basic medium for IVM (OMM) was M199 (Gibco BRL, Paisley, UK) supplemented with 2.2 mg/ml $NaHCO_3$, 0.1% polyvinyl alcohol (PVA) and 100 µM cysteamine [10,11]. Depending on the treatment, recombinant human FSH (hFSH)-Org 32489 (Organon, Oss, The Netherlands) was added

to a final concentration of 0.05 IU/ml. The IVF medium was modified Tris-buffered medium [12] containing 1 mM caffeine and 0.1% (w/v) BSA (Fraction V, fatty acid free). For washing of the presumptive zygotes, IVC-wash medium, NCSU-23 [13] supplemented with 0.4% BSA and 20 mM HEPES, was used. The IVC medium was NCSU-23 supplemented with 0.4% BSA. All media were pregassed in a CO_2 incubator for at least 2 h before use.

Selection and culture of COCs

Ovaries were collected from adult sows at a local slaughterhouse, and were transported to the laboratory within 2 h in a thermoflask. After dissection of the surrounding tissue, the ovaries were flushed under running tapwater and kept at 30°C in saline supplemented with penicillin and streptomycin. COCs were aspirated from 2 to 6 mm follicles using an 18 g winged infusion set needle attached to a 50 ml polystyrene conical tube by means of a suction pump under pressure. The follicular contents were allowed to sediment and washed three times with a Tyrode's lactate-HEPES medium [14] supplemented with 0.1% (w/v) PVA. Oocytes surrounded by a compact cumulus cell mass were collected in HEPES-buffered M199 (Gibco BRL, Paisley, UK) supplemented with penicillin and streptomycin and washed three times in OMM.

IVM, IVF and IVC

In vitro maturation, IVF and IVC took place at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After selection, 35-50 COCs were transferred into a four-well culture dish (Falcon, Becton Dickinson, UK) containing 500 µl of OMM, which had been previously covered with warm mineral oil and equilibrated. During IVM, COCs were cultured for a 40 h period in OMM with or without hFSH. After culture, cumulus cells were removed by repeated pipetting, and the denuded oocytes were washed twice in IVF medium. Thirty-five to 50 oocytes were placed in 50 µl drops of IVF medium previously covered with mineral oil and equilibrated. Fresh extended (BTS extender) semen from two boars, irrespective of breed, was obtained from the local AI station. Five-hundred microliters of semen from each boar was pooled together in a 15 ml polystyrene conical tube (Greiner, Frickenhausen, Germany) and 2 ml of equilibrated IVF-medium was added. After centrifuging at 700 x *g* for 5 min at 25°C, the supernatant was removed and the sperm pellet was resuspended in 1 ml IVF-medium. This procedure was then repeated. The concentration was adjusted to 10^6 motile sperm/ml, and the sperm suspension warmed to 39°C before being adding to the IVF drops. For fertilization, 50 µl of the diluted sperm suspension were added to each 50 µl drop containing the oocytes, giving a final sperm concentration of $5x10^5$ sperm/ml. The sperm and oocytes were co-incubated for 24 h. The presumptive zygotes were then washed in IVC-wash medium, transferred to 500 µl IVC medium covered with mineral oil, and cultured in groups of 35-50 for 6 days.

Experiments

Four experiments were performed to investigate the effect of FSH during IVM on nuclear progression, fertilization, and embryo development.

Experiment 1

During IVM, COCs were cultured for 40 h in OMM without hFSH (control group), for 40 h in OMM with hFSH (FSH 0-40 h group), or for 20 h in OMM with hFSH, followed by 20 h in OMM without hFSH (FSH 0-20 h group). Oocytes from all treatment groups were washed three times after the first 20 h maturation period, with medium used in the second maturation period. At termination of IVM, the percentage of oocytes at germinal vesicle (GV), metaphase I (MI), and MII stage was assessed, and the expansion of the cumulus cells was observed. The experiment consisted of eight replicates.

Experiment 2

COCs were cultured for 20, 27, 33 and 40 h using the same treatments as described in Experiment 1. At each time point, the percentage of oocytes at GV, MI, and MII stages was determined to assess nuclear progression. The experiment consisted of four replicates. Additionally, an experiment was performed in which COCs were cultured under the same conditions for 0, 12, 16 and 20 h with or without FSH. This additional experiment consisted of three replicates.

Experiment 3

COCs were cultured for 40 h using the same hFSH treatments as described for Experiment 1, then fertilized in vitro. Twenty-four hours after the onset of IVF, the nuclear stage of oocytes was assessed. The experiment consisted of four replicates.

Experiment 4

COCs were cultured for 40 h with the same hFSH treatments as described for Experiment 1, then fertilized in vitro, and placed into culture for 6 days in IVC medium. Two and 7 days after onset of IVF the presumptive zygotes were examined for cleavage and blastocyst formation, respectively. At the end of the culture period embryos were fixed, stained with 4,6-diamino-2-phenyl-indole (DAPI), and the number of nuclei in each blastocyst was counted. The experiment consisted of six replicates.

Assessment of nuclear maturation and fertilization

The nuclear status of the oocytes was determined by DAPI staining. The oocytes were fixed in phosphate buffered saline (PBS) with 2% (w/v) paraformaldehyde, washed with PBS, stained with 2.5% (w/v) DAPI, and mounted on slides [15]. The nuclear state of the stained oocytes was assessed under a fluorescence microscope. Oocytes in which diffuse or slightly condensed chromatin could be identified were classified as being in the GV stage. Oocytes that possessed clumped or strongly condensed chromatin which formed an irregular network of individual bivalents (prometaphase) or a metaphase plate but no polar body were classified as being in the MI stage. Oocytes with either a polar body or two bright chromatin spots were classified as being in the MII stage. With respect to fertilization, oocytes with two pronuclei or with one pronucleus together with one decondensed sperm head, or cleaved embryos with two to four normal blastomeres were considered normally fertilized. Oocytes with more than two pronuclei or more than one

penetrated sperm head were considered as polyspermic, and oocytes without penetrated sperm heads were considered as unfertilized.

Statistical analysis

Statistical analysis was conducted with SPSS software (SPSS, IL, USA) using an analysis of logistic regression [16] following a binomial distribution. The data from Experiments 1, 3 and 4 were analyzed by the model: $\ln \pi/(1-\pi) = \alpha$ + treatment, and data from Experiment 2 were analyzed following the model: $\ln \pi/(1-\pi) = \alpha$ + treatment + time, where π = frequency of positive outcome, and α the intercept. Treatment and time are independent categorical variables in these models. Data from blastocyst cell numbers were analyzed by Student's t-test.

Table 1. Effect of duration of hFSH exposure on nuclear maturation rates of porcine oocytes after 40 h of culture (Experiment 1)

Treatment	Total oocytes N[1]	Stage of oocytes % (mean ± S.E.M.)			
		GV	MI	MII	Degenerated
Control (no FSH)	640	18 ± 3^{a}	12 ± 2^{a}	58 ± 5^{a}	$11 \pm 2^{a,b}$
FSH 0-40 h	642	12 ± 3^{b}	$9 \pm 1^{a,b}$	67 ± 4^{b}	12 ± 2^{a}
FSH 0-20 h	611	3 ± 2^{c}	5 ± 2^{b}	84 ± 4^{c}	8 ± 2^{b}

Within columns, values with different superscripts (a, b, c) are significantly different ($P<0.05$); [1]The experiment was replicated eight times.

Results

Effect of FSH on nuclear maturation and cumulus cell expansion (Experiment 1).

The effect of FSH supplementation on nuclear progression of porcine oocytes after 40 h of IVM, is shown in Table 1. Both FSH (0-20 and 0-40 h) treatments decreased the percentage of GV-stage oocytes ($P=0.0016$ and <0.0001, respectively) and increased the percentage of MII-stage oocytes ($P=0.0039$ and <0.0001, respectively), when compared to the control group. Exposure of COCs to FSH for only the first 20 h of IVM resulted in more MII-stage oocytes at the end of culture, than exposure to FSH for the entire 40-h culture period ($P< 0:0001$), and resulted in fewer GV-stage oocytes than either of the other treatments. Images of COCs at the start of culture, and after 40 h of culture with or without FSH are shown in Figure 1. After IVM, COCs in the control group were clumped together, and cumulus cells appeared black and shrunken (Fig. 1b), while COCs in both FSH groups showed expanded cumulus (Fig. 1c).

Effect of FSH on the kinetics of nuclear maturation (Experiment 2)

At the start of culture, oocytes in both groups were in the GV stage. The effect of FSH on the kinetics of nuclear progression from 12 to 20 h of culture is presented in Table 2. Over time, a decrease in the percentage of GV-stage oocytes occurred in the control

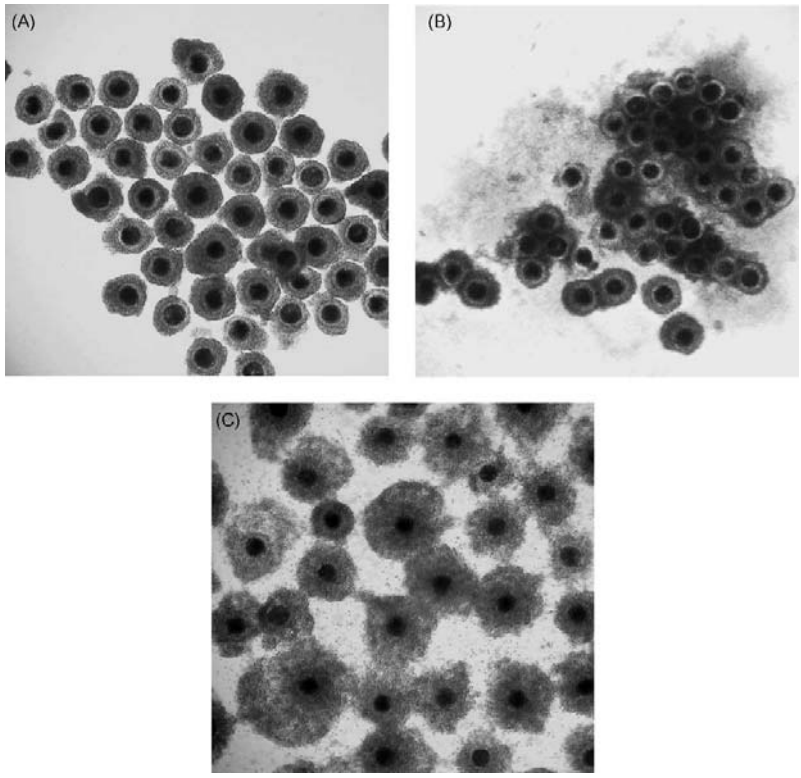

Figure 1. Cultured porcine COCs at the onset of culture (A), after 40 h of culture in maturation medium without FSH (B) and with 0.05 IU FSH/ml (C) (magnification: 30x).

Table 2. Effect of hFSH on kinetics of nuclear progression of porcine oocytes after 12, 16 and 20 h of culture (Experiment 2)

Treatment	Time of culture (h)	Total oocytes N[1]	Stage of oocytes % (mean ± S.E.M.)			
			GV	MI	MII	Degenerated
Control (no FSH)	12	158	75 ± 3^{a}	20 ± 4^{a}	1 ± 1^{a}	4 ± 2^{a}
	16	146	61 ± 3^{b}	29 ± 1^{a}	6 ± 4^{a}	5 ± 3^{a}
	20	131	49 ± 5^{c}	34 ± 7^{b}	12 ± 4^{b}	5 ± 1^{a}
FSH	12	134	85 ± 3^{a}	7 ± 1^{a}	3 ± 2^{a}	5 ± 3^{a}
	16	143	80 ± 5^{a}	10 ± 1^{a}	3 ± 2^{a}	7 ± 2^{a}
	20	129	75 ± 2^{a}	12 ± 4^{a}	2 ± 2^{a}	10 ± 2^{a}

Within the same column, values with different superscripts (a, b, c) are significantly different ($P<0.05$).
[1]The experiment was replicated three times.

group (P< 0.0001), while no significant decrease of GV-stage oocytes was observed in the group exposed to FSH (P=0.1157). The effect of FSH on the kinetics of nuclear progression stages during 20-40 h of culture is depicted in Fig. 2. At 20 h of culture fewer GV-stage oocytes (P=0.0022) were seen in the control group than in either of the FSH groups. In the culture period from 20 to 27 h, the percentage of GV-stage oocytes in the FSH 0-20 h group decreased more rapidly (P=0.0005) than was observed in the control or the FSH 0-40 h groups. The decrease of GV-stage oocytes in the control group was similar to that in the FSH 0-40 h group. No differences were observed among the treatment groups in formation of MII-stage oocytes until 33 h of culture. At the end of culture, more (P=0.0001) MII-stage oocytes were present in the FSH 0-20 h group than the control or FSH 0-40 h groups. The percentages of MII-stage oocytes were 60±8%, 66±5% and 85±3% in the control, FSH 0-40 and FSH 0-20 h groups, respectively.

Effect FSH during maturation on fertilization (Experiment 3)

The effect of FSH during maturation on IVF is presented in Table 3. The penetration rates in all treatment groups were similar, with the proportion of fertilized oocytes ranging from 26 to 29%. More polyspermic oocytes were found in the FSH 0-40 h group than in the control group (P=0.0137). The number of degenerated oocytes in the control group was higher (P=0.0044) than in the FSH 0-40 h group, but similar to the FSH 0-20 h group.

Effect of FSH during maturation on embryo development (Experiment 4)

The effects of FSH during maturation on subsequent cleavage and embryo development rates are presented in Table 4. Cleavage rates were higher for the FSH 0-20 and FSH 0-40 h groups than the controls (P=0.0001 and 0.0042, respectively). Also, cleavage rate was higher for the FSH 0-20 h group than the FSH 0-40 h group (P=0.0002). At 2 days after the onset of fertilization, the majority of cleaved embryos in all groups were at the four-to eight-cell stage and granulation in the cytoplasm appeared in the center of the blastomeres. Both FSH groups had a higher number of blastocysts than the control group (P<0.0001), while more blastocysts were found in the FSH 0-20 h group than in the FSH 0-40 h group (P=0.0066). Across treatment groups, the mean number of cells per blastocyst was similar and ranged from 20 to 70 cells per embryo.

Table 3. Effect of presence of hFSH during in vitro maturation of porcine oocytes on in vitro fertilization as assessed 24 h after the onset of IVF (Experiment 3)

Treatment	Oocytes in IVF N[1]	Fertilized oocytes % (mean ± S.E.M.)			Unfertilized oocytes % (mean ± S.E.M.)			
		Total	Normally fertilized[2]	Poly-spermic[2]	GV	MI	MII	Degenerated
Control (no FSH)	433	29±8[a]	93±2[a]	7±2[a]	14±4[a]	4±1[a]	42±0[a]	12±3[a]
FSH 0-40 h	502	26±10[a]	82±5[b]	19±5[b]	0±0[b]	1±1[b]	66±11[b]	6±2[b]
FSH 0-20 h	489	27±8[a]	89±4[a,b]	11±4[a,b]	2±0[b]	2±1[b]	61±9[b]	8±2[a,b]

Within columns, values with different superscripts (a, b) are significantly different (P<0.05). [1]The experiment was replicated four times. [2]Percentage of oocytes penetrated.

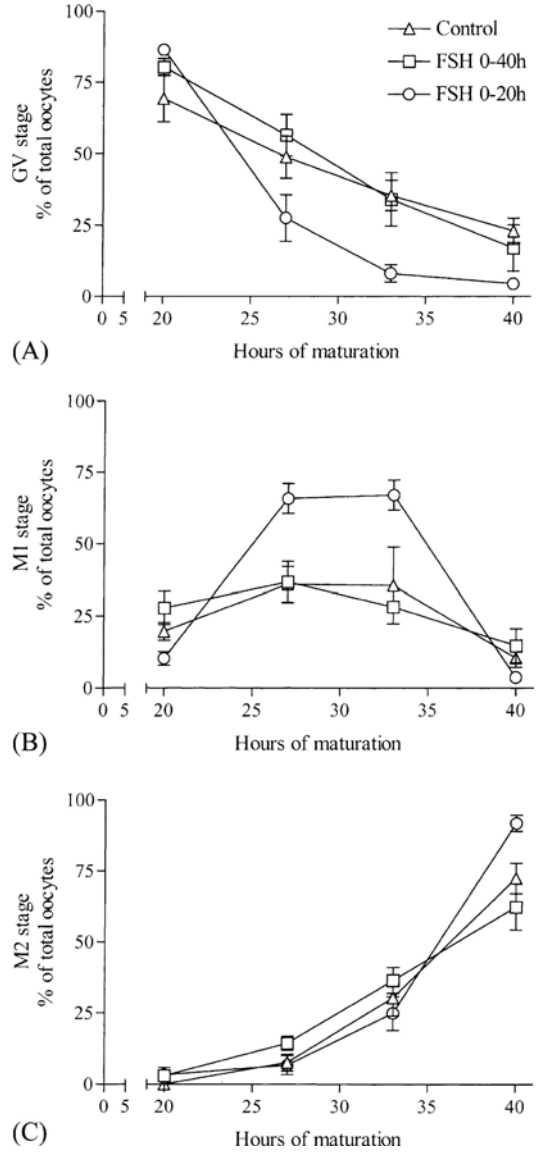

Figure 2. Kinetics of meiotic progression in porcine oocytes from 20 to 40 h of culture (Experiment 2). Nuclear stages of oocytes were scored as GV(A), MI (B), or MII (C). Sow COCs were cultured for 40 h in maturation medium without FSH (control; triangles) or with FSH (0.05 IU/ml) (FSH 0-40 h; squares), or cultured for 20 h in maturation medium with FSH (0.05 IU/ml) followed by culture in maturation medium without FSH (FSH 0-20 h; circles).

Table 4. Effect of the presence of FSH during IVM of porcine oocytes on the cleavage rate and blastocyst formation following IVF (Experiment 4)

Group	Oocytes N[1]	Cleaved embryos at 2 days % (mean ± S.E.M.)	Blastocysts at 7 days %(mean ± S.E.M.)	Cells per blastocyst (mean ± S.E.M.)
Control (no FSH)	815	21 ± 3[a]	7 ± 2[a]	37 ± 2[a]
FSH 0-40 h	711	29 ± 5[b]	15 ± 3[b]	38 ± 1[a]
FSH 0-20 h	727	38 ± 5[c]	20 ± 4[c]	38 ± 1[a]

Within columns, values with different superscripts (a, b, c) are significantly different (P < 0:05). [1]The experiment was replicated six times.

Discussion

The data presented clearly demonstrate that IVM of sow oocytes in the presence of FSH not only enhances nuclear maturation but also improves the developmental capacity of the oocytes as reflected by the enhanced rate of blastocyst formation. The presence of FSH during the first half or the entire culture period enhanced MII formation and induced cumulus cell expansion. Similar effects on nuclear progression [4-7] and on cumulus cell expansion [6,17-19] after exposure of pig COCs to FSH during the whole maturation period have been reported. Generally, we observed a higher proportion of oocytes that progressed to the MII stage in the absence of FSH as compared to the aforementioned studies. This might be due to the use of sow oocytes instead of oocytes from prepubertal gilts. Recently, it has been reported that oocytes from sows have higher maturation potency than oocytes from prepubertal gilts [20]. Our data on the kinetics of meiosis showed that addition of FSH retards GVBD, with the most apparent effect occurring during the first 20 h of maturation.

Thereafter, retardation by FSH has almost disappeared. Remarkably, omission of FSH after 20 h resulted in an acceleration of the progression of meiosis, resulting in more oocytes that reached the MII stage by 40 h. Presence of eCG and hCG during the first 20 h, or during the entire maturation period of 40 h for COCs from prepubertal gilts resulted in the same proportion of MII oocytes [2]. Retardation of nuclear maturation by FSH is in agreement with previous studies in bovine [21,22] and rodent [23-25] oocytes showing transient inhibition of maturation by FSH. The mechanism by which FSH induces oocyte maturation is not completely known. However, there are many reports indicating that cumulus cells play a mediating role in the FSH action on oocyte maturation. Recently, it has been shown that bovine oocytes obtained from small and medium sized follicles do not contain mRNA of FSH receptors [26]. In addition, culture of murine denuded oocytes in the presence of FSH [27] did not affect oocyte maturation, indicating that the effect of FSH on meiosis is exerted through cumulus cells. In contrast, Mattioli et al. [4] observed acceleration of nuclear progression when porcine oocytes were cultured in maturation medium supplemented with FCS and FSH.

The proportion of penetrated oocytes and those showing polyspermy were low compared with other studies [3-5,7,8]. It has been reported that oocytes from adult animals showed lower penetration rates, but also lower rates of polyspermy than oocytes from prepubertal gilts [20]. Although polyspermy rates were low, FSH treatment resulted in a higher level of polyspermic oocytes. It is not likely that this is due to an abnormal distribution of cortical granules, as MII-stage oocytes from both control and FSH treated groups showed

a cortical granule distribution nicely underlying the plasma membrane (Schoevers, personal observation).

FSH treatment during half or all of IVM of sow COCs resulted in enhanced cleavage following fertilization, whereas the presence of FSH during the first half of the culture period resulted in the highest yield of blastocysts. The enhanced blastocyst formation may be due to the higher percentage of MII oocytes at the end of culture, although it appeared that only a small percentage of MII oocytes were penetrated following in vitro fertilization. Expression of the blastocyst rate as the ratio of blastocyst and cleaved embryos revealed approximately 30% for the control and approximately 50% for both FSH treatments. Apparently, addition of FSH during the maturation of sow oocytes increases the competence of cleaved embryos to develop to the blastocyst stage, indicating that FSH not only enhances nuclear maturation but also promotes cytoplasmic maturation of sow oocytes. In vitro maturation of cow oocytes in the presence of FSH does not affect the proportion of MII oocytes, but cleavage and blastocyst formation is increased [22]. As the blastocyst to cleaved embryo ratio was similar for both FSH treatments, we may state that exposure of sow COCs to FSH is only necessary for the first 20 h of culture. Further exposure to FSH may have a negative influence on the cytoplasmic maturation of sow oocytes as determined by cleavage and development rates. Using pronucleus formation as parameter for cytoplasmic maturation, Funahashi and Day [2] found higher proportions of oocytes with male and female pronuclei after 20 h of culture with gonadotrophic hormones as compared with culture with hormones throughout the maturation period. Rath et al. [8] did not observe an effect on the blastocyst formation as expressed by the ratio of blastocysts to cleaved embryos following the in vitro maturation of prepubertal gilt oocytes in the presence of FSH. The relative high percentage of blastocysts obtained in our study, as compared to the study of Rath et al. [8], is probably due to the use of sow oocytes. In bovine species it has been shown that oocytes from prepubertal calves have less developmental competence [28,29].

In conclusion, our results indicate that FSH initially retards GV breakdown, but overall has a stimulatory effect on the nuclear and cytoplasmic maturation of sow oocytes.

Acknowledgments

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CHAPTER 5

The effect of oviductal epithelial cell co-culture during in vitro maturation on sow oocyte morphology, fertilization and embryo development

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Abstract

In vitro embryo production in the sow is challenged by poor cytoplasmic maturation, low sperm penetration and low normal fertilization, leading to the development of poor quality blastocysts containing a small number of nuclei. In prepubertal gilt oocytes, the presence of porcine oviductal epithelial cells (pOEC) during maturation increases cytoplasmic maturation and blastocyst development. These aspects, as well as blastocyst quality, may be improved when adult sow oocytes are matured with pOEC. Therefore, the effect of the presence of pOEC on sow oocyte morphology, fertilization and the progression of embryo development was evaluated. The pOEC were cultured in M199 for 18 h, then cultured in NCSU23 for 4 h before the oocytes were added. Oocytes from 2 to 6 mm follicles were matured in 500 ml NCSU23, with eCG and hCG, for 24 h, and then cultured with or without pOEC, in NCSU23 without hormones, for 18 h. In vitro fertilization took place in modified Tris-buffered medium, for 6 h, and the presumptive zygotes were then cultured for 162 h in NCSU23. Morphology of the IVM oocytes was compared to that of immature oocytes and in vivo matured MII oocytes from slaughtered sows in estrus. The in vitro matured oocytes had a greater diameter and a wider perivitelline space than the immature and in vivo matured MII oocytes ($P<0.01$). Penetration, polyspermy and pronucleus formation did not differ between the pOEC and Control groups, although the total penetration rate was higher for the Control oocytes (26% versus 39%; $P<0.01$). Fewer blastocysts developed in the pOEC group than in the Control group (19% versus 27%; $P<0.01$), but blastocyst growth was accelerated, leading to a higher percentage of hatched blastocysts (3% versus 10%; $P<0.01$). Finally, the average blastocyst cell number was higher in the pOEC group (47 versus 40; $P<0.05$) and a greater percentage of blastocysts contained a superior number of nuclei. In conclusion, the addition of pOEC during the second half of in vitro maturation resulted in fewer blastocysts formed, but of those blastocysts that did form, the quality was improved.

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Introduction

In vitro embryo production (IVP) is an important tool for study of the regulation of oocyte maturation and early embryonic development in the pig. During the past decade effective porcine IVP systems have been developed, leading to blastocyst development rates comparable to that of other domestic species such as the bovine and ovine [1-3]. Unlike other domestic species though, pig IVP is challenged by serious problems such as incomplete cytoplasmic maturation [1,4], leading to the development of blastocysts with reduced cell numbers and compromised viability [5]. Two recent studies have also demonstrated distinct differences in the developmental competence of prepubertal gilt and adult sow oocytes [4,6]. Although polyspermy has generally been regarded as the major obstacle in porcine IVF, these studies revealed greatly reduced incidences of polyspermy in oocytes from adult sows, but this was accompanied by lower rates of penetration and normal fertilization. Nevertheless, a higher proportion of oocytes developed to blastocysts from sow oocytes than from prepubertal gilt oocytes [4]. These studies indicate different requirements for the in vitro maturation and in vitro fertilization environments for prepubertal gilt and sow oocytes.

After ovulation, the oocyte resides in the oviduct a few hours before fertilization occurs. In vitro matured prepubertal gilt oocytes display different morphological and fertilization characteristics from those of ovulated oocytes [7]. A preliminary study by Day et al. [8] indicated that in vitro matured prepubertal gilt oocytes exposed to the estrous oviduct had a more similar morphology to ovulated oocytes, associated with lower polyspermic penetration. Co-culturing the oocytes with homologous oviductal epithelial cells [2,9,10] or adding oviductal fluid to the culture medium [11] increased normal fertilization and reduce the incidence of polyspermy in oocytes from prepubertal gilts. The effect of porcine oviductal epithelial cells (pOEC) during maturation on subsequent embryo development was examined by Bureau et al. [2] who found a pOEC-induced increase in the rate of blastocyst development compared with the pOEC-free control group. Whether pOEC co-culture also improves blastocyst quality by increasing the number of nuclei has not yet been established.

In vitro produced porcine embryos are retarded in their developmental progression compared to their in vivo counterparts as judged by the number of nuclei per blastocyst [5,11-14]. Improved cytoplasmic maturation of prepubertal gilt oocytes [1,13] results in improved blastocyst development rates, but the relation to blastocyst quality or number of nuclei is not fully established. Hatching of in vitro produced blastocysts is also rarely achieved, and requires the presence of serum and amino acid supplementation of the culture medium [15-18]. In the mouse, IVM/IVF derived blastocysts with reduced cell numbers are less viable after transfer and rarely lead to pregnancies [19-22]. This brings to light the necessity for acceleration of growth or cell division and increase of cell numbers of in vitro blastocysts in order to achieve blastocysts of quality equal to those produced in vivo.

The addition of pOEC during in vitro maturation is known to increase normal fertilization [2,8-10], but their effect on cytoplasmic maturation of sow oocytes, related to blastocyst development and specifically blastocyst quality, has not been determined. In this study we hypothesized that pOEC co-culture during IVM would improve cytoplasmic maturation of the oocyte, leading to increased fertilization rates, blastocyst quality and development. We therefore investigated the effects of pOEC co-culture during in vitro

maturation on oocyte morphology, fertilization parameters, the subsequent progression of embryo development and resultant blastocyst quality.

Materials and methods

Culture media

For oviduct trimming, oocyte searching and selection, 25 mM Hepes-buffered Tyrode's medium containing 0.1% polyvinyl alcohol (TL-Hepes-PVA) was used. The pOEC were harvested and washed in Hepes-buffered M199 (Gibco BRL, Paisley, UK). Culture of the pOEC took place in M199 supplemented with 2.2 mg/ml NaHCO3 with 75 mg/ml potassium penicillin G and 50 mg/ml streptomycin sulfate (PenStrep; Sigma, St. Louis, MO, USA). Cumulus-oocyte-complexes (COCs) were washed in IVM-wash medium consisting of NCSU23 medium [18] supplemented with 10% porcine follicular fluid (pFF), 0.57 mM cysteine, 25 mM β-mercaptoethanol and 20 mM Hepes. The maturation medium for the first 24 h of in vitro maturation, designated IVM-I, was NCSU23 with 10% pFF, 0.57 mM cysteine, 25 mM β-mercaptoethanol, 10 IU/ml eCG (Chorulon, Intervet, Boxmeer, The Netherlands) and 10 IU/ml hCG (Folligonan, Intervet, Boxmeer, The Netherlands). For the second 18-20 h of maturation (IVM-II), the same medium was used as for IVM-I, but without eCG and hCG and supplemented with PenStrep. The fertilization medium (IVF-medium) was modified Tris-buffered medium (113.1 mM NaCl, 3.0 mM KCl, 20.0 mM Tris, 11.0 mM D-glucose, 7.5 mM $CaCl_2.2H_2O$ and 5.0 mM Na-Pyruvate) containing 1 mM caffeine and 0.1% BSA (Sigma, St. Louis, MO, USA). In vitro culture of the zygotes then took place in NCSU23 medium supplemented with 0.4% BSA (IVC-medium).

Preparation of porcine oviductal epithelial cells

Oviducts attached to ovaries with recent corpora were used for obtaining epithelial cells. The age of the corpora hemorrhagica was estimated following previous experience using oviducts from sows slaughtered soon after of ovulation. Such oviducts display vigorous cilliary and secretory activity, known to be indicative of viability [23,24]. The method described below was developed during a prestudy in this laboratory. Two oviducts were collected from two different sows at the slaughterhouse and transported to the laboratory at 27-30 ^{o}C within 3 h postmortem. The oviducts were then dissected free of the ovaries and other tissues, and washed twice in 0.9% saline with 75 mg/l potassium penicillin G and 50 mg/l streptomycin sulfate at 30 ^{o}C before sectioning. Approximately 3 cm from the infundibulum (ampullar region) two sections of 1 cm each were cut off each oviduct and washed once more in room temperature saline. The sections were carefully wiped to remove excess saline and placed in room temperature M199-Hepes with 10% FCS and PenStrep. The pOEC were obtained by slicing the oviduct longitudinally and scraping the inside using a scalpel blade, keeping the oviduct immersed in the medium. The pOEC were then flushed in and out of a 21 g needle three times in order to separate the cells into smaller clumps. The cells were then centrifuged at 300 x g for 3 min, the supernatant removed, the pellet resuspended and the procedure repeated twice. After the final centrifugation, the soft pellet was resuspended in 1 ml M199-Hepes with 10% FCS and PenStrep and 20 ml of this cell suspension was transferred to 500 µl M199 with 10% FCS and antibiotics in a 4-well culture dish. The dish was then incubated at 38.5 ^{o}C in 5% CO_2 for 18 h. After the incubation period, the contents of each well were removed and

placed in a 15 ml polystyrene conical centrifuge tube. Each of the wells, which contained some attached oviductal cells, were then washed three times using 500 µl IVM-wash medium. After the final wash, 500 µl IVM-II medium was added and covered with warmed mineral oil. The pOEC were then washed three times by centrifugation at 300 x g using prewarmed IVM-wash medium. After the final wash, the pellet was resuspended in 80 ml IVM-II medium and 20 ml of this suspension was added to each well. The pOEC culture dish was then incubated for 4 h before the COCs were added for IVM-II.

Oocyte maturation in vitro

Ovaries, and attached oviducts, were collected from sows after slaughter and transported to the laboratory in an insulated container within 3 h postmortem at 27-30 ^{o}C, the temperature of the ovaries upon collection at the slaughterhouse. Upon arrival, the ovaries were washed under running tap water at 27-30 ^{o}C, then dried gently using paper towels and placed in 0.9% saline with 75 mg/l potassium penicillin G and 50 mg/l streptomycin sulfate at 27-30 ^{o}C until aspiration. COCs were aspirated from 2 to 6 mm follicles using an 18 g winged infusion set needle attached to a 50 ml polystyrene conical tube by means of a suction pump under pressure. Follicular contents were allowed to settle at room temperature after which the supernatant was discarded and the sediment resuspended with TL-Hepes-PVA. This procedure was repeated twice. Oocytes surrounded by three or more layers of compact cumulus cells were selected irrespective of ooplasm appearance and washed twice in IVM-wash medium. Then 45-50 oocytes were cultured in each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 500 µl of IVM-I medium which had previously been covered with mineral oil and equilibrated for 2 h at 38.5 ^{o}C and 5% CO_2 prior to use. After 24 h of maturation, the COCs were washed twice in IVM-wash medium and placed in IVM-II medium, after which they were cultured for an additional 18-20 h.

Fertilization and embryo culture in vitro

Fresh BTS-extended (Beltsville Thawing Solution) [25] semen from two randomly selected boars, irrespective of breed, was obtained from the local AI station. The spermatozoa were allowed to cool to room temperature for 30 min before processing. 500 ml sperm from each boar was pooled together in a 15 ml polystyrene conical tube (Greiner) and 2 ml room temperature IVF-medium (previously equilibrated for 18 h at 38.5^{o}C in 5% CO_2) was added. After centrifuging at 700 x *g* for 5 min at room temperature, the sperm pellet was resuspended to 1 ml IVF-medium. This procedure was then repeated, the concentration adjusted to 8 x 10^4 motile sperm/ml, and the sperm suspension warmed to 38.5 ^{o}C for 15 min before being added to the IVF drops.

After maturation was completed, the expanded cumulus cells were removed by pipetting. The denuded oocytes were washed twice in IVF medium, and 45-50 oocytes were placed in 50 ml drops of IVF medium that had previously been covered with mineral oil and equilibrated at 38.5 ^{o}C in 5% CO_2 in a 35 mm x 10 mm petri dish (Nunc, Roskilde, Denmark) for 1 h. For fertilization, 50 ml of the diluted sperm suspension were added to each 50 µl drop containing the oocytes, giving a final sperm concentration of 400,000 sperm/ml. The spermatozoa and oocytes were co-incubated for 6 h at 38.5^{o}C in 5% CO_2 humidified air. The oocytes were then washed in IVC-wash medium and transferred to IVC-medium for 12 h to 7 days.

Recovery of in vivo matured oocytes

Oocytes were recovered from the ovaries of GnRH-synchronized sows approximately 3± 8 h before estimated ovulation. Briefly, at 83-87 h after weaning, sows were treated with 50 mg of GnRH i.m. (Depherelin, Veyx-Pharma, Germany), to synchronize ovulation. Previous preliminary research [26] has shown that in sows treated around this time, ovulation occurs 35-41 h after GnRH administration. These sows were then slaughtered and the ovaries removed at approximately 38 h after GnRH treatment. Oocytes were aspirated from the follicles using an 18 g needle attached to a 10 ml syringe. The follicular contents of each individual syringe were placed in a 90 mm petri dish and approximately 10-12 ml of TL-Hepes-PVA was added to dilute the opaque follicular fluid in order to visualize the COCs clearly. The expanded COCs were then washed twice using TL-Hepes-PVA and placed in a solution of 0.2% hyaluronidase for 5 min to remove the majority of the cumulus cells. The remaining cumulus cells and corona radiata were removed by repeatedly passing the oocyte through a narrow bore glass pipette. The denuded oocytes were washed twice in TL-Hepes-PVA, and an image of each oocyte was recorded as described in Experiment 1. The oocytes were then fixed in 2% paraformaldehyde and stained with 4,6-diamino-2-phylindole (DAPI, Sigma, St. Louis, MO, USA; see Experiment 2) to determine the stage of nuclear maturation. Of this group, only oocytes at metaphase II were taken into consideration for morphological evaluations.

Experimental design

Experiment 1: morphological comparison of in vitro and in vivo matured oocytes

In the first experiment, a comparison of morphological characteristics of oocytes from four different origins were made: immature oocytes from 2 to 6 mm follicles (n=23); oocytes after 42-44 h in vitro maturation without pOEC (n=48); oocytes after 24 h in vitro maturation without pOEC and then 18 h with pOEC (n=35); and metaphase II oocytes from pre-ovulatory follicles (three sows; n=49). Immature oocytes were treated similarly to in vivo oocytes for the removal of the cumulus cells. All oocytes were placed in the same medium (TL-Hepes-PVA) for imaging to avoid any differences that might occur due to changes in osmolarity. Evaluations were made of the appearance of the ooplasm, the diameter of the oocyte, the thickness of the zona pellucida (ZP), the size of the perivitelline space and the zona-free diameter of the ooplasm. Images were recorded with a digital camera (Nikon Coolpix 990) at 200 x magnification on an inverted microscope (Olympus IM, Tokyo, Japan), and measurements were made from the images after printing (Fig. 1).

Experiment 2: the effect of pOEC co-culture during IVM on sperm penetration after in vitro fertilization

Oocytes collected from slaughterhouse ovaries, matured in the absence (42 h without pOEC) or presence of pOEC (24 h without and then 18 h with pOEC), were fertilized in vitro. The sperm penetration parameters were determined 18-20 h after the onset of IVF. Oocytes were washed in TL-Hepes-PVA and fixed in 2% paraformaldehyde for at least 4 days at 4^{o}C, then washed three times in TL-Hepes-PVA before being stained with 0.1 mg/ml DAPI, mounted and observed under epifluorescence at 400 x magnification (BH2-RFCA Olympus, Tokyo, Japan).

Penetration parameters were classified as follows: total penetration rate (TPN), oocytes containing an intact sperm head, a decondensed sperm head, one or more male pronuclei, syngamy, or two blastomeres each containing a normal nucleus in the presence of two polar bodies; penetration rate (PEN), as for TPN, but excluding intact sperm heads; male pronucleus formation (MPN), oocytes containing one or more male pronuclei or two blastomeres each containing a normal nucleus in the presence of two polar bodies; polyspermy, oocytes containing more than one decondensed sperm head or male pronucleus; normal fertilization, monospermic oocytes with two pronuclei and two polar bodies, or a decondensed sperm head, a female pronucleus and two polar bodies, or syngamy with two polar bodies, or two blastomeres each containing a normal nucleus in the presence of two polar bodies. The cytoplasmic index (CI) is defined as the ratio of the percentage PEN to the percentage TPEN. The index has a value between 0 and 1, with higher values implying greater cytoplasmic proficiency of the oocytes.

Experiment 3: in vitro embryo development of in vitro fertilized oocytes following IVM in the presence or absence of pOEC

Oocytes were matured and fertilized as in Experiment 2, but presumptive zygotes were subsequently cultured up to 168 h after insemination. The cleavage rates were assessed at 48 h post-insemination. Only embryos consisting of two to eight evenly sized blastomeres were included. Blastocyst formation and hatching was determined on Day 5, Day 6 and Day 7 after insemination (Day 0). Blastocysts produced were then fixed and stained to determine the number of nuclei. Briefly, blastocysts were washed in TL-Hepes-PVA and fixed in 2% paraformaldehyde for at least 4 days at 4 ^{o}C. They were then washed three times in TL-Hepes-PVA before being stained with DAPI and mounted. The number of nuclei was determined by viewing the blastocysts under epifluorescence. An image of each blastocyst was recorded using a digital camera, and the cells were then counted after printing of the images (Figure 2).

Statistical design

At least three replicate trials were carried out for each experiment. Morphological differences were analyzed by ANOVA, followed by the Bonferroni post hoc test. Differences in the rates of total penetration (TPN), penetration (PEN), male pronucleus formation (MPN), polyspermy, cleavage (CR) and blastocyst formation were analyzed by Fisher's exact test. Blastocyst cell numbers were analyzed by t-test and are presented as mean ± SEM. A difference with $P<0.05$ was considered to be statistically significant.

Results

Experiment 1

Differences in the distribution of the ooplasm between immature, in vitro matured and in vivo matured oocytes were observed (Fig. 1). The majority (98%) of in vivo matured oocytes displayed a continuous clear space in the cortex of the ooplasm, approximately 12-15 mm deep. Clear spaces 7-10 mm in depth were also seen in the ooplasm of the in vitro matured oocytes, but these were distributed semicontinuously in only 40-60% of the ooplasm cortex. Immature oocytes had an evenly distributed ooplasm with no clear spaces.

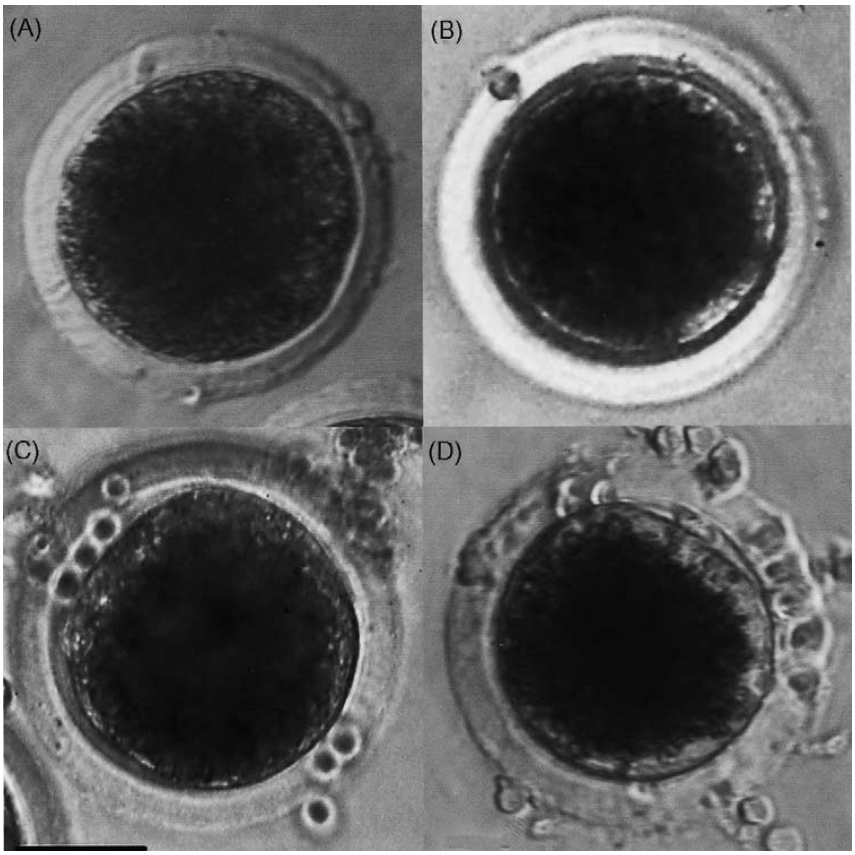

Figure 1. Digital micrographs (200 x magnification) of (A) an immature sow oocyte from a 2 to 6 mm follicle. (B) An MII oocyte matured in vitro. (C) An MII sow oocyte matured in vitro in the presence of porcine oviductal epithelial cell co-culture. (D) An in vivo MII sow oocyte recovered from preovulatory follicle approximately 30-35 h after an artificially induced LH-peak. Bar=50 µm.

Experiment 2

In vitro maturation of the oocytes from adult slaughterhouse sows, irrespective of the treatment, resulted in more than 97% MII oocytes by 42-44 h of IVM (data not shown). The effect of pOEC co-culture on in vitro fertilization is presented in Table 2. A distinction was made between the total penetration rate (TPEN) and the penetration rate (PEN). The TPEN results displayed in Table 2 indicate that significantly more oocytes had been penetrated by spermatozoa in the Control, than in the pOEC group. Of the sperm in the vitellus of the pOEC oocytes a greater proportion had undergone decondensation (CI: 0.44 versus 0.67; for Control and pOEC, respectively) during this time period (18 h) signifying a greater degree of cytoplasmic support of fertilization in this group. No differences were found in the rate of polyspermy nor the percentage of male pronucleus formation.

Table 1. Morphological characteristics of sow oocytes before (immature oocytes) and after IVM in the absence (IVM-II-control) or presence of porcine oviductal epithelial cells (IVM-II-pOEC), compared to in vivo matured MII oocytes

Treatment	Oocytes measured (n)	Oocyte diameter (μm ± SEM)	Oocyte diameter without ZP (μm ± SEM)	Thickness of ZP (μm ± SEM)	Size of perivitelline space (μm ± SEM)
Immature	23	146.85 ± 0.92[a]	113.14 ± 1.2	14.06 ± 0.28	2.80 ± 0.22[c]
IVM-II-control[1]	48	149.45 ± 0.60[b]	112.66 ± 0.65	14.15 ± 0.20	4.25 ± 0.21[d]
IVM-II-pOEC	35	150.43 ± 0.64[b]	114.10 ± 0.71	14.31 ± 0.37	4.40 ± 0.30[d]
In vivo MII	49	146.96 ± 0.50[a]	112.14 ± 0.61	14.82 ± 0.19	2.60 ± 0.12[c]

Different superscripts (a, b) within the same column, P<0.01; different superscripts (c, d) within the same column, P<0.001. [1]IVM-II-control: no pOEC co-culture during IVM.

Experiment 3

Porcine OEC co-culture did not have any significant effect on the progression of early embryo development up to Day 5 blastocyst development (Table 3). The percentage of zygotes that had cleaved by Day 2 of in vitro culture (34 and 29% for the Control and pOEC groups, respectively) appeared to be equivalent to the TPEN rate (Table 2; 36% for the Control, and 29% for the pOEC). In the pOEC group, the percentage normal fertilization was also equal to the percentage of zygotes that had progressed to the 4-cell stage by Day 2 after IVF (Table 3). These embryos could be considered to be synchronous and thus at a similar stage of development with their in vivo counterparts [27,28].

As depicted in Table 1, the diameter of the in vitro matured oocytes was significantly greater (P<0.01) than that of immature oocytes from 2 to 6 mm follicles and the in vivo matured MII oocytes. Neither the oocyte diameter without ZP, nor the thickness of the ZP differed between any of the groups. In vitro maturation, with or without pOEC, resulted in a significant increase in the size of the perivitelline space (P<0.01 for in vivo MII versus IVM-II-control and IVM-II-pOEC; P<0.001 for pre-IVM versus IVM-II-control and IVM-II-pOEC). Co-culture of the oocytes with pOEC did not result in any significantly different morphological observations.

Table 2. The effect of pOEC co-culture, during IVM, on fertilization parameters 18 h after IVF of sow oocytes[1]

Treatment	Oocytes (n)	Total penetration % (TPEN)	Penetration % (PEN)	Cytoplasmic Index**	Polyspermy % (of TPEN)	MPN % (of PEN)	Normal fertilization % (of Total)
IVMII-control[1]	193	36[a]	17	0.44	4	82	15
IVMII-pOEC	174	29[b]	21	0.68	8	81	18

Different superscripts (a, b) within the same column, P<0.01. [1]IVM-II-control: no pOEC co-culture during IVM. Cytoplasmic index is defined as the ratio of the percentage PEN to the percentage TPEN.

Furthermore, similarities could be found between the percentage normal fertilization (15% versus 18% for Control and pOEC groups, respectively) and the blastocyst development on Day 5 (15% for both of the groups). These blastocysts appeared to be developing at the same rate as their in vivo complement [27,28]. On Day 6, significantly more embryos were at the blastocyst/expanded blastocyst stage in the Control group than in the pOEC group (24% versus 19%; $P<0.01$). Similarly, on Day 7 more Control embryos had developed to blastulation compared with those in the pOEC group (27% versus 19%; $P< 0.01$).

In addition, of the pOEC co-cultured blastocysts, 10% had hatched or were hatching, compared to 3% in the Control group ($P<0.05$), indicating that the rate of embryo growth was also accelerated by the pOEC co-culture. In accordance with the higher hatching rate for pOEC co-cultured blastocysts, these blastocysts on average contained significantly more nuclei than the Control blastocysts (47 ± 2 versus 40 ± 2; $P<0.05$). Figure 3 demonstrates the distribution of blastocysts according to the number of nuclei contained: blastocyst cell numbers in the Control group were spread symmetrically over the median providing equal-sized intervals to contain the middle quartiles of the cell number data, whereas the distribution of the pOEC co-cultured blastocyst cell numbers were skewed to the right indicating the larger percentage (of the total number of blastocyst in this group) of pOEC co-cultured blastocysts with greater cell numbers. These data therefore indicate that the ratio of blastocysts containing a superior number of nuclei (of the total number of blastocysts in the group), was greater in the pOEC group than in the Control group.

Discussion

In the first experiment of this study we compared morphological characteristics of sow oocytes matured in vitro, in the presence or absence of pOEC, and in vivo. Metaphase II oocytes collected from preovulatory follicles, after an artificially induced LH peak, were compared to MII in vitro matured oocytes and considered to represent their `in vivo complement.' Clear spaces were observed in the cortex of the ooplasm in both the in vivo and in vitro matured oocytes, but not in the immature oocytes which displayed a dense evenly granulated ooplasm. Pubertal gilt oocytes recovered from the oviduct after ovulation have clear areas in the cortex cytoplasm, in contrast with a dense evenly granulated ooplasm evident in in vitro matured oocytes from prepubertal gilts [7]. These differences in the distribution of the ooplasm content may be due to modifications in the cytoskeleton of the oocyte during the final maturation phases in vivo, resulting in an altered organization of organelles within the ooplasm. Nagashima et al. [29] found that oocytes with unevenly granulated ooplasm, after IVM, had greater developmental competence. As the appearance of the pOEC-matured ooplasm did not differ from that of the IVM-Control, and was largely similar to the in vivo matured oocytes, it seems probable that the changes in the distribution of organelles take place in the pre-ovulatory follicle during maturation, or during in vitro maturation. These changes in the aforementioned oocyte characteristics may therefore not be due to the effect of oviductal contact as previously suggested [7], but may indeed be owed to incomplete differentiation of prepubertal oocytes as suggested by Marchal et al. [4].

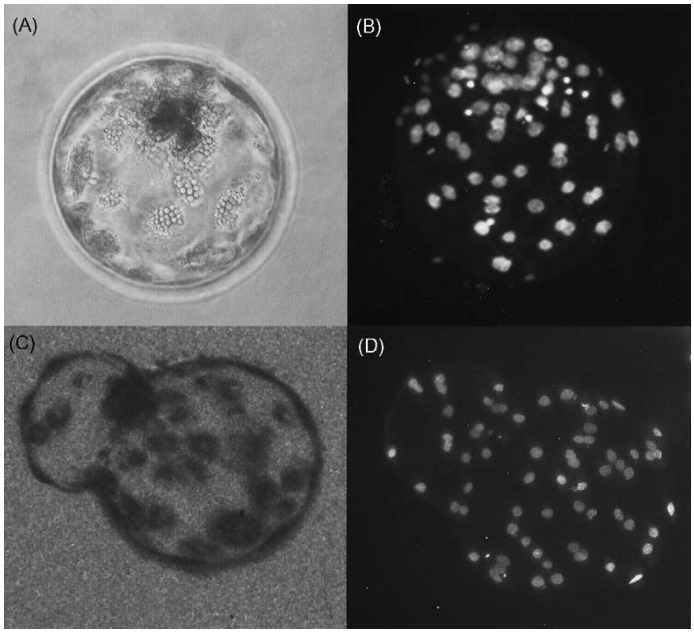

Figure 2. Digital micrographs (200 x magnification) of in vitro produced Day 7 blastocysts derived from in vitro matured sow oocytes fertilized and cultured in vitro. (A) An expanded Control blastocyst. (B) An expanded Control blastocyst stained with DAPI for cell number determination. (C) A hatching pOEC-blastocyst. (D) A hatching pOEC blastocyst stained with DAPI.

Table 3 The effect of pOEC co-culture, during the second half of IVM, on porcine embryo development from Day 2 to Day 7 following IVF

		Cleavage Rate on Day 2 (%)				Blastocyst Rate (%)			
	n	2-cell	4-cell	8-cell	Total	Day 5	Day 6	Day 7	Day 7 Hatching (% D 7 Blastocysts)
Control*	358	6	22	6	34	15	24[a]	27[a]	3[a]
POEC	391	5	18	5	29	15	19[b]	19[b]	10[b]

Different superscripts (a, b) within the same column, P < 0:01. *Control: no pOEC co-culture during IVM.

The perivitelline space of the in vitro matured oocytes was wider than that of the in vivo matured oocytes, and probably accounts for the larger diameter of the in vitro matured oocytes. An increase in the size of the perivitelline space is usually associated with oocyte activation [7]. In mouse, hamster and human oocytes it has been shown [30,31] that a 'partial cortical reaction' takes place in oviductal oocytes prior to fertilization; this might play an important role in the conditioning of the ZP prior to the ZP reaction. These oocytes also have a fully developed perivitelline space. Although prefertilization cortical granule release has not yet been identified in pig oocytes, oviductal oocytes have previously been shown to have a wider perivitelline space than in vitro matured

prepubertal gilt oocytes [7].

Although higher rates of blastocyst formation are found when using sow oocytes, sperm penetration rates are lower compared with oocytes from prepubertal gilts [4,6]. This serves as indication of possible differences in the requirements for both oocyte maturation and IVF conditions for sow as opposed to prepubertal gilt oocytes. In this study we did not find any increase in penetration rates for the pOEC oocytes and the explanation might be found in the absence of the cumulus cells during IVF. Extended physical contact between the oviductal cells and the COC [8] are necessary for achieving higher penetration rates, and it is probable that the effect of the pOEC is lost due to the commonly used procedure of oocyte-denudation before IVF. In vivo, after ovulation, the cumulus surrounding the oocyte is largely intact and groups of ovulated oocytes clump together in a mass of mucified expanded cumulus [32]. It is in this state that spermatozoa encounter the COCs. In the cow and pig, denudation of the oocyte prior to IVF often leads to reduced penetration rates [33,34]. While this practice may be of benefit in prepubertal gilt IVF, where polyspermy is common, it might very well have the opposite effect in sow IVF.

Oviductal proteins are known to be associated with the zona pellucida, perivitelline space and vitelline and blastomere membranes of ovulated oocytes and oviductal embryos [35-37], suggesting a role during fertilization and early embryonic development. The exact function and effect of these proteins, and other oviductal compounds, on oocyte morphology and function have not yet been fully elucidated, but the results presented in Experiment 2 demonstrate an effect of pOEC on the progression of fertilization. In this study, both the total penetration rate (TPEN) and penetration rate (PEN) were taken into account to obtain a clearer picture of the oocyte penetration and the ability of the ooplasm to support sperm decondensation at the designated time point of 18 h after the start of IVF. The cytoplasmic index was hypothesized to provide information on the 'normal' progression of fertilization and served as an indicator of the cytoplasmic proficiency of the oocyte and hence the quality of the resultant zygotes. The CI for the Control group was lower than that of the pOEC group, indicating delay in the decondensation of the sperm found in the vitellus and thus suboptimal functioning of the ooplasm. Factors found in oviductal fluid are known to modulate protein synthesis in zygotes and embryos [36], and pOEC-produced secretions during in vitro maturation may therefore have contributed to the enhanced functioning of the cytoplasm of pOEC-matured oocytes. The parameter generally used to signify the cytoplasmic maturity of the oocyte (percentage pronucleus formation) was not different for the two groups, and provided no correlation with oocyte or embryo quality.

The fact that blastocyst development seemed to have plateaued in the pOEC-treated group and was lower than that of the Control group was in contrast to our hypothesis that pOEC treatment would increase blastocyst development, and also contrary to the results obtained by Bureau et al. [2] who found that pOEC co-culture of oocytes enhanced the subsequent percentage of oocytes developing to blastocysts. This difference appeared even more pronounced by Day 7, by which time 27% of the Control and 19% of the pOEC oocytes placed into maturation had developed to blastocysts. This apparent suppression of blastocyst growth in the pOEC group may be explained by the in vivo embryo development condition, where faster growing blastocysts secrete estrogen which inhibits the growth of late developing embryos [38,39]. Keeping in mind that the Day 7

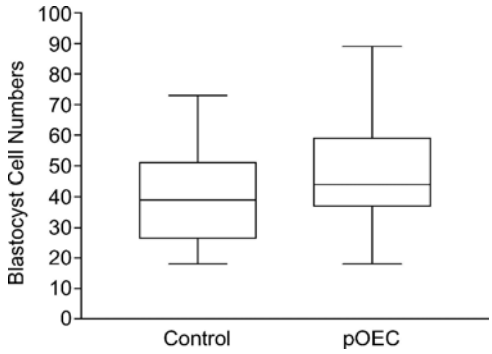

Figure 3. The effect of pOEC during the second half of IVM on the distribution of the number of nuclei contained in subsequent blastocysts following in vitro culture until Day 7 of in vitro fertilized sow oocytes.

pOEC co-cultured blastocyst rate was equal to the penetration rate (PEN), this evidence appears to corroborate the view that pOEC treatment enhances the ability of the oocyte to support and facilitate normal fertilization with the resultant development of superior quality blastocysts. Taking into account the percentage of blastocysts that had hatched, or were hatching, in the pOEC group, the progression of blastocyst growth, especially, seemed to have been accelerated in the pOEC group. This was confirmed by the average number of nuclei per blastocyst which was significantly higher for the pOEC group than for the Control group, as well as the larger proportion (of the total number) of blastocysts in pOEC group containing more nuclei. This indicated that the pOEC blastocysts were of superior quality to the Control blastocysts as forecast by the cytoplasmic index. Hatching of blastocysts is facilitated by events related to protein synthesis earlier in embryo development [15]. The pOEC treatment may thus modulate events in the ooplasm enabling the oocyte to elicit the eventual hatching of the blastocyst. The cytoplasmic index forecast the greater potential of oocytes in the pOEC group and it thus appears that the CI is more predictive of oocyte, and subsequent blastocyst quality, than the percentage pronucleus formation or normal fertilization.

In all, these results indicate that the presence of pOEC during IVM enhances the quality of cytoplasmic maturation of the oocyte, and subsequent blastocyst cell proliferation or tempo of growth. Although the percentage oocytes developing to blastocysts in the pOEC group was reduced, the blastocysts forming in this group were of superior quality as judged by their greater number of nuclei. Addition of pOEC during the second half of maturation also enhances the inherent hatchability of the resultant blastocysts within this serum-free environment. The secretions of the pOEC might provide beneficial factors such as energy substrates, growth factors [36,37] or scavenger ions and other macromolecules involved in the functioning of the ooplasm, but the precise role of pOEC in in vitro oocyte maturation and subsequent embryo development is unclear and remains intriguing.

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CHAPTER 6

Quality of porcine blastocysts produced in vitro in the presence or absence of Growth Hormone

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Abstract

GH receptor (GHR) mRNA is expressed in bovine in vitro produced embryos up to the blastocyst stage and GH improves the quality of bovine embryos by increasing blastocyst cell numbers and reducing the incidence of apoptosis as evaluated by DNA strand-break labelling. Porcine in vitro produced blastocysts have lower cell numbers than in vivo blastocysts and exhibit higher incidences of apoptosis. Therefore we investigated the effects of 100 ng GH/ml NCSU23 medium during in vitro culture of presumptive in vitro fertilized sow zygotes on embryo development and blastocyst quality (defined by diameter, cell number, apoptosis and survival after non-surgical transfer). In vivo produced blastocysts were analysed concurrently as a reference value. GHR was expressed in embryos from the 2-cell to blastocyst stages. GH had no effect on blastocyst development or cell numbers, but increased the mean blastocyst diameter. The incidence of apoptosis, detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL), was decreased by GH, but when non-TUNEL-labelled apoptotic fragmented nuclei were included, no difference was seen. GH appeared to slow down the progression of apoptosis though. In vivo produced blastocysts presented no apoptotic nuclei, and contained higher cell numbers and larger diameters. Pregnancy rates on day 11 were similar for all groups, but survival was poorer for in vitro than in vivo produced blastocysts. In this study GH appeared to be beneficial only from the blastocyst stage, but the presence of GHR from early cleavage stages nevertheless indicates a role for GH throughout porcine embryo development and deserves further investigation.

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Introduction

Growth hormone (GH) is a polypeptide hormone, mainly produced by the adenohypophysis [1, 2]. Its regulatory effect on cell proliferation [2, 3] follicular growth [4, 5] and metabolism [6] is widely recognized. The exact role of GH during embryogenesis is unknown, but GH receptor (GHR) mRNA has been identified in the mouse, bovine and human from the fertilized oocyte up to the blastocyst stage [7-9]. This suggests a functional role of GH in preimplantation embryo development. Most recently the positive influence of GH on oocyte maturation in the bovine [10] and embryo development in both the mouse and bovine [10-13] has been identified. Addition of GH to in vitro culture (IVC) media stimulates growth and proliferation of in vitro produced bovine and mouse embryos by exerting distinct effects on the metabolic processes of the embryo [12, 14] GH also increases blastocyst yield and cell numbers in the mouse and bovine [8, 12] and enhances post-thaw survival of bovine blastocysts cultured with GH [13] Furthermore, culture of bovine embryos in the presence of GH greatly reduces the incidence of double-strand DNA breaks in resultant blastocysts [15]. These promoting effects of GH on the developmental competence of preimplantation embryos in the mouse and bovine indicate a rudimentary role of GH during the early stages of in vitro embryo development, which could also be pertinent to embryo development in other species, such as the pig.

Many porcine embryo in vitro production laboratories are now as standard obtaining averages of 30% or more blastocysts from in vitro matured oocytes [16-18], but despite these promising yields the quality of the resultant blastocysts remains questionable. Embryo quality is difficult to define, but chronologically and morphologically normal cleavage of the embryo is regarded as significant indicators of in vitro produced blastocyst quality and viability [19]. The use of gross morphological criteria is subjective and not devoid of error due to possible bias of the evaluator [20, 21], and moreover, in the porcine no guidelines have been set by which in vitro produced blastocysts can be evaluated. Current parameters by which embryo quality can be measured include the nuclear and chromosomal status of the blastomeres contained in an embryo, indicative of apoptosis or programmed cell death. Late-stage apoptosis markers, such as nuclear condensation and fragmentation, are evaluated by methods such as 4,6-diamino-2-phenylindole (DAPI) staining for nuclear morphology, whereas the final stages of apoptosis are assessed by terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling (TUNEL), which enables in situ biochemical detection of single-and double-strand DNA breaks. Apoptosis is regarded as a side effect of in vitro embryo culture in the bovine [15] but also in the pig [22, 23]; in the latter little or no apoptotic DNA fragmentation is found in blastocysts produced in vivo. The degree of apoptosis in blastocysts, prevalent in any given in vitro embryo production system, can therefore be used as a tool for indicating the effectiveness and suitability of the system for the needs and developmental potential of the embryos involved. Ultimately, the definitive test of embryo viability remains the survival of embryos after transfer. Up to now the transfer of in vitro produced pig blastocysts has mainly been performed surgically [24-26], but survival rates remain unsatisfactory. Pregnancy rates, comparable with those of surgical embryo transfer have been achieved after non-surgical transfer of in vivo produced blastocysts [27-29], but to the best of our knowledge non-surgical transfer of in vitro produced blastocysts has not been reported. Furthermore, data to correlate embryo morphological selection criteria and post-transfer survival rates are not available to date,

and still need to be established in the actively developing field of in vitro pig embryo production.

Improvements in porcine in vitro embryo production regimens to enhance blastocyst viability, as well as the estimation thereof, are an all-important concern. Therefore, we investigated the effect of GH on in vitro preimplantation embryo development in comparison with in vivo produced embryos. The quality of the embryos was assessed by determining the diameter, blastocoel volume and cell number, the type of apoptosis, and survival after non-surgical transfer. In addition, the expression of mRNA for GHR was determined in early-stage embryos as an indication of the presence of the necessary molecular machinery.

Materials and Methods

Experimental design

RT-PCR was used firstly to ascertain the presence of the necessary molecular apparatus for potential utilization of GH, i.e. GHR mRNA. For this, embryos were produced in vitro and those at the 2-, 4-and 8-cell stages and Day 6 blastocysts were analysed. Upon confirmation of the presence of GHR, the effect of GH on in vitro embryo development was assessed. A total of 1263 (Control) and 1347 (GH) oocytes divided over seven replicates were evaluated for stage of development at 48 h (Day 2), 5 days (Day 5) and 6 days (Day 6) after the addition of sperm for in vitro fertilization (IVF). Of the seven in vitro embryo production replicates, three replicates were destined for blastocyst quality evaluation. These three replicates consisted of 268 oocytes in the Control and 278 oocytes in the GH group. From these oocytes 91 Control and 89 GH blastocysts were produced, of which 68 Control and 83 GH displayed the morphological characteristics required for non-surgical embryo transfer, and were analysed for quality parameters. In vivo derived blastocysts (n 54) were randomly selected for embryo quality analyses from pooled blastocysts (n 144) recovered from 26 donors. The remainder of the in vitro produced blastocysts were used for other studies.

The remaining four of the seven in vitro replicates were destined solely for non-surgical embryo transfer purposes. A total of 995 Control and 1069 GH oocytes subsequently led to 313 Control and 357 GH blastocysts. Of these blastocysts, a total of 200 per treatment group were transferred to 16 recipients (n 8 Control and n 8 GH). In addition, 80 extra embryos per group (20 per transfer replicate) were also transported to the embryo transfer site as a backup for eventual embryo losses during removal from the transport Eppendorf tubes. Of the remaining non-transferred blastocysts, 27 Control and 20 GH were set aside for ethidium homodimer (EthD-I) staining. For the transfers of in vivo derived blastocysts a total of 953 embryos (morula to expanded blastocyst stages) were recovered from 57 donors. Of these, 575 early to expanded blastocysts were allocated for transfer to 23 recipients. The number of blastocysts surviving after transfer was evaluated on Day 11 of the pregnancy, i.e. 6 days after transfer.

Animals, donors and recipients

All animal experiments were approved by the Ethical Committee for animal experiments of Wageningen University. Synchronization and embryo recovery was performed according to Hazeleger et al. [30]. Briefly, a total of 85 crossbred gilts, 7–11 months old (TOPIGS; Vught, The Netherlands), were used as donors and 39 multiparous crossbred sows (TOPIGS) as recipients (n=8 for Control; n=8 for GH; n=23 for In vivo). Ovulation was synchronized using PG600 (Intervet International BV, Boxmeer, The Netherlands) for first oestrus induction and 2 x 500 µg Estrumate (Schering-Plough, Maarssen, The Netherlands) 8 h apart around Day 13 of the first luteal phase. The next day follicular development was induced with 700 IU equine chorionic gonadotrophin (eCG) (Folligonan; Intervet International), followed by 600 IU human chorionic gonadotrophin (hCG) (Chorulon; Intervet International) after 72 h to induce ovulation. The gilts were artificially inseminated 24 and 36 h after hCG administration. Donors were killed 7 days (168 h) after hCG (120 h after estimated ovulation) for collection of Day 5 embryos. Immediately after stunning, bleeding and removal of the genital tract, the embryos were flushed from the uterus horns using Dulbecco's PBS (DPBS) (BioWhittaker, Verviers, Belgium) supplemented with 1% heat-inactivated lamb serum (GIBCO, Paisley, Strathclyde, UK) and 1% PenStrep (penicillin-G 100 IU/ml and streptomycin sulphate 100 mg/ml; BioWhittaker) at 37°C. The flushing medium was then filtered, and the embryos retrieved by rinsing the filter with Dulbecco's PBS (DPBS). An average of 16.3 ± 4.5 (mean ± S.D.) blastocysts was recovered per donor (19.5 ± 4.0 corpora lutea). The embryos were then directly transported to the laboratory in a temperature-controlled insulated container kept at 25°C. All non-fertilized oocytes and degenerated zygotes, as well as morulae and hatching or hatched blastocysts were discarded upon stereomicroscopic evaluation. The remaining unexpanded and expanded blastocysts were prepared for non-surgical transfer.

IVC media

For oocyte and embryo searching and selection, 25 mM Hepes-buffered Tyrode's medium containing 0.1% polyvinyl alcohol (TL-Hepes-PVA) was used. The in vitro maturation (IVM) medium used for the first 22–24 h of oocyte IVM (IVM-I) was BSA-free North Carolina State University 23 medium (NCSU23) supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.8 mM cysteine, 25 mM β-mercaptoethanol, 10 IU/ml eCG and hCG (Folligonan and Chorulon). The second IVM (IVM-II) culture period (18–20 h) occurred without eCG and hCG added to the medium. pFF was collected from follicles 2 to 6 mm in diameter, centrifuged at 1900 *g* for 30 min (4°C), filtered through 0.8 mm syringe filter (Millipore SA, Molsheim, France) and stored at –20°C until use. Cumulus-oocyte complexes (COCs) were washed in IVM-wash medium (IVM-II medium with 20 mM Hepes). Modified Tris–buffered medium (mTBM) was used as fertilization medium. This medium consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate. Caffeine (1 mM) and 0.1% BSA (w/v) (A-6003; Sigma, St Louis, MO, USA) were supplemented to mTBM for use as IVF medium. The in vitro embryo culture medium was NCSU23 containing 0.4% BSA (w/v) with 100 ng/ml bovine GH (NIH-B18; National Institute of Diabetes, Digestive and Kidney Diseases National Hormone and Pituitary Programme, NIH, Bethesda, MD, USA). Culture without GH served as a control. Before being placed in the IVC medium, presumptive zygotes were washed in IVC-wash medium, which is similar to the IVC medium, but containing

20 mM Hepes. All culture took place under oil (Heavy Mineral Oil for IVF; Reproline GmbH, Rheinbach, Germany).

Recovery of oocytes and IVM

Oocyte recovery, IVM, IVF and embryo culture proceeded as previously described [31]. Briefly, ovaries were collected from sows of unknown reproductive status at a local slaughterhouse and transported to the laboratory in insulated containers. Excess connective tissue and oviducts were cut from the ovaries, and they were then washed once at 30°C under running tap water. The ovaries were then placed in a beaker of pre-warmed sterile saline, supplemented with penicillin and streptomycin, and held at 30°C until aspiration. COCs were aspirated from 2–6 mm follicles with an 18-gauge needle fixed to a vacuum pump via a 50 ml conical tube. Contents were collected into the tube and allowed to settle for 10 min at room temperature. The supernatant was removed and sediment was resuspended in TL-Hepes-PVA at room temperature and allowed to settle. This treatment was repeated once more and the content of the tube was observed under a stereomicroscope on a heated stage (38.5°C). COCs surrounded by two or more layers of compact cumulus investment and containing oocytes of equal size were selected, washed twice in IVM-wash medium which had been pre-warmed to 38.5°C prior to use, and transferred in groups of 40–50 to a four-well dish containing 500 µl equilibrated IVM-I medium under oil (Heavy Mineral Oil for IVF) in each well. The four-well dish was then incubated for 24 h at 38.5°C in 5%CO_2 in humidified air. After 22–24 h all the oocytes were washed twice in IVM-wash medium and placed in 500 µl IVM-II medium for an additional 16–18 h of culture.

IVF and embryo culture

After maturation the oocytes were placed in a wash dish containing pre-warmed equilibrated IVF medium. Using a micropipette, the contents of the dish were vigorously pipetted for 30 s to remove the expanded cumulus cells. The denuded oocytes were washed once more in IVF medium before being placed in 50 µl droplets of equilibrated IVF medium, in groups of 40–50, and incubated at 38.5°C in 5%CO_2 for 30 min until the addition of the sperm.

Fresh room temperature Beltsville thawing solution-extended [32] semen was diluted 1:2 in IVF medium (also at room temperature). Sperm were centrifuged in a conical tube for 4 min at 700 g. The supernatant was removed and sperm resuspended in IVF medium and centrifuged again. Following resuspension the sperm concentration was determined and adjusted to achieve a final concentration ±1000 motile cells/oocyte. After warming to 38.5 °C for 30 min, 50 µl of the sperm suspension were added to the 50 µl IVF droplets containing the oocytes and co-incubated with the oocytes for 20–24 h at 38.5°C at 5% CO_2 in air. Twenty-four hours after insemination, the oocytes were removed from the IVF droplets and washed twice in IVC-wash medium. They were then gently pipetted to remove excess sperm attached to the zona pellucida and transferred in groups of 40–50 into 500 µl IVC medium under oil in a four-well dish. At 48 h after the addition of sperm for IVF, cleavage rate was determined (structures judged to be degenerated or uncleaved were not removed), and on Days 5 and 6 of embryo culture, blastocyst formation was evaluated.

Determination of cleavage, blastocyst size, volume and fixation

To determine the efficiency of the IVC systems, embryos were scored morphologically on Days 2, 5 and 6 after the addition of sperm for IVF (Day 0). On Day 2 the percentage of cleaved embryos displaying two to eight evenly sized blastomeres was recorded; embryos with fragmented or uneven-sized blastomeres were categorized as degenerated. The percentage blastocysts, expressed on the basis of the number of oocytes placed into maturation, was evaluated on Day 5 and Day 6. At the termination of the culture period on Day 6, all blastocysts displaying a clear inner cell mass were selected for further evaluation. Both in vivo and in vitro derived blastocysts were measured for diameter using a graduated eyepiece, fixed individually in 2% paraformaldehyde and stored at 4°C until further processing. Day 5 in vivo produced embryos were regarded to be the equivalent of Day 6 in vitro blastocysts due to the retardation of growth experienced in in vitro produced embryos [33, 34]. Blastocysts were categorized as more developed large expanded blastocysts (Large) when no perivitelline space was visible which corresponded to a diameter of 180 μm. Blastocysts of lesser development and diameter were categorized as small blastocysts (Small). Blastocyst volume was determined using the formula: $4/3 \times \pi \times (\text{diameter}/2)^3$.

TUNEL and cell counting

Biochemical detection of DNA strand breaks was performed on each individual blastocyst by using TUNEL (fluorescein isothiocyanate (FITC)-conjugated dUTP and TdT; Roche, Mannheim, Germany) according to the manufacturer's instructions, with 0.05 μg/ml DAPI (Sigma) as a counterstain. After 3 or more days of fixation the zona pellucida becomes elastic, subsequently allowing complete flattening under the coverslip upon mounting. Fixed blastocysts were washed twice in TL-Hepes-PVA and then permeabilized for 15 min on ice in 0.1% Triton X-100 (0.1% sodium citrate in PBS). Following two more rinses in TL-Hepes-PVA, blastocysts were incubated in microdrops (25 μl per 1–12 embryos) of the TUNEL reaction mixture, for 1 h under oil in a humidified atmosphere in the dark. After TUNEL culture the embryos were washed twice in TL-Hepes-PVA and then stained with DAPI (5 μl/ml) for 5 min at room temperature in the dark. The embryos were then mounted in a minimal amount of DAPI fluid, flattened completely by applying firm pressure to the cover slip, and examined (x 200 magnification) using a fluorescence microscope (BH2-RFCA; Olympus, Tokyo, Japan) to assess the total number of nuclei and the proportion showing DNA fragmentation. Overlap of nuclei was negligible in all groups. Nuclear morphology (i.e. fragmentation) was assessed whilst viewing through the microscope, but for determining cell numbers two digital images (Nikon Coolpix 990; Nikon Corporation, Tokyo, Japan) of each blastocyst were recorded: (i) using the UV filter for the DAPI image, and (ii) using the FITC filter for the TUNEL image. The number of nuclei was counted after printing of the images.

Five embryos from each treatment group were treated with DNAse before TUNEL staining as a positive control for TUNEL labelling; for a negative control the terminal transferase enzyme was omitted during TUNEL labelling. To rule out necrosis, a number of embryos from each group (in vitro Control n=27; in vitro GH n=20) were stained with 4 mM EthD-I (Molecular Probes Europe BV, Leiden, The Netherlands) before TUNEL and DAPI staining. In the case of the in vivo derived blastocysts all were triple stained with EthD-I, TUNEL and DAPI.

Nuclear morphology assessment for apoptosis

Nuclei were classified according to three nuclear morphologies: (i) 'healthy' interphase nuclei with uniform DAPI staining and a clear outline but without TUNEL staining, also including mitotic nuclei; (ii) fragmented nuclei with no TUNEL labelling (FT-); (iii) TUNEL-labelled nuclei which were condensed (T+) and/or fragmented (FT+). Embryos containing only fragmented non-TUNEL stained nuclei, and no other apoptotic morphologies, were designated under the 'fragmented' classification. The total nuclei count consisted of all nuclei, whether they displayed apoptosis or not. TUNEL, fragmented and total apoptotic indices were calculated for each embryo as follows: TUNEL index = (no. of TUNEL-positive nuclei, either fragmented or condensed)/(total no. of nuclei) x 100; fragmented index = (no. of TUNEL-negative fragmented nuclei)/ (total no. of nuclei) x 100; total apoptotic index = (TUNEL-positive nuclei, either fragmented or condensed)+(no. of TUNEL-negative fragmented nuclei)/ (total no. of nuclei) x 100.

Non-surgical embryo transfer

Transfer of in vivo and in vitro blastocysts took place as previously described [24], except that in addition to expanded blastocysts, non-expanded blastocysts were transferred as well. Recipients were prepared similarly and synchronously to the donor animals described above. On the day of transfer in vitro produced blastocysts displaying a clear inner cell mass, and with less than 25% of the blastocyst volume containing extruded cells, were placed in Eppendorf tubes, containing transfer medium (D-PBS with 10% lamb serum) at 38.5°C, in batches of 25. They were then allowed to cool to 25°C for transport to the transfer location. In vivo derived embryos were collected as previously described (see Animals), and transported to the transfer station similarly to the in vitro produced embryos. Upon arrival at the transfer station (±2 h after initial collection) the embryos were removed from the Eppendorf tubes, placed in a small Petri dish and photographed using a Polaroid camera (Polaroid Microcam; Polaroid Europe BV, Enschede, The Netherlands) to document the blastocyst diameters prior to transfer. Each batch of 25 embryos was then aspirated into the tip of the transfer catheter (Swinlet; Institute for Pig Genetics, Beuningen, The Netherlands). The transfer procedure consisted of careful passage of the instrument through the cervical folds into the uterine body. The catheter, containing the embryos, was then passed through the instrument and the embryos were deposited in the uterine body with, 0.1 ml transfer medium. Recipients were not sedated during the transfer procedure.

On Day 11 after ovulation (6 days after transfer) the recipients were killed to evaluate the survival of transferred embryos. Blastocyst numbers were recorded following recovery from the uterus. Recipients were regarded as having been pregnant following the recovery of one or more blastocysts from the uterus.

RT-PCR

RT-PCR was used to assess the presence of the GHR mRNA in early in vitro produced embryos at the 2-, 4 and 8-cell stage, and the blastocyst stage on Day 6 of embryo culture. The embryos were washed four times in TL-Hepes-PVA, placed in Eppendorf tubes in groups of ten and frozen at –80°C until use. For each developmental stage, 30 embryos, divided over three replicates, were analysed.

Poly(A)$^+$ RNA was isolated, following the manufacturer's instructions, from groups of ten 2-, 4- and 8-cell embryos and Day 6 blastocysts using a Dynabeads® mRNA Direct™ Micro kit (DYNAL, A.S., Oslo). Briefly, 100 µl of Lysis/binding buffer (100 mM Tris-HCl, pH = 8.0, 500 mM LiCl, 10 mM EDTA, 1% (w/v) Lithium dodecysulfate (LiDS), 5 mM dithithreitol) was added to the frozen samples and pipetting was repeated to obtain complete lysis. Twenty µl prewashed Dynabeads® Oligo $(dT)_{25}$ was added to each tube and mixed thoroughly. After 5 min incubation at room temperature the beads were separated using Dynal MPC-E-1 magnetic separator. The beads were washed in 100 µl washing buffer (10 mM Tris-HCl, pH=8.0, 0.15 M LiCl 1 mM EDTA, 0.1% (w/v) LiDS) and two times in 100 µl washing buffer 2 (10 mM Tris-HCl, pH=8.0, 0.15 M LiCl, 1 mM EDTA). The mRNA was then eluted from the beads by incubating in 20 µl RNAse free water at 65°C for 2 min. Reverse transcription was done in a total volume of 20 µl containing 10 µl of the sample RNA, 4 µl 5X reverse transcriptase buffer (GIBCO BRL, Breda, The Netherlands), 8 units RNAsin (Promega), 150 units Superscript II reverse transcriptase (GIBCO BRL, Breda, The Netherlands), 0.036 U random primers (Life Technologies BV, Leiden, The Netherlands) and final concentrations of 10 mM DTT and 0.5 mM of each dNTP. The mixture was incubated for 1 hour at 42^0C, for 5 min at 80^0C, and stored at -20^0C. Minus RT blanks were prepared under the same conditions but without reverse transcriptase.

Oligonucleotide primers used for amplification of the GH receptor (GHR) were based on the porcine GHR cDNA sequence as described in the Genbank database by (Genbank accession number X54429, 1995). Amplification of the cDNA was performed in two stages with GHrL1 (5'-TGTCAGAGCATCTCAGAGTC-3', sense, position 105-124) and GHrR1 (5'- GTCTCTAGTTCAGGTGAACG-3', antisense, position 187-206). The second round of PCR was performed to increase the recovery of the final product. Reactions were carried out in 200 µl tubes (Eurogentec, Seraing, Belgium) using 1 µl cDNA as template in 25 µl PCR mixture containing final concentrations of 2 mM $MgCl_2$, 200 µM of each dNTP, and 0.5 µM each of primers and 0.625 units Taq DNA polymerase (HotStarTaq, Qiagen, Valencia, USA) in 1xPCR buffer. The thermal cycling profile for the first round was: initial denaturation and activation of the polymerase for 15 minutes at 94^0C, followed by 40 cycles of 15 sec at 94^0C, 30 sec at 55^0C and 45 sec at 72^0C. Final extension was for 7 min at 72^0C. For the second round of amplification 1 µl of the first round product was transferred to another 200 µl tube containing 24 µl PCR buffer as above, and amplified for 30 cycles according to the same profile. All PCRs were performed in a 24-well thermocycler (Perkin-Elmer, Gouda, The Netherlands). Ten µl of the second round product was resolved by 1% Agarose gel containing 0.4µg/ml ethidium bromide. A 100 basepair (bp) ladder (GIBCO BRL, Breda, The Netherlands) was included as a reference for fragment size. An image of the gel was taken using a digital camera (Olympus C-4040, New York, USA) and stored in digital form. A standard sequencing procedure (ABI PRISM 310 Genetic analyser, Applied Biosystems) was used to verify the analytical specificity of the PCR product, and compared with the Genbank database as described by [35].

Statistical analyses

All experiments consisted of a minimum of three replicates. Statistical analysis of embryo development data was carried out using Fisher's exact test for the cleavage and blastocyst-formation rates, as well as for the incidence of apoptosis. After testing for normality (Kolmogorov–Smirnov test with Lilliefor correction) and testing for equal variances (F-test for two groups and Bartlett's test for multiple groups) the means of blastocyst diameters, nuclei count and nuclear damage were compared by unpaired t-test, or ANOVA (followed by Bonferroni multiple pair wise comparison) where appropriate. Data are presented as means ± SEM. Differences at $P<0.05$ were considered significant. All analyses were done using the statistical analysis program GraphPad Prism (Graphpad Software, San Diego, CA, USA).

Results

Expression of GHR mRNA

Amplification of cDNA from different stages of porcine preimplantation embryo development, primed with GHR-specific primers, resulted in one abundant PCR product after two rounds of amplification in all the samples (Fig. 1). Sequence analysis on the amplified GHR band showed 100% homology with the sequence described in the Gen-bank database.

Embryo development

There were no differences in the percentage of embryos displaying 2, 4 or 8 evenly sized blastomeres on Day 2, or blastocysts developing on Day 5 and Day 6 of IVC after IVF, in the two treatment groups (Fig. 2). The proportions of fragmented/degenerated embryos and uncleaved oocytes also were not different between the treatment groups (data not shown). Overall, 34% of oocytes placed in IVF had undergone cleavage up to the 8-cell stage on Day 2 of IVC, and almost completely developed to the blastocyst stage on Day 6, constituting a 97 and 103% conversion of embryos to the blastocyst stage in the Control and GH treatment groups respectively.

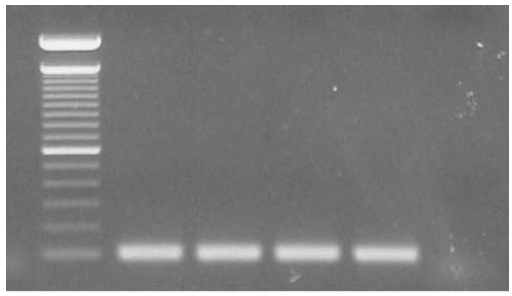

Figure 1. The PCR products, after amplification of cDNA with GHR-specific primers, of 2-, 4- and 8-cell embryos and Day 6 in vitro produced porcine blastocysts (D6 Bl) after two rounds of amplification. The PCR product had an expected size of 102 bp (bp M basepair size marker). No specific PCR products were detected when a water control or a minus RT blank (data not shown) was used as a template for the PCR.

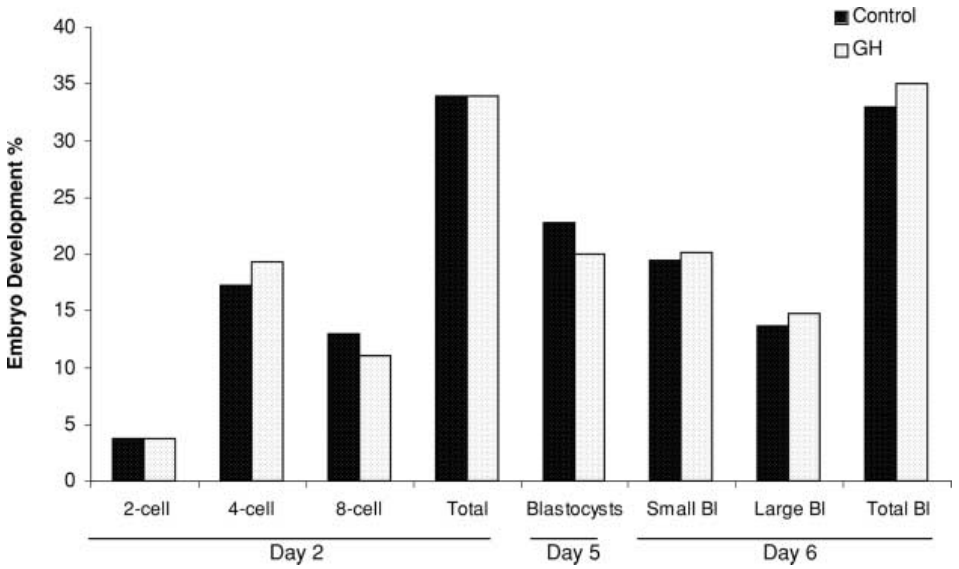

Figure 2. Porcine embryo development on Days 2, 5 and 6 of IVC in the absence (Control; n=1236) or presence (GH; n=1347) of 100 ng/ml GH (Small Bl=Small blastocysts; Large Bl=Large blastocysts; Total Bl=total blastocysts formed). Embryo development is expressed as the percentage of oocytes in IVM.

Diameter, volume and nuclei count of in vitro Day 6 and in vivo Day 5 blastocysts

In general, treatment of embryos with GH significantly increased the blastocyst diameter, but in vitro derived blastocysts irrespective of treatment were smaller in diameter than their in vivo counterparts (Table 1). The proportion of Large:Small blastocysts was similar in all groups. Blastocysts classified as Small did not differ in diameter according to either treatment or source of embryo. Large blastocysts in the Control group were on average smaller than those treated with GH, which again were smaller than in vivo produced Large blastocysts. The blastocoel volume did not differ for treatment within the Small blastocysts group. Large in vitro derived blastocysts had a significantly smaller volume than in vivo blastocysts, and this was similar when all blastocysts from each treatment group were taken into account. Similarly, blastocysts cultured in the presence of GH had an equal mean number of nuclei to embryos cultured in the absence of GH (41±1 and 40±2, for GH and Control respectively), which was significantly lower than that of in vivo produced blastocysts (51±3). GH treatment also did not benefit the number of nuclei contained in Large blastocysts, but in vivo derived Large blastocysts had a greater number of nuclei than both in vitro derived groups ($P<0.001$; ANOVA). The total number of nuclei ranged from 18 to 73 and 18 to 71 in the Control and the GH-treated groups respectively, and from 22 to 102 in the in vivo group.

Table 1. The effect of GH treatment during porcine in vitro embryo culture on different embryo quality parameters in Day 6 in vitro produced blastocysts, as compared with in vivo produced Day 5 blastocysts.

	Small blastocysts (< 180 µm)			Large blastocysts (> 180 µm)			All blastocysts		
Treatment	Control	GH	*In vivo*	Control	GH	*In vivo*	Control	GH	*In vivo*
n blastocysts	40	48	32	28	35	22	68	83	54
% of all blasts	58.8	57.8	59.3	41.2	42.2	40.7	-	-	-
Diameter (µm±SEM)	159.4 ±1.4[a]	161.2 ±1.1[a]	159.1 ±2.0[a]	195.1 ±3.5[a]	201.5 ±4.4[b]	226.9 ±5.1[c]	174.1 ±2.7[a]	178.2 ±2.9[b]	196.9 ±5.6[c]
Volume ($\mu m^3 x10^6$±SEM)	2.1 ±0.3[a]	2.2 ±0.3[a]	2.2 ±0.3[a]	4.0 ±0.3[a]	4.5 ±0.3[a]	6.8 ±0.3[b]	2.9 ±0.2[a]	3.2 ±0.2[a]	4.6 ±0.2[b]
No. of nuclei (mean±SEM)	38 ±2[a]	40 ±2[a]	34 ±1[a]	42 ±2[a]	42 ±2[a]	65 ±3[b]	40 ±2[a]	41 ±1[a]	51 ±3[b]

[a,b,c]Within rows for each stage of development, values with different superscripts are significantly different: P<0.001 (ANOVA). Number of nuclei: [a,b,c]Within rows for each stage of development, values with different superscripts are significantly different: P< 0.05 (ANOVA).

Apoptosis in Day 6 in vitro and Day 5 in vivo produced blastocysts

No indications of apoptosis (either morphological or biochemical) were found in blastocysts that had been produced in vivo. Large blastocysts in the in vitro produced Control group were more predisposed to biochemical apoptosis labelling by TUNEL than Small blastocysts, whereas GH-cultured blastocysts, irrespective of size (Table 2), contained fewer TUNEL-labelled nuclei overall. No necrosis was found in any embryos in any of the three groups. In the Control group, the majority of blastocysts containing nuclei presenting apoptotic morphology (fragmentation) in the absence of TUNEL labelling were found in the Small group, with only a small percentage of Large Control blastocysts presenting this characteristic. An equal proportion of the GH-treated Small and Large blastocysts contained TUNEL-positive apoptotic nuclei. Differences in appearance were apparent for fragmented TUNEL-positive nuclei (FT+) and fragmented TUNEL-negative nuclei (FT-) as presented in Fig. 3: FT+ consisted of either two larger nuclear fragments, or one large and one small fragment; whereas FT 2 consisted of mostly a larger number of highly condensed very small fragments. The total incidence of apoptosis (TUNEL-positive and fragmented only blastocysts) did not differ between the treatment groups nor did the size of the blastocysts (Table 2). GH treatment significantly reduced the percentage of apoptotic nuclei (apoptotic index) in Large blastocysts containing ≥ 1 apoptotic nuclei, but not in Small apoptotic blastocysts. Also, the percentage of either TUNEL-labelled or fragmented nuclei per blastocyst did not differ for treatment group or blastocyst diameter. When taking into account both Small and Large in vitro produced blastocysts, Fig. 4 shows the differences in apoptosis morphology between the Control and GH group blastocysts cultured with or without GH. The manner in which apoptosis was manifested differed greatly between the Control and the GH-treated blastocysts, of which the latter contained fewer TUNEL-labelled apoptotic nuclei.

Embryo transfer

As shown in Table 3, the non-surgical transfer of in vitro produced blastocysts resulted in pregnancy rates comparable with those achieved when in vivo produced blastocysts were transferred. Although GH-treated blastocysts had a higher pregnancy rate than Control

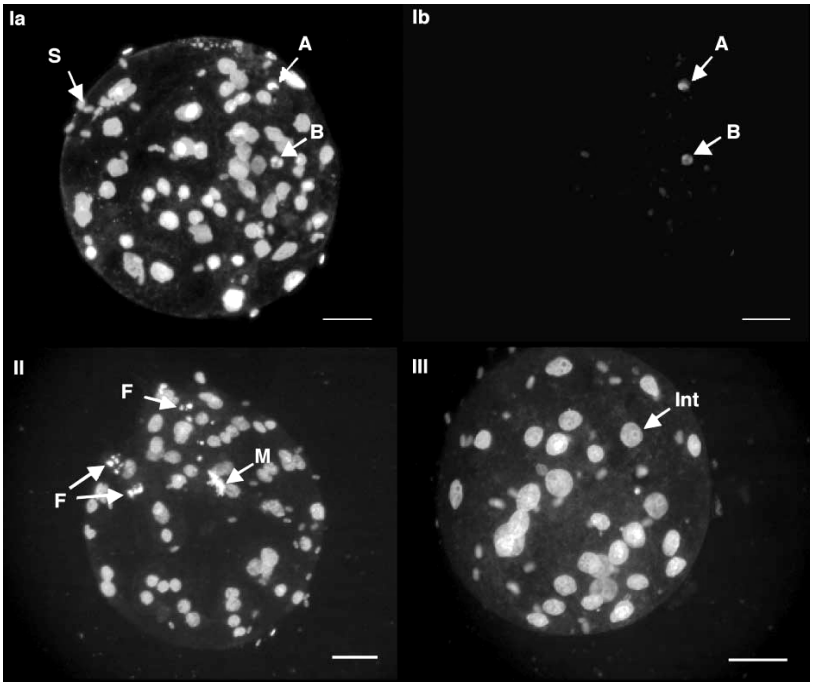

Figure 3. Phenotypic appearance of normal or apoptotic nuclear fragmentation in Day 6 in vitro produced porcine blastocysts. (Ia) DAPI-filter image of a blastocyst containing A: condensed nucleus, B: fragmented nucleus, S: sperm on zona pellucida; (Ib) corresponding FITC-filter image of the blastocyst in (Ia) showing A: condensed TUNEL-positive nucleus and B: fragmented TUNEL-positive nucleus; (II) DAPI-filter image of a blastocyst containing F: fragmented TUNEL-negative nuclei and M: mitotic nucleus; (III) DAPI-filter image of a healthy blastocyst containing Int: normal interphase nuclei (Bar: 35 μm).

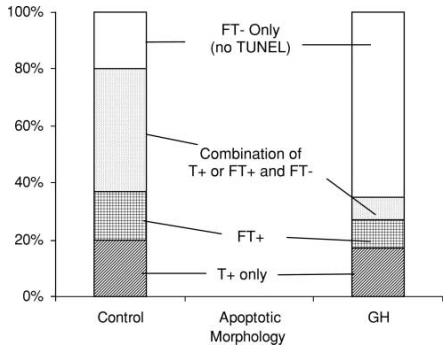

Figure 4. The distribution of different apoptotic morphologies among the cohort of in vitro produced porcine blastocysts containing ≥1 apoptotic nuclei and cultured in vitro with (n=83) or without (n=68) GH (FT-: TUNEL-negative fragmented nuclei; T+: TUNEL-positive condensed nuclei; FT+: TUNEL-positive fragmented nuclei).

blastocysts, the difference was not significant. Furthermore, the number of blastocysts surviving transfer was greatly reduced in the in vitro blastocyst litters. Albeit that care was taken to randomly distribute blastocysts according to size, retrospective analysis of blastocyst diameters (images were recorded before transfer as described in the section on Non-surgical embryo transfer) revealed that both the mean diameter ($P<0.01$) and volume ($P<0.001$) of the batches of transferred embryos were significantly greater for those that led to the establishment of pregnancies, irrespective of in vivo or in vitro treatment group. All non-pregnant batches consisted of 'Small' blastocysts on average.

Discussion

In the present study we have examined the effect of GH on in vitro embryo development in the pig. In addition, to gain more insight into the functional aspects of blastocyst quality in the pig we compared in vitro with in vivo derived blastocysts with regard to blastocyst diameter, volume, cell number, apoptosis and survival following non-surgical embryo transfer. In general, in vitro produced porcine embryos are known to be retarded in their development when compared with their in vivo counterparts and also contain fewer nuclei [34, 36]. Following the positive effects that GH has on bovine in vitro embryo development and quality [12, 13, 15] it presented a promising tool for improving the quality of in vitro produced embryos in the porcine. The present data, though, show for the first time that GH addition during in vitro porcine embryo culture does not improve blastocyst formation or cell numbers, nor reduce apoptosis, but alters the pattern of apoptosis in Day 6 blastocysts. Another novel finding is related to the diameter-expanding effect of GH, which points to modulation of the metabolic activity of GH-treated blastocysts.

First, despite the presence of GHR during early in vitro embryo development in the pig, no effect of GH on rate of blastocyst formation, or blastocyst cell numbers, was found. Although both the maternal and the embryonic genome express GHR (porcine embryonic genome is activated at the 4-cell stage [37-39]), the question now arises whether the GHR is functional in pre-blastocyst stage pig embryos. In the bovine [9, 12] and mouse [11, 40] the functionality of the GHR from early cleavage stages has been clearly illustrated where GH significantly improved cleavage and blastocyst development rates. The absence of any GH-mediated effect on porcine embryo development could be due to the specific metabolic needs of the porcine embryo. In the mouse it has been reported that GH significantly stimulates glucose uptake in blastocysts in a dose-dependent manner [8, 14] by direct recruitment of glucose transporters. In preimplantation prepubertal gilt embryos, glucose has recently been shown to inhibit early development in vitro while a significant increase in glucose uptake was found at the blastocyst stage [41]. This effect is similar in the mouse [42], hamster [43] and rat [44] where glucose is known to suppress embryo development before compaction or before the blastocyst stage. As porcine embryos do not utilize glucose until the blastocyst stage [41], pre-blastocyst stage embryos could not take advantage of the glucose-uptake-promoting effect of GH, and hence no cell proliferation effect of GH was seen.

Table 2. Apoptosis in Day 6 porcine Small or Large blastocysts cultured in the presence or absence of GH following IVM and IVF of sow oocytes.

	Control		GH	
	Small blastocysts (<180 μm)	Large blastocysts (>180 μm)	Small blastocysts (<180 μm)	Large blastocysts (>180 μm)
Number (N) of blastocysts	40	28	48	35
% TUNEL (DNA damaged)	35.0^{A*}	60.7^{a*}	14.6^{B}	20.0^{b}
% Fragmented Only	20.0*	3.5^{A*}	33.3	28.6^{B}
% Total† Apoptosis	55.0	64.3	47.9	48.6
TUNEL Index	5.5 ± 0.8	6.2 ± 1.1	4.2 ± 0.9	5.5 ± 1.0
	(n=496)	(n=684)	(n=214)	(n=265)
Fragmented Index	5.0 ± 1.1	3.7 ± 0.0	4.9 ± 1.0	4.0 ± 0.5
	(n=242)	(n=53)	(n=677)	(n=383)
Total† Apoptotic Index	6.2 ± 0.7	7.9 ± 1.3^{d}	5.0 ± 0.8	4.8 ± 0.6^{e}

Within rows and between treatment groups: $^{A,B}p<0.05$; $^{a,b}p< 0.01$;$^{d,e}p<0.001$; Within rows and within treatment groups: *p<0.05; N = total number of blastocysts per category containing≥1 apoptotic nuclei; n = total number of nuclei in blastocysts containing ≥1 apoptotic nuclei †Total = combination of TUNEL and Fragmented Only

Table 3. Pregnancy rates and embryo characteristics after non-surgical transfer of in vitro produced (IVP) and in vivo produced porcine blastocysts.

	In vivo Blastocysts	Control IVP blastocysts	GH IVP blastocysts
No. of transfers	23	8	8
No. embryos transferred in total	575	200	200
Mean blastocyst diameter (μm ± SEM)			
Pregnant	192.30 ± 2.1*	186.42 ± 2.87*	187.41 ± 2.59*
Non-pregnant	173.10 ± 1.1	175.77 ± 2.44	177.14 ± 2.21
Mean blastocyst volume ($\mu m^3 x 10^6$ ± SEM)			
Pregnant	3.7 ± 0.2**	3.4 ± 0.2**	3.5 ± 0.2*
Non-pregnant	2.7 ± 0.3	3.0 ± 0.2	3.1 ± 0.2
No. of embryos recovered in total	28	2	4
% pregnant Day 10	30 (7/23)	25 (2/8)	38 (3/8)

Within columns Pregnant differs significantly from Non-pregnant: *p<0.01; **0.001

The main effect of GH was seen in the modulation of apoptosis, which has been prominent in the literature during the past 2 years. In the human [45], mouse [46] and bovine [47] the occurrence of apoptosis in preimplantation embryos has been identified both in vivo and in vitro. It is likely that blastocyst stage apoptosis acts to eliminate damaged cells no longer required (i.e. undifferentiated trophoblast cells inappropriately present in the inner cell mass), or developmentally incompetent, and is thought to be a part of the cellular 'quality control' within the developing embryo [48]. In this study and other studies from our laboratory [49] in vivo produced sow embryos presented none of the classical features of apoptosis such as nuclear condensation and blebbing/fragmentation (karyorhexis), or DNA fragmentation (karyolysis). This is in contrast to the findings of Long et al. [23], who found apoptotic nuclei in the majority of expanded in vivo (70.8%) and in vitro (90.3%) produced blastocysts. The differences in results may be due to donor age, as all in vivo derived blastocysts in this study were collected from older gilts than those of Long et al. [23] and oocytes for in vitro embryo production were harvested from slaughterhouse sows, and not prepubertal gilts. Sow and prepubertal gilt oocytes differ in their ability to support normal fertilization, and subsequent blastocyst quality is superior in sow embryos produced in vitro [16, 50]. Although no comparisons have been made between sow and gilt blastocysts regarding the incidence and degree of apoptosis it is highly likely that they would differ in this aspect as well. This study, in concurrence with previous studies from our laboratory [49], thus strongly indicates that under ideal in vitro circumstances porcine blastocysts should not contain any apoptotic cells.

In this current experimental design, in agreement with bovine studies [15] GH significantly reduced the incidence of karyolysis as detected by TUNEL, as well as the apoptotic index (percentage apoptotic nuclei in each blastocyst presenting ≥1 apoptotic nuclei). However, the combined levels of karyolysis and karyorhexis (total incidence of apoptosis) were not decreased by GH addition to the culture medium. In the majority of papers investigating apoptosis in embryos only karyolysis is taken into account when determining the incidence of apoptosis. The fact that some nuclei display apoptotic morphology (karyorhexis) in the absence of TUNEL staining may have different reasons and deserves careful consideration. Evaluation of both morphological and biochemical characteristics supports the comprehensive assessment of apoptosis, first to distinguish the stage of apoptosis progression and secondly because different apoptotic pathways have specific substrate targets during the execution phase of apoptosis [51]. During the final stages of apoptosis, karyolysis is thought to precede karyorhexis [45, 52, 53], which means that a larger proportion of non-GH-treated blastocysts displayed earlier stages of apoptosis than the GH-treated blastocysts. In the light of the recently published glucose requirements of pig embryos [41] it may thus be hypothesized that GH exerts its effect only from the early blastocyst stage onwards where it then prevents or reduces the onset of 'new' apoptosis as blastocyst development progresses. On Day 6 of embryo development, GH-treated blastocysts are thus 'healthier' as they are not suffering from the cumulative effects of IVC induced apoptosis.

Alternatively, it is a known fact that dissociation between apoptosis and DNA fragmentation can occur in blastocysts as well as other cell types [54-57]. It is thought to be linked to the pathway or specific trigger of apoptosis. In studies investigating apoptosis in the bovine [53]) and apoptosis and the metabolism of human [58], mouse [59] and rat embryos [54] nuclear fragmentation in the absence of TUNEL labelling has been reported. A mitochondrial protein, bcl-2, involved in the modulation of caspase activity, has been

shown to protect the rat blastocyst from chromatin degradation due to hyperglycaemic culture conditions, but not from nuclear fragmentation [60]. In both the mouse and bovine blastocysts [15, 61] GH increases the expression of bcl-2 which is known to suppress caspase-3 cleavage, and inhibits TUNEL-detected apoptosis in this way [62, 63]. Caspase-3, via caspase-activated DNAse, leads to DNA fragmentation, whereas caspase-6, via lamin/nuclear-mitotic apparatus protein, brings about chromatin condensation and fragmentation [51, 54]. It is thus conceivable that, in porcine embryos, GH reduces caspase-3-dependent apoptosis but does not influence caspase-6-dependent apoptotic pathways. The potential interaction of GH and glucose on early in vitro produced porcine embryos should be ascertained to establish the relationship with specific apoptotic pathways in subsequent blastocysts.

Embryo diameter is as yet an unexplored gauge for evaluating blastocyst quality, as only one report exists in which larger in vivo produced pig blastocysts led to a higher percentage of pregnancies and litter size after nonsurgical transfer [30]. In this respect GH had indeed slightly improved blastocyst quality by increasing the mean diameter of the blastocysts, albeit that the cell numbers had not been improved. In contrast to the in vivo produced blastocysts, the diameter of both the groups of in vitro blastocysts had increased without a concurrent increase in cell number. This finding could point to anomalies in either the cell proliferation activity of the in vitro produced blastocysts or the osmotic/secretory activity of the blastomeres responsible for blastocoel formation. When taking into account the blastocyst volume, although not statistically significant, the increase in diameter induced by GH constitutes a 10% increase in blastocyst volume. This effect could indicate superior metabolic activity of the larger, as opposed to the smaller, in vitro derived blastocysts. The trophectoderm plasma membrane sodium pump, Na^+/K^+ -ATPase, plays a critical role in the formation and maintenance of the blastocoel, which in turn is essential for preparing the embryo for implantation [review, 64]. A direct relationship exists between glucose consumption and blastocoel formation/expansion, which reflects the energy demands of Na^+/K^+-ATPase [65, 66]. GH could thus influence blastocoel expansion through its glucose transport effects [8, 14], but also possibly by its direct activation of the sodium pump as is seen in adipocytes [67]. In our experimental design the full effect of GH is not yet clear, but optimization of the energy substrates within the embryo culture medium for each stage of embryo development could create a more suitable setting for examining the role of GH more effectively.

The fact that all the pregnancies in this study resulted from the transfer of Large blastocysts points to the idea that blastocysts may differ in quality due to embryonic metabolism effects rather than cell number, as previously explained. Due to the small number of transfers, it is not yet clear whether any other factors (such as apoptosis or cell number) investigated in this study, apart from diameter and volume, are involved in post-transfer survival. This study provides the first proof that the diameter and volume of the porcine blastocysts, irrespective of origin, are of key importance when selecting blastocysts for transfer. Further studies investigating the metabolism and also the structure of Large and Small blastocysts will provide further insight into the quality of such blastocysts, and the correlation with blastocyst diameter or expansion.

In summary, our study shows that GH can improve in vitro produced blastocyst quality by enhancing blastocyst expansion and positively modulating either the time course or pathway of IVC induced apoptosis. Culture of in vitro produced embryos in the presence of GH thus improves their similarity to in vivo derived blastocysts in both morphological

and biochemical aspects. Nevertheless, in vitro produced pig blastocysts remain inferior in quality to their in vivo derived counterparts as judged by the poor survivability after transfer. More transfers, either surgical or non-surgical, will shed more light on the intrinsic viability of in vitro produced porcine blastocysts.

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CHAPTER 7

Morphology-related cytoskeleton dynamics in porcine blastocysts produced in vitro or in vivo, and its consequences on embryo viability.

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Abstract

The blastocyst yield from porcine oocytes matured and fertilized in vitro is similar to that achieved in the bovine, but the post-transfer survival of is much lower compared with their in vivo counterparts. This may be due to the lower cell number of in vitro blastocysts, which is also correlated with the presence or extruded cells or irregularities of the trophectoderm layer. Anomalies in the actin microfilament (MF) content and distribution in porcine in vitro matured oocytes and early embryos are thought to impair cell division thereby causing low cell numbers in resultant blastocysts. In this study we compared in vitro and in vivo produced sow blastocysts for morphology, actin cytoskeleton and blastocyst inner cell mass and trophoblast cell number. We then evaluated the ability of blastocysts to re-expand after cytochalasin-B (CytB) treatment, and assessed the survival of in vitro produced quality classified blastocysts following non-surgical transfer. Results indicated a correlation between blastocyst morphology and actin quality. Class A blastocysts contained grade I actin, whereas the morphological anomalies (Class B) was correlated with poor actin quality (grade II and III). The ability of blastocysts to re-expand after actin depolymerization was positively correlated with morphology of the blastocyst, and associated mainly with the quality of the actin cytoskeleton. Class B blastocysts did not survive CytB challenge. More recipients receiving Class A blastocysts were pregnant on Day 20 than those receiving Class B blastocysts. One recipient farrowed five healthy piglets from Class A blastocysts, providing a yet early, but encouraging verification of the enhanced viability of quality classified blastocysts and the value of cytochalasin-B as means of evaluating blastocyst quality.

Manuscript in preparation

Introduction

The pig's importance as a food-producing animal, as well as a biological model in human biomedical applications [1], has prompted increased efforts to improve the efficiency of reproductive technologies in the pig. Despite the establishment of several systems to generate embryos in vitro, the inferior quality of resultant blastocysts limits the improvement of other reproductive techniques, such as embryo transfer and stem cell technology, which are dependent on the blastocyst as source of material. Culture media have been adapted to suit the specific individual needs of the pig oocyte and embryo [2], leading to improved blastocyst development and the birth of piglets [3, 4]. Despite these achievements, not much progress in the improvement of embryo viability has been made since the adoption of its use a decade ago. Using blastocyst yield as the ultimate parameter for evaluating the success of a given porcine IVP system is not without its pitfalls, especially when taking into account the problems with cytoplasmic maturation and polyspermy [5-7]. Zygotes derived from polyspermic oocytes readily develop to blastocysts, but their viability after transfer is severely restricted [8]. In vitro produced blastocyst differ distinctly from their in vivo counters parts, with the former containing much lower numbers of nuclei. This believed to be due to deficiency and abnormalities in actin filament distribution [9], as early as the first cleavage division. The distribution of actin filament is affected by the culture environment, which in turn influences actin-dependent migration of organelles and also subsequent cell division [10]. Parameters used in the pig to define embryo quality include total and inner cell mass cell number, chromosomal abnormalities, and apoptosis [11]. These invasive techniques, requiring destruction of the embryo, have indeed proven differences between in vivo and in vitro (prepubertal gilt only) derived blastocysts, but none have been utilised to distinguish good from poor quality embryos within the population of in vivo or in vitro produced embryos. Blastocyst morphology and kinetics of development are the only non-invasive techniques, of which the former has only recently been explored for use in pig embryos in a study completed in our laboratory [12]. In that study, we observed that morphological abnormalities such as extruded cells or disruptions in the trophoblast layer were indeed associated with a lower total blastocyst cell number.

The cytoskeletal stabilizer, cytochalasin-B, has been used extensively in studies dealing with microfilament dynamics in oocytes and embryos, as well as micromanipulation of embryo development [13-15]. Cytochalasin-B inhibits the polymerization of actin, thus preventing cytokinesis without affecting karyokinesis [16, 17]. When used in bovine in vitro produced embryos the effects of cytochalasin-B are completely reversible, and do not inhibit mitotic progression after removal from the inhibitors [18]. Pig embryos exposed to cytochalasin-B at the 2-4 cell stage re-assemble actin filaments and undergo blastocyst formation similar to that of controls [19]. Use of cytochalasin-B at the blastocysts stage is mainly employed when preparing embryos for vitrification, where it stabilizes the cytoskeleton and thereby improves post-thaw embryo survival.

Transfer of in vivo produced pig blastocysts has mainly been performed surgically, with reasonable success rates; at present, 60% of transfers lead to pregnancies, with up to 60% of transferred embryos surviving to farrowing [20]. Although surgical transfer of IVP blastocysts has led to pregnancies and live births [3, 4], it required the transfer of vast numbers (50 to 100) of randomly chosen IVP blastocysts resulting in less than 20 % survival to farrowing [3]. Surgical transfer of porcine embryos is not allowed in the Netherlands due to the invasive nature of the procedure, which has necessitated the

development of non-surgical ET (nsET). Being a less invasive procedure, requiring neither surgery nor sedation [21], nsET promises to be a valuable tool for both research and commercial purposes, due to its on-farm applicability and reduced need for special facilities. Pregnancy rates similar to that of surgical ET have been achieved after the non-surgical transfer of in vivo produced blastocysts, but with slightly lower piglet rates [21, 22]. Non-surgical transfer of randomly selected in vitro produced blastocysts have lead to pregnancies up to Day 11, but embryo survival rates were very poor as judged by the low number of conceptuses recovered from the uterine tract [11]. Explanations for these poor survival rates are most likely found to the inherent poor quality of in vitro produced blastocysts, but needs to be further evaluated.

The objectives of this study were therefore to compare in vitro and in vivo produced sow blastocysts in terms of: (1) the classification of morphological abnormalities and associated architecture of the actin cytoskeleton, (2) blastocyst cell numbers (inner cell mass and trophoblast cells), (3) the ability of classified blastocysts to re-expand following cytochalasin-B treatment, and (4) to assess the viability of blastocysts produced in vitro and classified according to morphology, in terms of their survival following non-surgical transfer.

Materials and Methods

Animals, donors and recipients

All animal experiments were approved by the Ethical Committee for animal experiments of Wageningen University. Synchronization and embryo recovery was performed according to [21]. Briefly, 90 to 120 crossbred gilts (over 6 to 8 replicates), 7 to 11 months old (TOPIGS, Vught, The Netherlands), were used as donors and 21 multiparous crossbred sows (TOPIGS) as recipients (n=11 for Grade A; n=10 for Grade B; n=24 for In vivo). Ovulation was synchronized using PG600® (Intervet International BV, Boxmeer, The Netherlands) for first oestrous induction and 2 x 500 µ cloprostenol (Estrumate®, Schering-Plough, Maarssen, The Netherlands) 8 h apart around Day 13 of the first luteal phase. The next day follicular development was induced with 700 IU eCG (Folligonan; Intervet International BV), followed by 600 IU hCG (Chorulon; Intervet International BV) after 72 h to induce ovulation. The gilts were artificially inseminated 24 and 36 h after hCG administration. Donors were slaughtered seven days (168 h) after hCG (120 h after estimated ovulation) for collection of Day 5 embryos. Immediately after stunning, bleeding and removal of the genital tract, the embryos were flushed from the uterus horns using DPBS (Dulbecco PBS; BioWhittaker, Verviers, Belgium) supplemented with 1% heat-inactivated lamb serum (GIBCO, Paisley, England) and 1% PenStrep (penicillin-G 100 IU/ml and streptomycin sulphate 100 µg/ml; BioWhittaker) at 37°C. The flushing medium was then filtered, and the embryos retrieved by rinsing the filter with DPBS. The embryos were directly transported to the laboratory in a temperature controlled insulated container kept at 25°C. For transfer, all non-fertilized oocytes and degenerated zygotes, as well as morulae and hatching or hatched blastocysts were discarded upon stereomicroscopic evaluation and the remaining blastocysts were prepared for non-surgical transfer. For morphological evaluation and cytochalasin-B treatment, a representative sample (5 to 10 per replicate; 6 to 8 replicates) of expanded blastocysts stage embryos were randomly selected from the pooled batches of embryos (150 to 200 per replicate) recovered from donors prepared for transfer purposes.

In vitro culture media

For oocyte and embryo searching and selection, 25 mM HEPES-buffered Tyrode's medium containing 0.1% polyvinyl alcohol (TL-HEPES-PVA) was used. The medium (IVM-I) used for the first 22-24 h of oocyte in vitro maturation was BSA-free North Carolina State University 23 medium (NCSU23) [2] supplemented with 10% (v/v) pFF, 0.8 mM cysteine, 25 µM ß-mercaptoethanol, 10 IU/ml eCG and hCG (Folligonan and Chorulon, Intervet international, The Netherlands). The second IVM (IVM-II) culture period (18-20 h) occurred without eCG and hCG added to the medium. Porcine follicular fluid (pFF) was collected from morphologically healthy follicles 3 to 6 mm in diameter, centrifuged at 1900 *g* for 30 min (4ºC), filtered through 0.8 µm syringe filter (Millipore SA, Molsheim, France) and stored at -20ºC until use [12]. Cumulus-oocyte complexes (COCs) were washed in IVM-wash medium (IVM-II medium with 20 mM HEPES). Modified Tris-buffered medium (mTBM) was used as fertilization medium. This medium consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate. Caffeine (1 mM) and 0.1 % BSA (w/v) (A-6003, Sigma, St. Louis, MO) were supplemented to mTBM for use as IVF medium. The in vitro embryo culture medium was NCSU23 containing 0.4 % BSA (w/v). Before being placed in the IVC medium, presumptive zygotes were washed in IVC-wash medium, which is similar to the IVC-medium, but containing 20 mM HEPES.

Recovery of oocytes and in vitro maturation

Oocyte recovery, in vitro maturation, fertilization and embryo culture proceeded as previously described [23]. Briefly, ovaries were collected from sows at a local slaughterhouse and transported to the laboratory in insulated containers. Excess connective tissue and oviducts were cut from the ovaries, and they were then washed once at 30°C under running tap water. The ovaries were subsequently placed in a beaker of pre-warmed sterile saline, supplemented with 1% PenStrep, and held at 30ºC until aspiration. COCs were aspirated from 3 to 6 mm non-atretic follicles with an 18-gauge needle fixed to a vacuum pump via 50 ml conical tube. Contents were collected into the tube and allowed to settle for 10 minutes at room temperature. The supernatant was removed and sediment was resuspended in TL-HEPES-PVA at room temperature and allowed to settle. This treatment was repeated once more and the content of the tube was observed under a stereomicroscope on a heated stage (38.5 °C). COCs surrounded by two or more layers of compact cumulus investment and containing oocytes of equal size were selected, washed twice in IVM-wash medium which had been pre-warmed to 38.5ºC prior to use, and transferred in groups of 40 to 50 to a four-well dish containing 500 µl of equilibrated IVM-I medium in each well. The four-well dish was then incubated for 24 h at 38.5 ºC in 5% CO_2 in humidified air. After 22 to 24 h all the oocytes were washed twice in IVM-wash medium and placed in 500 µl IVM-II medium for an additional 16 to 18 h of culture.

In vitro fertilization and embryo culture

After maturation the oocytes were placed in a wash dish containing pre-warmed equilibrated IVF medium. Using a micropipette, the contents of the dish were vigorously pipetted for 30 sec to remove the expanded cumulus cells. The denuded oocytes were washed once more in IVF medium before being placed in 500 µl wells of equilibrated IVF

medium, in groups of 40 to 50, and incubated at 38.5ºC in 5% CO_2 for 30 min until the addition of the sperm.

Fresh room temperature Beltsville Thawing Solution (BTS)-extended [24] semen was diluted 1:2 in IVF medium (also at room temperature). Sperm were centrifuged in a conical tube for 4 min at 700 *g*. The supernatant was removed and sperm resuspended in IVF medium and centrifuged again. Following resuspension the sperm concentration was determined and adjusted to achieve a final concentration approx. 1000 motile cells/oocyte. After warming to 38.5 ^{o}C for 30 min, the appropriate volume of the sperm suspension (range 4 to 10 µl) was added to the 500 µl IVF wells containing the oocytes and co-incubated with the oocytes for 20 to 24 hours at 38.5ºC at 5% CO_2 in air. Twenty-four hours after insemination, the oocytes were removed from the IVF wells and washed twice in IVC-wash medium. They were then gently pipetted to remove excess sperm attached to the zona pellucida and transferred in groups of 40 to 50 into 500 µl of IVC medium a four-well dish. At 48 h after the addition of sperm for IVF, cleavage rate was determined (structures judged to be degenerated or uncleaved were not removed), and on Day 6 of embryo culture, blastocyst formation was evaluated.

Determination of blastocyst development

Embryos were scored morphologically on Day 6 after the addition of sperm for IVF (Day 0). The percentage blastocysts, expressed on the basis of the number of oocytes placed into maturation, was evaluated on Day 6 (29%; 294/858). Day 6 in vitro produced blastocysts were regarded to be the equivalent of Day 5 in vivo produced blastocysts due to the retardation of growth experienced in embryos produced in vitro from in vitro matured oocytes [12, 25, 26].

Blastocyst morphology

Of in vivo produced blastocysts 90% (1447/1608) recovered from all donor females, for both transfer and cytoskeleton analysis purposes, were of normal morphology and at the expanded blastocyst stage of development. Both in vivo and in vitro derived blastocysts were individually assessed for morphological appearance and characteristics, as previously reported [12]. Blastocysts were strictly evaluated according to their morphological appearance using a stereo microscope at 160 to 200X magnification. Blastocysts that were symmetrical and spherical with a clear inner cell mass, intact trophoblast and containing no visible extruded blastomeres were categorized as having a normal morphology, and designated as Class A. When there was doubt about the presence or absence of abnormalities, the blastocyst was classified as Class B. Abnormal blastocysts were designated as Class B, and included embryos with one or more extruded blastomeres present under the zona pellucida, and/or anomalies of the trophoblast layer, considered to be loss of integrity between adjacent blastomeres.

Cytochalasin-B (CytB) treatment

After selection and classification, blastocysts were incubated at 38.5^{o}C in 5% CO_2 in humidified air in IVC-medium containing 7.5 µg/ml CytB (C-6762, Sigma), previously dissolved in food grade ethanol as a 5 mg/ml stock solution. In vitro produced blastocysts were exposed to CytB until collapse of the blastocoel cavity, and contraction of the

embryo which was regarded to be indicative of the full depolymerization of microfilaments. This process took approx 40 min for in vitro produced blastocyst, 40 to 90 minutes for blastocysts produced in vivo. After the CytB incubation time, the blastocysts were washed 4 times in IVC-medium (without CytB) before being placed into culture in IVC medium (5% CO_2 in humidified air) for 16 to 18 h in order to facilitate re-expansion of the blastocoel. Blastocysts were then labeled with Ethidium-homodimer (EthD-I), before being divided into 're-expanded' or 'non-re-expanded'. When the blastocoel cavity had retained at least 80% of its original volume, its was regarded to have re-expanded, and when it occupied less than 80% of its original volume it was classified as 'non-re-expanded'. All blastocysts were then fixed in 3% (w/v) paraformaldehyde in PBS, until further processing.

Molecular labeling of blastocyst cytoskeleton

In order to detect blastomeres with compromised cell membrane, live blastocysts were incubated for 10 min (39º C, 5% CO2 in humidified air) in 500 µl IVC-medium to which 2 µM EthD-I (Molecular probes, Leiden, The Netherlands) was added. The embryos were then washed three times in phosphate buffered saline (PBS), and fixed in 3% paraformaldehyde. To detect microfilaments oocytes were washed three times in PBS with 0.1% PVA, before being incubated for 30 min with 0.165 µM Alexa Fluor™488-Phalloidin (Molecular Probes) in a solution of 0.1% Triton X-100 in PBS. Blastocysts were washed in PBS and stained with 0.1 µg/ml 4,6-diamino-2-phenyl-indole (DAPI) (Molecular probes) as a counterstain,. Stained embryos were gently mounted on a glass slide with anti-fade mounting medium (Vectashield, VectorLab, Burlingame, USA) to retard photo bleaching, and the cover slip sealed with nail polish. The slides were kept in the dark at room temperature until analysis.

Multiphoton confocal laser scanning microscopy

Fluorescent signals in all embryos were visualized using a Bio-Rad Radiance 2100MP Multiphoton Excitation Microscope (MPEM) combined with a Confocal Laser Scanning Microscope (CLSM; Bio-Rad, Hertfordshire, UK) equipped with a Nikon TE300 inverted microscope (Uvikon, Bunnik, Netherlands). Excitation of DAPI was achieved by 100 fs multi-photon excitation at 780 nm using a mode-locked Titanium:Sapphire laser (Tsunami; Spectra-Physics, Mountain View, CA) pumped by a 10 W solid laser (Millennia Xs; Spectra-Physics), whereas Alexa Fluor ® 488 and Ethidium homodimer were excited by a Argon and Helium/Neon lasers at 488 and 543 nm.

The images were obtained as multi-channel colors, written to CD-rom and visualized using Confocal Assistant™ 4.02 (© Todd Clark Brelje, USA) and Adobe® Photoshop 7 (Adobe Systems Inc., Mountain View, CA, USA). Embryos were subjectively scored according to their actin cytoskeleton quality according to Tharasanit et al. [27] (Figure 1. Images A to D). Grade I was typified by sharp staining of the cell borders with an abundant microfilament complement in the cytoplasm; grade II staining was characterized by gross maintenance of the cell outline, but with clumped or scant microfilaments visible in the cytoplasm. In a grade III cytoskeleton the integrity of the cells were lost, with actin filaments visible mainly as aggregated clumps. In order to be classified as a blastocyst containing grade I actin, no more than 25% blastomeres presenting type II was allowed.

Blastocyst cell number was evaluated by merging all scanned images layers. When overlap of nuclei occurred, the layers of overlap were sequentially viewed to increase and assure accuracy of the nuclei count. The inner cell mass (ICM) was located from the phalloidin images, in which the position of the ICM was clearly visible due to the intense staining of the ICM boundary, combined with the tightly packed natured of the ICM cells (Figure 1. Image E). The ICM cell numbers were then counted by simultaneously viewing the phalloidin and DAPI images.

Non-surgical embryo transfer

Transfer of in vitro blastocysts took place as previously described [20]. Recipients were prepared similarly and synchronously to the donor animals as described above. Day 6 in vitro produced embryos were transferred to Day 5 recipients, and Day 5 in vivo produced blastocysts were transferred synchronously to Day 5 recipients as well. On the day of transfer in vitro produced blastocysts, grouped according to quality score (described under Results), were placed in eppendorf tubes, containing transfer medium (D-PBS with 10% lamb serum) at 38.5°C, in batches of 30. They were then allowed to cool to 25°C before transport to the transfer location. Upon arrival at the transfer station (<3 h after initial collection) the embryos were removed from the eppendorfs, and placed in a small petri dish containing the transfer medium. Each batch of 30 embryos was then aspirated into the tip of the transfer catheter (Swinlet-prototype, Wageningen University, The Netherlands). The transfer procedure consisted of careful passage of the instrument through the cervical folds into the uterine body. The catheter, containing the embryos, was then passed through the instrument and the embryos were deposited in the uterine body with <0.1 ml transfer medium. Recipients were not sedated during the transfer procedure.

From the day of transfer, recipients were checked for oestrus each day and from Day 14 onwards occasionally checked for pregnancy. On Day 20 after ovulation the recipients were scanned using transrectal ultrasonography (Scanner 150S, Pie Medical Equipment BV, Maastricht, The Netherlands) to visualize the presence or absence of corpora lutea or follicles. Recipients were regarded to have been pregnant on Day 20 when animal had not returned to oestrus, corpora lutea were present on the ovaries and foetal fluid was observed. The recipients were regularly observed for estrous behaviour until farrowing.

Statistical analysis

All experiments consisted of a minimum of 3 replicates. Statistical analysis of embryo development data were carried out using Chi Square or Fisher's Exact test, where appropriate. After testing for normality (Kolmogorov-Smirnov test with Lilliefor correction) and testing for equal variances (F-test for two groups and Bartlett's test for multiple groups) the means of blastocyst nuclei count and nuclear damage were compared by unpaired t-test, or ANOVA (followed by Bonferroni multiple pair wise comparison) where appropriate. Data are presented as the mean ± SEM. Differences at $P \leq 0.05$ were considered significant. All analyses were done using the statistical analysis program SPSS.

Experimental Design

In the first instance, blastocysts produced in vitro (n=50) or in vivo (n=38) were scored for morphological appearance and separated according to each classification, as described below. The respective classification groups were then fixed in 3% paraformaldehyde until processing for immunocytochemical staining of microfilaments and nuclear chromatin for cytoskeleton analysis. A second group of blastocysts from each origin (n=211 for in vitro, and n=53 for in vivo), and each morphological classification, was subjected to the actin polymerization inhibitor, cytochalasin-B, after which they were placed back into standard IVC-medium to evaluate their ability to re-expand to the original status. At the termination of culture, each classification group of blastocysts was further divided into re-expanded or non-re-expanded groups before fixing in 3% paraformaldehyde. As a control for the actin filament-depolymerization ability of CytB, a small group of blastocysts (n=6) was subjected to CytB treatment, and then immediately fixed (Figure 1 Image F). In order to evaluate differences in cytoskeleton between the morphological classification groups, and after CytB treatment, all blastocysts were labelled with phalloidin and DAPI and to monitor subcellular changes in the actin cytoskeleton and nuclear material, and EthD-I for evaluating nuclear membrane integrity. In the final part of the study, in vitro produced blastocysts were selected according to the newly-established quality scoring criteria and transferred non-surgically to embryonic stage synchronous recipient females.

Results

Morphological evaluation of blastocysts

Blastocysts produced both in vivo and in vitro typically presented one of two abnormalities, i.e. extruded blastomeres only or anomalies in the trophectoderm layer (referred to as Troph Anom) in the presence or absence of extruded cells (Figure 2 Images A to D). The inner cell mass was clearly visible in all in vitro and in vivo produced blastocysts. Scoring of Class A in vitro produced blastocyst was accurate to 90%, an in vivo to 97% (Table 1). The falsely scored Class A blastocysts contained extruded blastomeres which were not visible until treatment with CytB, when upon collapse of the blastocoel due to the actions of CytB, the previously non-detected extruded cells were clearly visible. After subtraction of the number of falsely scored embryos, the corrected total of blastocysts that had re-expanded 18h after removal from the CytB treatment was 94% for IVP and 100% for in vivo produced Class A blastocysts. In Class B blastocysts, 80% of in vitro and 63% in vivo produced blastocysts were accurately classified as containing one or more extruded blastomeres, while the remaining blastocysts were of Class A morphology. Blastocysts containing extruded cells were less able to re-expand following CytB treatment with only 15% and 21% of IVP and in vivo derived embryos re-expanding after 18h of culture.

Table 1. Re-expansion rate of morphologically classified Day 6 in vitro and Day 5 in vivo produced porcine blastocysts following treatment with cytochalasin-B.

Origin	Quality Class	Morphology	n†	% of Total (n)	% False (n)	% Re-expanded post-CytB	
						Total	Corrected††
In vitro	Class A	Normal	104	49 (104/211)	10 (10/104)	85^{a} (88/104)	94^{a} (88/94)
	Class B	Extrusions	66	31 (66/211)	20 (13/66)	33^{b} (22/66)	15^{b} (9/66)
	Class B	*Troph Anom	41	20 (41/211)	15 (6/41)	37^{b} (15/41)	22^{b} (9/41)
In vivo	Class A	Normal	31	58 (31/53)	3 (1/31)	97^{x} (30/31)	100^{x} (30/30)
	Class B	Extrusions	19	36 (19/53)	37 (7/19)	58^{y} (11/19)	21^{y} (4/19)
	Class B	*Troph Anom	3	6 (3/53)	0 (0/3)	67^{y} (2/3)	67^{y} (2/3)

$^{a,b/x,y}$Within columns values with superscripts differ significantly ($P<0.05$); † Classification in vitro embryos expressed as percentage of all blastocysts produced, whereas in vivo is expressed as percentage of all blastocysts evaluated; †† Number of falsely scored embryos was subtracted from the total number re-expanding; *Troph Anom: trophectoderm layer anomalies

When blastocysts contained anomalies of the trophoblast layer, 45 and 67% respectively for in vitro and in vivo produced blastocysts, re-expanded. Of blastocysts produced in vitro, 85% were accurately scored for having anomalies of the trophectoderm layer, and 100% for in vivo derived embryos. The falsely scored blastocysts in this group could not be identified after CytB treatment, but upon re-expansion 18 h after CytB treatment these blastocysts could be accurately identified.

Blastocyst cell number

Blastocysts classified as Class A morphology contained significantly higher total cell numbers than Class B embryos, irrespective of the origin of the embryo as presented in Table 2. In vivo produced Class A blastocysts contained higher total and trophoblast cell numbers ($P<0.05$) than in vitro produced blastocysts, but a similar number of inner cell mass cells ($P>0.05$). Class B embryos did not differ for total, trophoblast or inner cell mass cell numbers irrespective of the embryos origin, but both these numbers were lower than those of Class A embryos. The ratio of inner cell mass : trophoblast cells differed according to the blastocyst classification, but not between in vivo or in vitro produced embryos.

Actin cytoskeleton of untreated Grade A and Grade B blastocysts

The actin cytoskeleton of Class A in vitro and in vivo produced blastocysts was largely similar in appearance and distribution (Figure 1 Images A and B). Actin quality of grade I was present in 73% of in vitro and 70% of in vivo Grade A blastocysts, with the remaining embryos containing grade II actin. No instances of grade III actin were observed. Actin microfilaments (MF) were localized at the contiguous cell borders in the trophoblast and inner cell mass cells, where they exhibited intense phalloidin staining. In vivo derived blastocysts showed very prominent MF staining on the boundary of the ICM, whereas it was less marked in the ICM of in vitro produced blastocysts.

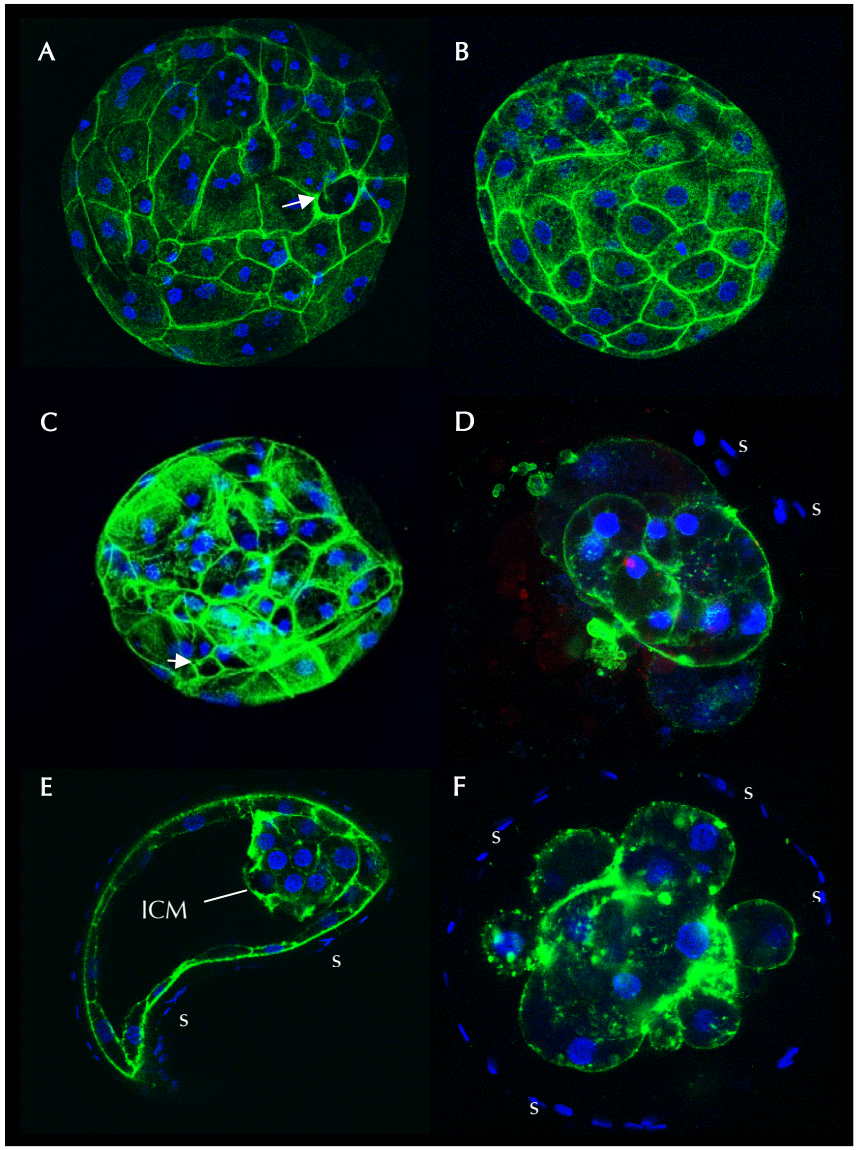

Figure 1. Multiphoton excitation microscopy images of in vitro and in vivo porcine blastocysts to show the actin microfilament (MF) architecture (green) and nuclei (blue). A to D: actin cytoskeleton quality grading. Grade 1 actin (A) in a Class A in vitro produced Day 6 blastocysts and (B) in a Class A in vivo produced Day 5 blastocyst, is typified by sharp phalloidin staining at the contiguous cell borders; Grade II actin in a Class B in vitro produced blastocyst (C) is characterized by less uniform distribution of actin in cell borders, with small clumps in the cytoplasm; note the irregular size and shape of blastomeres; D) a non-re-expanded Class B in vitro blastocyst displaying grade III actin in which the cell borders are poorly distinguishable and the cytoplasm has areas lacking actin staining with agglomerated clumps of MF; the arrow heads in A and C denote a round cell containing a nucleus but no MF within the cytoplasm. E) an in vivo produced blastocyst showing inner cell mass with clear peripheral actin border. F) a blastocyst fixed shortly after collapse of the blastocoel cavity following cytochalasin B treatment; note visible cell border and maintenance of cell volume, but with clumped actin in cytoplasm; s indicates perm on the zona pellucida.

A dense network of MF was present under the outer plasma membrane with fibers spanning the length and width of the blastomere and terminating at the cell border (stress fibers) present on the basal blastocoel-side of trophoblast cells (Figure 3. Image B). Less MF were seen within the inner cell mass cells, where they appeared as infrequent MF foci. Small vacuoles in the intracytoplasmic MF were often seen in one or more blastomeres, irrespective of the their origin and were not associated with the nucleus or lipid vesicles (Figure 3. Image A). In general, in vitro and in vivo blastocyst contained lipid droplets in the peri-nuclear area, but those in blastocysts of Class B were smaller in number, but larger in size than those present in blastocysts of Class A morphology.

Of Class B blastocysts, 55% of in vitro and 53% of in vivo blastocysts contained grade II actin in the embryo proper, which was significantly higher than that of Class A blastocysts. The remainder blastocysts contained grade I actin, with extruded cells exhibiting grade III actin. The cytoskeletal morphology for Class B embryos differed between in vitro and in vivo produced blastocysts. The former typically exhibited blastomeres of varying sizes and irregular shapes (Figure 1. Image C), and was characteristic of, but not limited to, Class B blastocysts with trophoblast anomalies. In vivo produced blastocysts, on the other hand, contained mainly evenly sized and shaped blastomeres (Figure 1. Images A and B). The ICM contained little intracellular MF which was mainly observed as aggregates. Extruded blastomeres contained mainly grade III actin, but scant or no actin was also observed in a number of extruded cells (Figure 3. Images C and D). The nuclei in the extruded cells were mainly intact, as judged by their exclusion of EthD-I staining. Blastocysts with extruded cells and/or anomalies in the trophoblast layer also did not contain any dead cells within the embryo proper. Round blastomeres containing a nucleus but no actin filaments was frequently seen in Class B blastocysts, but also occurred in Class A blastocysts although to a much lesser extent (Figure 1. arrows).

Actin cytoskeleton of CytB-treated Class A and Class B blastocysts

Treatment of blastocysts with CytB caused generalized depolymerization of actin microfilaments, upon which the actin aggregated into larger clumps (Figure 1 Image D). Cell borders were still visible, and individual blastomeres could easily be distinguished. Incubation of treated blastocysts in the absence of CytB, allowed re-polymerization of the actin to its original status in the greater majority of Class A embryos, irrespective of embryo origin, as evaluated by blastocoel restoration (Table 1). Of in vitro produced Class A blastocysts re-expanding after CytB treatment, the majority contained grade I and a small percentage grade II actin (Figure 4). Despite the apparent normality of the cytoskeleton, 20% of blastocysts contained one to four dead cells. Of blastocysts that did not re-expand after culture, 74% were incorrectly classified and contained extruded cells. These embryos also displayed mainly grade III actin, with a small percentage of grade II. Blastocysts that had not re-expanded despite containing grade I actin, presented no obvious abnormalities. Class B in vitro produced blastocysts, non-re-expanded after culture, contained mainly grade III actin, irrespective of the presence of extruded cells or anomalies in the trophoblast layer (Figure 3 Images C and D). When Class B blastocyst

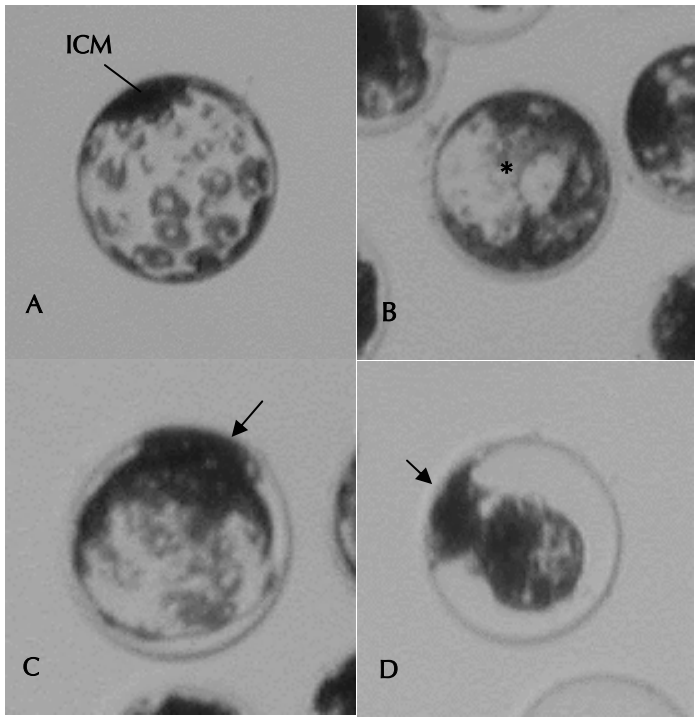

Figure 2. Digital micrograph of A) a Class A in vitro blastocysts with normal morphology; B) a Class B blastocyst with an anomaly of the trophectoderm layer as indicated by the asterisk; C) a Class B blastocyst with an extruded cell as indicated by the arrow; D) a Class B blastocyst with collapsed blastocoel shortly after treatment with cytochalasin-B, exposing a previously hidden extruded cell (arrow) (ICM = inner cell mass).

Table 2. Inner cell mass and trophoblast cell number of quality graded in vitro or in vivo produced porcine blastocysts

		n	ICM	Trophoblast	Total	ICM:trophoblast
In Vitro	Class A	27	12 ± 1^{a}	40 ± 2^{a}	52 ± 2^{a}	$1{:}4^{a}$
	Class B	27	4 ± 1^{b}	31 ± 2^{b}	35 ± 3^{b}	$1{:}9^{b}$
	Total	54	8 ± 1*	35 ± 2*	44 ± 2*	1:6
In Vivo	Class A	21	14 ± 1^{a}	51 ± 3^{c}	64 ± 3^{c}	$1{:}4^{a}$
	Class B	10	5 ± 1^{b}	37 ± 3^{b}	42 ± 3^{b}	$1{:}8^{b}$
	Total	31	11 ± 1	46 ± 3	57 ± 3	1:5

[a,b]Within columns values with superscripts differ significantly: P<0.01; *Within columns In vitro differs from In vivo: P<0.05

did undergo re-expansion after removal from CytB, they contained mainly grade I actin, but dead cells were also present in 33% of the embryos. In vivo produced Class A blastocysts, all of which had re-expanded, contained grade I actin in 85% of the cases. The remaining 25% which contained grade II actin, did not show any other obvious abnormalities. Class B in vivo re-expanded blastocysts contained all three grades of actin quality, but when grade I was present the blastocysts had been wrongly classified as Class B in 67% of the cases. Non-expanded blastocysts also contained all three grades of actin quality, and those with grade I also contained extruded blastomeres. Dead cells were not often seen, accept in non-expanded Class B embryos that originally presented loss of integrity between adjacent blastomeres. In general, Class A blastocysts regained their pre-CytB structure except for the presence of dead cells in 20% of the in vitro produced embryos. These dead nuclei also presented typical apoptotic morphology as they were fragmented or condensed (Figure 3 Image D), although non-EthD-1 stained apoptotic nuclei were also observed (Figure 3 Image C). No dead cells were seen in any of the in vivo produced Class A blastocysts.

Non-surgical Embryo transfer

Of 11 recipients receiving Class A blastocysts, 5 remained pregnant until Day 20, in comparison with only 2 out of 10 recipients receiving Class B embryo. One Class A recipient farrowed 6 normal piglets, of which one was stillborn following suffocation in the cervical canal. All piglets were male.

Table 3. Pregnancy results following non-surgical transfer of quality scored Class A and Class B in vitro produced porcine blastocysts.

	Class A Blastocysts	Class B Blastocysts	In vivo Control
n transfers	11	10	24
n embryos/recipient	30	30	30
Pregnant on Day 20 (%)	5 (46)	2 (20)	15 (63)*
Farrowed (%)	1 (9)	0 (0)	N/A
N offspring	6 (all male)	0 (0%)	N/A

*Pregnant on Day 35

Discussion

In this study we have demonstrated that classification of sow blastocysts produced in vitro, according to their morphological appearance, can identify those embryos which are similar in quality to their in vivo counterparts. In vivo produced blastocysts, harvested from the reproductive tract of the donor female, rarely displayed any morphological abnormalities thereby setting the standard for 'normal' morphological of the blastocyst. Morphological observation of the embryos by light microscopy revealed that 50% of in vitro produced blastocysts contained extruded cells, or anomalies in the trophoblast layer. The remaining 50% contained no abnormalities, and were also similar in their cytoskeletal architecture to in vivo produced blastocysts, as assessed by phalloidin staining of the actin microfilaments. Selection of the in vitro produced blastocysts prior to transfer, subsequently lead to the first live offspring farrowed after non-surgical transfer of in vitro produced blastocysts.

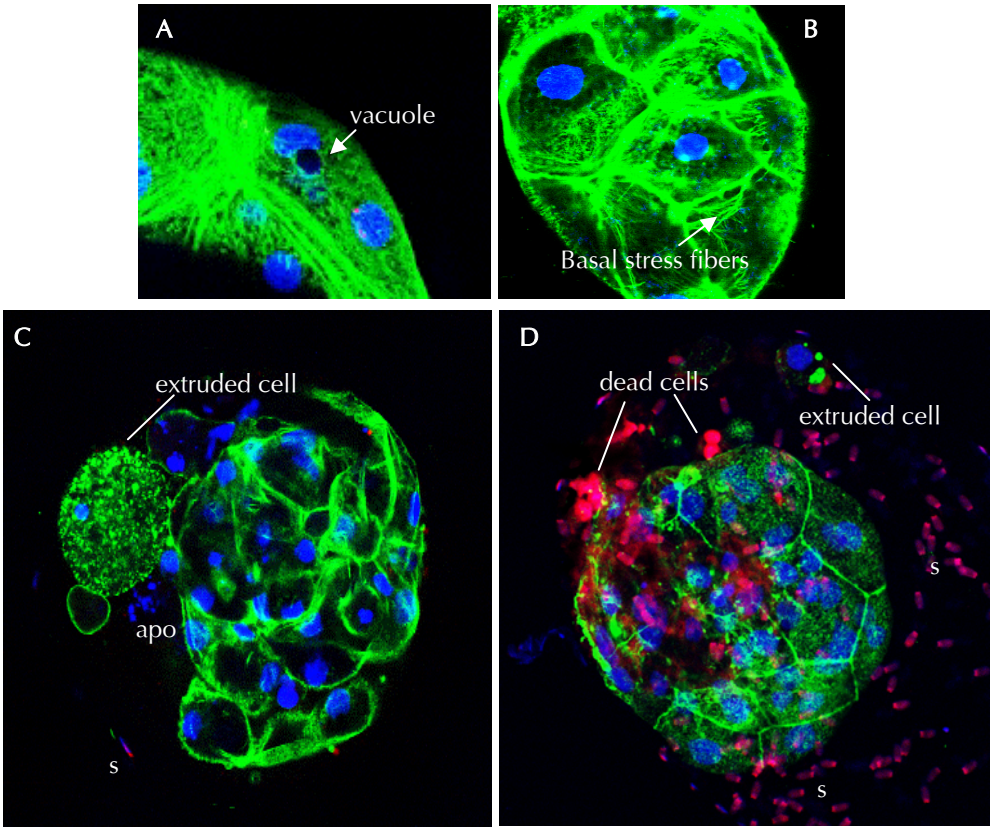

Figure 3. Multiphoton excitation microscopy images of in vitro and in vivo produced porcine blastocysts to show the actin microfilament (MF) architecture (green), nuclei (blue) and dead nuclei (red). A) a Class A in vitro blastocyst with a vacuole in the microfilament network as indicated; B) a Class A in vitro blastocyst showing the stress fibers present along the basal region of the blastomere; C) a Class B in vitro blastocyst with a large extrusion containing grade III actin, while grade II actin is present in the embryo proper; apo indicates a fragmented apoptotic nucleus; D) an in vivo produced Class B blastocyst with loss of integrity between blastomeres in the trophectoderm layer; note the dead nuclei labelled in red; s indicates sperm on the zona pellucida.

In the pig, the assessment of quality of in vivo produced blastocysts is mainly focused on the stage of blastocyst development in relation to their age, and retarded embryos are omitted from selecting for transfer purposes [28]. The fact that in vivo produced blastocysts hardly ever contain abnormalities, has simplified the establishment of selection criteria, as reported in this study. In vitro produced blastocyst were only regarded to be of Class A quality, when no morphological abnormalities are evident and were classified as Class B when any anomalies were observed, as evident by extruded blastomeres and trophoblast layer anomalies. Although it seems very strict, compared with the human [29] and bovine [30] in which a small degree of fragmentation or extruded cells is allowed, the presence of any abnormalities had a negative influence on porcine blastocyst cell numbers, as we previously reported [12]. Due to the narrow or

almost absent perivitelline space in pig blastocysts, the presence of flattened extruded cells or fragments could not be easily assessed. A tenth of in vitro produced blastocysts were subsequently incorrectly classified as Class A when in fact they contained extruded blastomeres, and Class B blastocysts believed to contain extrusions or anomalies in the trophoblast layer, 15 to 20% were inaccurately evaluated and deserved Class A classification. In the bovine, embryos are placed into a hypertonic sucrose solution in which the viable cells shrink and cellular fragments or extruded cells appear more clearly in the perivitelline space [31]. A similar effect was obtained in this study, when the embryos were exposed to CytB which caused the collapse of the blastocoel, upon which the extruded cells flattened against the zona pellucida became visible.

Blastocysts produced in vitro from prepubertal gilt oocytes contain fewer cells in the inner cell mass and trophoblast layer, but also a lower ratio of ICM:trophoblast cells than in vivo derived blastocysts [25]. Akin to prepubertal gilt blastocysts, non-classified sow in vitro produce blastocysts also contain lower total, ICM and trophoblast cell numbers than their in vivo counterparts as presented in this study. In vitro culture conditions can retard cell proliferation in embryos developing from ovulated oocytes fertilized and cultured in vitro to the blastocyst stage [12]. Delay of developmental progression in embryos derived from in vitro matured oocytes occurs from the first cell division, whereas in ovulated in vitro fertilized embryos it possibly occurs only at a later stage of in vitro embryo development [12]. Importantly, the ratio of ICM:trophoblast cells did not differ between in vitro and in vivo produced embryos in the present experiments. In vitro and in vivo produced blastocysts of Class A quality did not differ in ICM cell number, and also had a higher ICM:Trophoblast cell ratio, whereas Class B blastocysts contained a very low number of ICM cells overall, and also a lower ratio of ICM:trophoblast cells. The allocation of cells to the ICM thus appears to be determined by the morphological 'health' of the blastocyst, and is detrimentally affected by the presence of any anomalies in the blastocyst irrespective of the origin of the embryo.

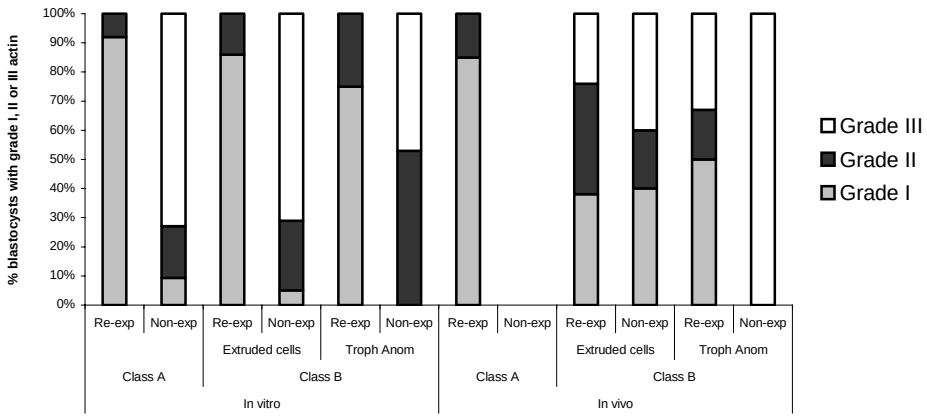

Figure 4. Actin skeleton quality of re-expanded and non-re-expanded Class A and B blastocysts, at 18 h of culture after removal from cytochalasin-B treatment.

The ability of embryos to undergo normal cell divisions has been correlated with both the actin content and distribution within the embryo [9, 10, 19]. The lower cell numbers experienced in embryos produced in vitro from prepubertal gilt oocyte has been ascribed to their smaller actin microfilament complement, and also to anomalies in the microfilament distribution which impairs blastomere cell division [9, 19]. The characteristics of the actin cytoskeletal architecture were similar for in vitro and in vivo blastocysts, and corresponded with that previously described by Albertini et al. [32]. In the present study, there was a direct association between actin quality and the morphology of the blastocyst. Class A in vitro and in vivo derived blastocyst showed grade I actin quality and only minor differences in the content of actin cytoskeleton were observed. In vitro produced blastocysts seemed to contain a slightly lower amount of actin, but as quantification of the actin content was not done in this study no conclusions can yet be made concerning this parameter. Class B embryos, on the other hand, presented grade II actin in the embryo proper of roughly half of the cases, which could be a reason for the lower cell numbers seen in Class B blastocysts. The observation that, in addition to the differences in actin quality, Class B blastocysts contained a large amount of enlarged lipid vesicles could also be indicative of their poor viability prognosis. Abnormally accumulated lipids present in sheep and bovine embryos produced in vitro, are enriched in polyunsaturated fatty acids (PUFAs) which constitutes a threat to sheep, bovine and pig embryos which preferentially avoid long-chain PUFAs in their endogenous reserves [33].

The actin quality was not only correlated with the morphology of the embryos, but also with their ability to re-expand following treatment with CytB. As far as we know this is the first report in which the response of blastocyst to CytB depolymerization of actin has been used as a test for evaluating blastocyst quality. The value of CytB treatment was twofold: firstly, collapse of the blastocoel cavity allowed for the re-classification of blastocysts when they contained a hidden extruded blastomere, and secondly, the ability of blastocysts to re-expand after removal from CytB showed a strong relation with the morphological Class assigned to the embryo and the quality of the cytoskeleton contained within the embryo. Cytochalasin-B challenge therefore supplies the opportunity to select blastocyst for quality and cytoskeleton according the their ability to re-expand after treatment, as blastocysts with abnormalities and a poor actin cytoskeleton cannot recover from the depolymerizing and collapsing effects of CytB.

When quality graded in vitro produced blastocysts were non-surgically transferred, a greater number of recipients were pregnant on Day 20 of gestation from Class A than from Class B blastocysts. In addition, the only female that remained pregnant and farrowed 5 live healthy piglets had received blastocysts of Class A quality. The low farrowing and embryonic survival rates for Class A transfers could be due to a number of embryo-related but also external factors. As transfer took place under field-conditions due to unforeseen circumstances, the embryo manipulation environment could not be strictly controlled. The main problem was maintenance of a constant temperature from transport to the transfer-site, until actual placement of the embryos into the uterus of the recipient. It was previously established that transport and storage at 25^{o}C was most suitable for porcine in vivo blastocyst [28, 34], although it has not been verified for in vitro produced blastocysts. Fluctuations up to 39^{o}C, caused by the light source of the stereomicroscope used to aspirate the embryos into the transfer catheter, could have been detrimental to embryo survival. On the other hand, correct synchronicity of the donor embryo and recipient reproductive tract are imperative for embryonic survival. Day 6 in vitro

produced sow blastocyst are largely comparable with Day 5 in vivo produced blastocyst with regard to morphology and cell number, as seen in this study, and thus transferred to recipients deemed suitable for Day 5 in vivo produced blastocysts. Although the synchronous transfer is suitable for in vivo derived embryos, different requirement may be relevant to in vitro produced embryos, especially as they contain fewer nuclei. These non-ideal circumstances surrounding the transfer procedures could also have contributed to the imbalance of sex ratio in the embryos developing to piglets. Male bovine embryos are known to show preferential survival over female embryos under adverse culture conditions [35, 36]. The fact that piglets were farrowed nevertheless verifies the technique of non-surgical transfer for in vitro produced embryos, and can only show improvement with further modification.

In conclusion, to our knowledge this study provides the first report of 1) a direct association between blastocyst morphology and the quality of the actin cytoskeleton, and 2) the application of cytochalasin-B challenge as a non-invasive technique to evaluate blastocyst quality in both in vitro and in vivo produced blastocysts. We have demonstrated that the greater majority Class A blastocysts contain grade I actin, whereas the presence of any anomalies in the gross morphology of the blastocyst to a large extent was correlated with poor actin quality. The ability of blastocysts to re-expand following depolymerization of the actin microfilaments was dependent on the morphology of the blastocyst, and thus associated mainly but not exclusively with the quality of the actin cytoskeleton. Class B blastocysts containing grade I actin in the presence of extruded cells or anomalies in the trophectoderm layer, did not survive CytB challenge, pointing to other unknown factors also contributing to the reduced resilience of the abnormal blastocysts. The fact that piglets were born from in vitro produced Class A blastocyst, despite the non-ideal transfer circumstances, provides a yet early, but encouraging verification of the enhanced viability of quality classified blastocysts.

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CHAPTER 8

Summarizing discussion

"In the shaping of a life, chance and the ability to respond to chance are everything."
Eric Hoffer, Philosopher, United States, 1902 – 1983

The application of advanced reproductive technologies in the pig, for both commercial and biomedical research purposes, requires large numbers of embryos at the various stages of development. Technological advancements in the genetic modification have generated much interest in the potential of the pigs as xenograft and embryonic stem cell donor [1]. These technologies require mature oocytes as well as embryos capable of developing to live offspring. The ultimate culture environment for pig embryos is the pig reproductive tract itself, but due to the complex nature of the female reproductive tract with its convoluted cervical canal, and long coiled nature of the uterine horns, the non-surgical retrieval of oocytes and embryos cannot be achieved. Surgical embryo collection is also not an option, due to animal welfare considerations and the labor intensive nature of the procedure. Ovaries collected from female pigs, slaughtered at abattoirs, provide a favorable alternative for the supply of large numbers of oocytes. Such oocytes, though, are still immature and require further maturation before they can be utilized for any of the purposes described above.

There are four factors believed to determine oocyte developmental potential, i.e. normal growth phase progression, adequate follicle cell support during maturation, completion of intracellular programming before fertilization and the functioning of oocyte checkpoint surveillance mechanisms [2]. Support of these processes, during in vitro culture, is of cardinal importance in promoting the ability of an oocyte to develop to a healthy individual. It wasn't until as recent as the early 1990s, that the first culture media capable of supporting oocyte maturation and embryo development to the blastocyst stage was developed for the pig [3]. These media were derived from that used for the culture of hamster embryos, and modified for the differential requirements of the pig embryo. Although piglets have been born from embryos produced entirely in vitro, from the dictyate stage oocyte to the blastocyst stage of embryonic development, the culture conditions do not model the conditions prevalent in vivo in the female reproductive tract. The percentage offspring produced from in vitro derived embryos remains less than 20%. A significant part of the embryonic mortality associated with in vitro production, appears to be a result of the inherent quality of the oocyte, combined with the deficient in vitro culture environments. Inadequate understanding of the intracellular processes fundamental to oocyte development form a significant cause of these imperfections and limits progress in the development of optimal in vitro embryo production methodologies. In pigs, the utilization of in vitro produced embryos is therefore dependent on the development of improved in vitro methodologies for oocyte maturation, fertilization and embryo culture.

The studies presented in this dissertation have explored various modifications of our core porcine in vitro embryo production system, by addition of selected biological supplements during in vitro oocyte maturation and the following phases of in vitro embryo production. Fundamental features of oocyte maturation and fertilization were studied in order to improve our understanding of the mechanisms critical to the overall developmental proficiency of resultant blastocysts. This chapter briefly summarizes the major findings, and their implications for future developments in porcine advanced reproductive technologies.

Oocyte donor age

When discussing porcine in vitro embryo production, a clear distinction should be made between the use of sow or prepubertal gilt oocytes. This stems from the inherent differences between the sow and prepubertal gilt oocytes which affect many aspects of oocyte and embryo development. Porcine in vitro oocyte maturation systems lack the in vivo dominant and preovulatory follicular development, which may contribute to the poor fertilization and embryo development results obtained in prepubertal gilt and sow oocytes. The later phases of follicular development, i.e. prematuration in the dominant follicle and final maturation in the preovulatory follicle are believed to advance cytoplasmic capacitation and determine the developmental capacity of the oocyte [4]. As prematuration can occur only in gonadotrophin-stimulated follicles [4], such as during the estrous cycle of the peri- or post-pubertal animal, the age of the donor animal is of key importance when exposing the immature oocyte to maturation conditions in vitro. The developmental potential of oocytes recovered from prepubertal animals is documented to be lower than that of adult animals in a number of species [5-7], including the pig [8-10], and is therefore possibly related to the lack of oocyte prematuration. In fact, in the bovine, the prepubertal oocyte is used as a negative model for studying oocyte developmental competence [11]. The most prominent characteristic associated with poor developmental competence of prepubertal oocytes is their poor ability to block polyspermy during fertilization [12], a trait not present in sow oocytes [8, 10]. Indeed, this is the 'trademark' abnormality experienced during prepubertal gilt IVP, for which no solution has been found to date.

As the greater majority of pig IVP laboratories have access only to prepubertal gilt ovaries, they are faced with a dilemma, but also a challenge, to overcome the seemingly insurmountable obstacle of polyspermy. Whereas nuclear maturation proceeds without much difficulty in prepubertal gilt oocytes, cytoplasmic differentiation is delayed or incomplete in vitro and believed to be the major cause of polyspermy. Ovulated oocytes collected from cyclic females and sow oocytes matured in vitro, are able to support up to 95% normal fertilization (<10% polyspermy) and close to 50% blastocyst development following IVF and culture in vitro [8, 10]. In contrast, only 40% normal fertilization, 40% polyspermy and 10 to 30% blastocyst development is achieved on average in prepubertal gilt oocytes. The intrafollicular environment from which the oocyte is harvested thus plays an elemental role in cytoplasmic maturation and developmental potential of the oocyte. Hence, the main challenge regarding prepubertal gilt IVP is the provision of an in vitro maturation environment which could augment the support of cytoplasmic maturation to at least the level attained in sow in vitro matured oocytes.

Oocyte maturation milieu

As mentioned earlier, the optimal culture environment for pig oocytes and embryos is the female reproductive tract. A compromise suitable for the in vitro culture situation may thus be fashioned by mimicking the prevailing in vivo conditions. Especially in the light of the modest and simple composition of porcine in vitro culture media, addition of in vivo-derived components were hypothesized to benefit the developing oocyte. In Chapter 2, we endeavored to create an in vitro maturation microenvironment more similar to that prevalent in vivo in the adult sow follicle by supplementation of the IVM medium with sow, as opposed to gilt follicular fluid. We found that sow follicular fluid was not only richer in estradiol and progesterone content, but also contained greater concentrations of growth hormone and LH, all of which are known to contribute to oocyte cytoplasmic maturation [13, 14]. In the 'sow-environment', the ability of the prepubertal gilt oocytes to support normal fertilization was improved and the quality of resultant blastocyst enhanced. In fact, these parameters were augmented to similar levels as those of ovulated oocytes used in the same experiment. Polyspermy, on the other hand, remained elevated to levels three times as high as those occurring in ovulated oocytes fertilized in vitro. Only when the oocytes were fertilized with an intact cumulus mass, did the incidence of polyspermy decrease and normal fertilization increase. Nevertheless, as long as the oocytes were matured with gilt FF blastocyst quality could not be improved. It therefore appears as though blastocyst quality is determined by an aspect of oocyte maturation, independent of its ability to promote normal fertilization and reduce polyspermy. Addition of sow follicular fluid to the maturation medium provides a simple means of improving the overall developmental efficiency of prepubertal gilt oocytes.

Although no major maturational difficulties are experienced with the use of sow oocytes, the developmental potential of sow oocytes is not yet optimal, as judged by the retardation in embryo developmental progression compared with embryos derived in vitro from ovulated oocytes (Chapters 6 and 7). Similar to prepubertal gilt blastocysts, cell numbers in sow blastocysts are also lower compared with those of in vivo derived blastocysts. Seeing that blastocyst quality, as evaluated by cell number, is determined by the oocyte maturation milieu, it is clear that sow oocytes could also benefit from improvements in cytoplasmic maturation. One similarity between prepubertal gilt and sow oocytes is the beneficial effects of a two-phase in vitro maturation system [15, 16] in which hormonal stimulation takes place only during the first part of IVM. This regimen improves the percentage of oocytes developing to metaphase II and increased blastocyst yield (Chapter 4). It has no effect on blastocyst cell number, and its value is therefore expressed in the more synchronous progression of oocyte nuclear maturation within the cohort of sow oocytes placed in to IVM. It therefore forms an integral part of the basic porcine IVM systems applied in both sow and prepubertal gilt IVP.

Sow oocytes, in contrast to prepubertal gilt oocytes, are not plagued by any abnormalities of excess, such polyspermy. In contrast, the main problem with sow oocytes found in this dissertation was low sperm penetration rates during IVF. Even though sperm concentrations 20x higher than those in gilt oocytes are used in the sow, with sperm-oocyte co-incubation times four times as long, penetration rates for sow oocytes rarely exceeded 40%. In an effort to improve the poor sperm penetration of sow oocytes, we designed an epithelial cell co-culture system (pOEC) in Chapter 5 to create an in vitro

maturation environment more similar to that found in vivo shortly after ovulation. In the female reproductive tract after ovulation, the oocyte comes into close contact with the oviductal epithelial cell layer, an environment known to have many beneficial effects on the oocyte's preparation for fertilization. Oviductal proteins are associated with the zona pellucida, perivitelline space and vitelline membranes of ovulated oocytes and oviductal embryos [17-19]. The exact functions of the oviductal secretions have not been fully established, but they are thought to contribute positively to oocyte final maturation and fertilization. When we co-cultured sow oocytes with pOEC, disappointingly we found no improvement in sperm penetration or normal fertilization rates. Blastocyst cell numbers were nevertheless improved, providing more evidence for the modulatory role that oocyte maturation microenvironment plays in the developmental outcome after IVF. When taking the whole pOEC co-culture system and combined results into consideration, its value for practical application is not entirely convincing. The fact will remain that the blastocyst cell numbers were improved, albeit by a margin of <15%, but the 30% reduction in the total blastocyst yield disqualifies it as a suitable methodology for improving in vitro developmental efficiency in sow oocytes.

A new gauge for cytoplasmic maturation?

Cytoplasmic maturation is a biologically fuzzy concept, and no clear-cut measures are available yet to gauge the degree of its progression. Although the glutathione content of the oocyte is a valuable indicator of cytoplasmic maturation [20], it cannot be measured in individual oocytes or correlated with any other aspects of oocyte morphology. The actin content of the oocyte cytoplasm is a factor worth mentioning, even though not directly studied in this dissertation, as its association with oocyte developmental potential has been established and shown to have far-reaching effects on the subsequent development of the embryo. Most developmental events, such as polar body release, nuclear migration, and embryo cleavage, are dependent on normal filamentous actin distribution [21-23]. It is also plays an important role in the distribution of some organelles, such as mitochondria [24] and Golgi complexes [25], ion channel regulation [26], and the expression of some mRNAs [27]. Ovulated oocytes [28] and oocytes recovered from preovulatory follicles display a continuous clear space in the cortical region of the ooplasm, whereas in vitro matured sow oocytes also have discontinuous clear areas present in only half of the ooplasm cortex, as reported in Chapter 5. When fertilizing oocytes by subzonal sperm injection, blastocysts developed mainly from those displaying heterogeneous cytoplasm [29]. In contrast prepubertal gilt in vitro matured oocytes have an evenly distributed, densely granulated ooplasm [28] similar to that of immature oocytes [Chapter 5]. These differences in the distribution of the oocyte cytoplasmic contents originate from the inherent differences in cytoskeletal morphology between prepubertal and adult oocytes [5], which influences actin-dependent migration of organelles, including the calcium stores of endoplasmic reticulum, to the periphery of the ooplasm [30, 31]. Prepubertal gilt oocytes contain a smaller pool of filamentous actin than their ovulated counterparts and show abnormalities in actin filament polymerization and distribution [32]. The anomalies of cleavage divisions caused by the actin-deficiency are thought to be a cause of lower cell numbers commonly found in prepubertal gilt blastocyst produced in vitro, but also in a proportion of sow blastocyst developing in vitro.

An actin-associated protein, called tropomyosin, is gaining interest during fertilization and subsequent stages of early mammalian development. Tropomyosin acts as an actin-binding protein associated with actin filament stabilization, and three isotypes have already been identified soon after fertilization [33] where it resides in the cortex region of newly-fertilized mouse oocytes. Tropomyosin may be involved in the regulation of organelle transport by serving as motor protein [34]. Any anomalies in its function could therefore have an impact on many functions within a cell, oocyte or embryo. In fact, disruption of tropomyosin expression in Drosphila oocytes inhibits mRNA localization [35]. It associates with the cleavage furrow later during cytokinesis in mammalian embryos, and is thought to function in a contractile role during cytokinesis of blastomeres [36]. Tropomyosin thus presents a possible target for identifying the cause of the proposed incomplete organelle migration, and its detrimental consequences in prepubertal gilt oocytes.

Playing devil's advocate on the overall wellbeing of current IVM systems

When taking into account the oocyte nuclear maturation kinetics data presented in Chapters 3 and 4, an important concern relating to the age of the MII oocyte at the time of IVF comes to light. Almost a third of all oocytes, irrespective of origin or maturation milieu, reached MII by 36 h of IVM. This leaves these oocytes 'overmatured' by 4 to 6 h, and thus quality-jeopardized, at the time non-capacitated sperm are added for IVF. Under ideal in vivo conditions the capacitated acrosome-intact sperm are awaiting the oocyte at the site of fertilization prior to ovulation. This in vivo mechanism maintains the stability of activation competence in the MII oocyte after ovulation [37]. If cortical granule exocytosis were to be initiated prematurely, prior to sperm penetration of the ZP, fertilizibility of the oocyte would be compromised, whereas premature progression to anaphase II (signifying parthenogenesis) followed by fertilization could jeopardize normal development of the zygote. Delayed fertilization in vivo causes a decrease in normal fertilization, and an increase in abnormal fertilization resulting in lower pregnancy rates and smaller litter sizes [38]. The temporal window for normal oocyte activation and fertilization is crucial for subsequent developmental potential, and may thus be a severely limiting obstacle during in vitro embryo production. Rapid changes in the oocyte cytoplasm take place during the pre- and post-ovulatory period, which narrows the window for optimal fertilization and subsequent normal development. Oocytes are deficient in activation competence prior to ovulation, and rather than losing competence, MII oocytes are hypothesized to become 'hypercompetent' for activation [37] and thus more sensitive to aberrations in activation following late sperm entry. Whether these 'overmature' oocytes form only a part or the whole of group of oocytes typically displaying aberrant chromosomal arrangements at 42 h of IVM, yet needs to be determined. How this temporal window for normal fertilization can be improved in current pig IVP systems will require careful scrutiny and deserves indepth investigation.

In vitro fertilization milieu - the blind leading the blind?

One area of porcine IVP which has been neglected to some extent, concerns the requirements for in vitro fertilization on both the oocyte and the sperm. Less than a decade ago, a fertilization medium was developed specifically for the pig (modified Tris-buffered medium; mTBM), and is now used with relative success in the greater majority of porcine IVP laboratories. Many of the procedures still standardly applied in pig IVF, such

as oocyte denudation, are based on the results of early studies before the advent of mTBM. Validation of these procedures under mTBM conditions are largely lacking in literature, and have only enjoyed a very modest amount of renewed interest during the past three years. The main aspects in need of attention are the status of the oocyte, i.e. presence or absence of cumulus cells, and sperm type, i.e. fresh or frozen-thawed, which are determining factors in the fertilization outcome both in vivo and in vitro.

In the pig, after ovulation, the cumulus surrounding the oocyte is largely intact and groups of ovulated oocytes clump together in a mass of mucified expanded cumulus [38]. Sperm-oocyte interactions occur at multiple levels on the COCs surface. During the final growth phase of the COC in the preovulatory follicle in vivo, cumulus cells synthesize the extracellular matrix (ECM) which consists of the mucified hyaluronic acid-rich cumulus mass and zona pellucida, and provides an important element of the creation of a fertilization-competent egg surface. Of specific importance to the prepubertal gilt oocyte, is the fact that ZP3 proteins have been detected both in the granulosa cells and the oocyte, and these sights of synthesis may influence the subcomponents of the zona pellucida matrix and their three-dimensional assembly. In the oviduct, sperm firstly encounter the oocyte's extracellular matrix, and secondly the zona pellucida and oocyte plasma membrane. It therefore seems logical that, as in the bovine, the pig oocyte should remain cumulus-intact for fertilization, but the procedure of oocyte denudation before in vitro fertilization is still standardly applied. The rationale for this procedure stems from early studies, in which sperm penetration consisted almost wholly of polyspermy [39-41]. This prompted researchers to denude the oocytes, which induced a degree of zona hardening and thus reduced the polyspermy rates by a small degree. In contrast to the early studies, recent reports [42, 43] as well as our results in Chapters 2 and 3, utilizing mTBM as IVF medium have shown a decline in penetration rate when oocytes were fertilized with an intact cumulus, but also a sharp reduction in polyspermy and an improvement in normal fertilization. In COCs matured with sow FF, polyspermy was reduced to less than 10%, which was similar to that of ovulated oocytes, but COCs matured in gilt FF nonetheless managed decrease polyspermic penetration by more than 50%, compared with oocytes denuded before fertilization. Increasing the sperm dose indeed improves sperm penetration rates in cumulus intact oocytes, but also increased polyspermy and decreased normal fertilization. This disappointing decrease in fertilization efficiency, and subsequent low blastocyst yield, limits its application in routine IVF systems.

The answer to the problems experienced with cumulus intact IVF might be found in the type and capacitation status of sperm used. The type of sperm applied for IVF, i.e. fresh or frozen-thawed, is known to have different requirements and effects during IVF leading to altered patterns of sperm penetration and fertilization [39, 41-45]. Yet, very few studies have examined the capacitation status of spermatozoa at the time of addition to the fertilization wells or drops [41, 42, 46]. In vivo, sperm are sufficiently prepared and functionally capacitated for fertilization by the time they reach the fertilization site in the oviduct [47-49] and upon encountering the newly ovulated COC, the sperm are fully equipped for penetrating the mucified cumulus mass surrounding the oocyte. In effect, the number of capacitated sperm at the site of fertilization in vivo number less than 100 per oocyte. During IVF, as applied in current porcine IVP systems, the sperm are generally not exposed to any capacitative treatments or pre-incubations and the large differences in sperm penetration between denuded and cumulus intact fertilized oocytes,

could thus possibly be attributed to the inappropriate capacitation status of the sperm. Following the standard wash steps performed for IVF in mTBM, at the start of IVF only 50-60% of sperm have an intact acrosome, and three hours later less than 10% of sperm have not undergone the acrosome reaction (Kidson, unpublished data). Only acrosome intact sperm can bind to the zona pellucida in the pig [48], and as only half of the sperm dose added to the oocytes is in the apparent correct capacitational status, it is no wonder that sub-optimal fertilization results are obtained during pig IVF. High concentrations of capacitated sperm during fertilization are believed to be a cause of polyspermy [38, 50] and indeed sow oocytes, appear to subject to threshold dose at which the oocyte cannot block multiple sperm entry any more (Kidson, unpublished data). Pre-treatment of sperm to suit specific IVF conditions in the pig is lacking in literature, compared with other domestic species such as the bovine, and represents a neglected field of study much in need of attention. By refining IVF conditions and establishing universal sperm capacitation techniques, the already encouraging results pertaining to embryo development and viability could be improved to a more satisfying degree.

Does polyspermy spell the end?

Polyspermy is a generic term describing the penetration of two or more sperm and can thus include di-, tri- and tetraspermy or even greater degrees of multiple sperm entry. Although polyspermy is widely believed to be a fatal condition, it is the location of the pronuclei before the first cell division which appears be the determining factor in the developmental fate of the zygote [51]. In Chapters 2 and 3 we observed four types of polyspermy (PPN), i.e. Types I to III, and Early PPN of which the latter two types had not been described in literature before. The pattern of polyspermy can be modulated by the maturational status of the oocyte (IVM conditions), as well as the dose and type of sperm used for IVF. In our studies we found that prepubertal gilt oocyte matured in gilt FF, exhibited a larger percentage of Type I polyspermy, in which all pronuclei participate in syngamy, than those matured in sow FF. The developmental outcome for Type I polyspermy is less optimistic than that of Type II polyspermic zygotes [51]. When one male and one female pronucleus are centrally located in apposition to one another, with supplementary pronuclei or sperm located eccentrically (Type II), the zygote is believed to be capable correcting ploidy and developing to term following embryo transfer [51]. This type of pronuclear arrangements was also found to be the most prevalent during polyspermy induced by in vitro fertilization conditions in our studies. The correction of ploidy was hypothesized to occur when any haploid cells died later during embryo development, leaving only diploid cells in the embryo. Blastocysts with extruded cells, in Chapters 2 and 3, may have originated from polyspermic zygotes, having eventually 'corrected' their original ploidy by discarding the unwanted supplementary pronucleus. Moreover, such extruded blastomeres in many cases appeared to contain pronucleus-like structures, and not interphase or condensed nuclei. In the treatment groups which had higher percentages of polyspermy, i.e. sow FF nude, and the gilt FF groups, a number of blastocysts appeared to contain no visible inner cell mass. Interestingly the groups containing this specific abnormality also were the only groups in which Type I polyspermy was observed. As piglets have been born from polyspermic zygotes [51], it is clear that this anomaly of fertilization does not spell the end. These hypotheses regarding the correlation between polyspermy, blastocyst morphology and survival after transfer need further careful investigation, which could in turn help to forecast the developmental potential of such embryos.

Embryo development and viability

When using modern culture media, such as mTBM and NCSU23, blastocyst development rates on par with that of the bovine can realistically be achieved [52]. This still only amounts to a rough average of 30% of all oocytes submitted to IVM, developing to the blastocyst stage. At first thought, it sounds as though 70% of all oocytes subjected to IVF are not capable of blastocyst development, but one needs to take into account all aspects of oocyte and zygote development in order to get a more accurate picture. Of ovulated oocytes subjected to IVF, in Chapters 2 and 3, close to 100% of normally fertilized zygotes developed to the blastocyst stage. This is comparable with the in vivo situation, where almost all ovulated oocytes survive and develop to the blastocyst stage by day 5 after ovulation. Reduction of the number of embryos only takes place during later development in the uterus. Sow or gilt oocytes matured in vitro, also readily develop to the blastocyst stage in percentages similar to the normal fertilization rate (Chapters 4 and 5). The problem with porcine IVP systems therefore seems to originate from the inherent poor developmental potential of the oocyte and/or suboptimal fertilization conditions. The embryo culture system is not without blame either, as in vivo matured and in vivo or in vitro fertilized oocytes, develop to blastocysts containing fewer nuclei than their uterine counterparts [Chapters 1, 2 and 7]. Recently it was reported that step-wise modification of the energy substrates improved the cell number and viability of in vitro produced prepubertal gilt blastocysts [53]. In their system, oocytes were fertilized with an intact cumulus mass, followed by incubation for two days in medium containing only Na-pyruvate and lactate as energy substrates conditioned by oviductal cells, and then for 4 days in culture medium with only glucose. Of 6 surgical transfers (50 blastocysts/recipient) all females became pregnant and farrowed on average 6 healthy piglets. It therefore seems as though the energy substrates are a contributing factor in the establishment of developmental potential of in vitro produced blastocysts.

In Chapter 6, when growth hormone (GH) was added to the sow embryo culture medium, contrary to reports in the bovine [54], we found no positive effects of GH on blastocyst yield or quality. This absence of effect could be due to the specific metabolic needs of the porcine embryo. Glucose has recently been shown to inhibit early prepubertal gilt embryo development in vitro, while a significant increase in glucose uptake takes place at the blastocyst stage embryos produced in vitro or in vivo [55]. In the mouse GH stimulates glucose uptake in blastocysts [56, 57] by direct recruitment of glucose transporters. Growth hormone also did not reduce the incidence of apoptosis in the blastocysts, as seen in the bovine [54]. Although only one or two blastomeres in an in vitro produced blastocyst are typically affected by apoptosis (Chapters 1 and 6), the fact remains that in vivo produced blastocysts do not present any apoptotic nuclei [58] and under ideal circumstances in vitro produced blastocysts should therefore also be apoptosis-free. The mere presence of glucose in the culture media at inappropriate stages of embryo development can directly trigger apoptosis, as seen in the mouse [59] and might be the cause of the low degree but persistent presence of apoptosis in porcine cultured blastocysts. Another clue to support this hypothesis is the presence of apoptosis in blastocysts produced in vitro from ovulated oocytes, as described in Chapter 2.

The most encouraging results derived from Chapter 6, was the observation that all pregnancies were established from largely expanded blastocysts, irrespective of their

origin or treatment. This provided the first clear marker for selection of blastocysts for transfer purposes. The presence of glucose at the blastocyst stage, combined with GH, had a positive effect on blastocoel expansion. This could be due to GH's effect via glucose transport in the Na^+/K^+ ATPase system [60, 61] which promotes blastocoel expansion, but also as a result of its direct activation of the sodium pump [62]. Although intriguing, the effects of GH on blastocoel expansion were marginal, and thus doubtful in their general applicative value for the pig embryo culture. Early pregnancy results (up to day 11) were comparable for control, GH-treated and in vivo derived embryos following non-surgical transfer, but survival of the in vitro produced blastocysts was very poor. Growth hormone supplementation of the embryo culture medium does therefore, under current culture conditions, not provide any specific benefit, but deserves further study as the growth hormone receptor mRNA is present throughout embryo development in the pig (Chapter 6) and therefore most likely has a elementary function.

Is (cellular) suicide (apoptosis) a cardinal sin?

In the previous section, we briefly touched upon the topic of apoptosis which was also the main theme of Chapter 6. Apoptosis, as evaluated by nuclear condensation and blebbing/fragmentation and DNA fragmentation (TUNEL labeling), has recently been used as a marker to evaluate preimplantation developmental potential in embryos [60, 63, 64]. In Chapter 2, we drew its validity as a universal embryo quality marker into question, due to the absence of correlation between apoptosis, blastocyst morphology, cell number and size. Indeed, the low number of apoptotic nuclei (typically 1 to 3/blastocyst; Chapters 2 and 6) found in porcine blastocysts produced in vitro suggests a negligible impact on embryo viability if any at all. However, its value as an indicator of the influence or suitability of a given culture environment, prevalence of pathological conditions or impact of culture medium supplements, should not be underestimated. The degree of apoptosis in blastocysts, prevalent in any given in vitro embryo production system, can therefore be used as a tool for indicating the effectiveness and suitability of the system to the needs and developmental potential of the embryos involved.

Detection of apoptosis in embryos, using the abovementioned techniques of TUNEL and nuclear morphology, does not allow for unambiguous identification of apoptotic cells. The term apoptosis refers to a myriad of intrinsic and extrinsic pathways which does have the 'suicide' of the cell as endpoint, but DNA fragmentation and nuclear blebbing occur only during the final phases of the process. If quantification of the extent of apoptosis is the main aim of the study, alternative biochemical means of identifying apoptotic cells should rather be considered. Mitochondrial membrane potential (caspase-activity) or expression and localization of apoptosis regulating-proteins (Bcl-2 family) could provide indications of the earlier stages of apoptosis and thus more appropriate and meaningful results.

The cause of apoptosis in porcine embryos produced in vitro is not entirely clear. As previously mentioned, inappropriate culture conditions could be one of the triggers, but could also have its origin in the maternal genome of the oocyte. In fact, it was recently reported that apoptosis does not require the paternal genome during preimplantation embryo development [65], with the machinery necessary for apoptosis thus being inherited from the oocyte. In addition to stored mRNA and proteins for early development, the oocyte also contributes maternally inherited components such as

mitochondria for mediating apoptosis [66]. Haploidy and retardation of development cause an increase in apoptosis in mouse embryos, and therefore pig embryos resulting from abnormal fertilization might show an increased degree of apoptosis. The extent to which haploidy-induced apoptosis can be generalized to other forms of aneuploidy is unclear at present, but warrants further investigation.

Blastocyst morphology and viability - the good, the bad and the ugly

Although it seems as though the blastocysts developing in vitro are derived from normally fertilized oocytes, they differ greatly in appearance and structure compared with in vivo produced pig blastocysts. Only one rigorous test can determine the ultimate quality of an embryo, and that is its survival following transfer to a suitable recipient. In Chapter 7, we combined and scrutinized the embryo development and evaluation data presented in the previous 5 chapters to identify the key elements central to blastocyst quality in the pig. In vivo produced blastocysts harvested from the reproductive tract of the donor female, rarely display any morphological abnormalities, thereby setting the standard for the normal morphological appearance of the blastocyst. In vitro produced blastocysts are therefore only regarded to be of Grade A quality, when no morphological abnormalities are evident. Although it seems very strict, compared with the human and bovine in which a small degree of fragmentation or extruded cells is allowed, the cell number and function of porcine blastocysts are negatively influenced by the presence of any abnormalities. When blastocysts were graded according to our criteria presented in Chapter 7, pregnancy rates following non-surgical transfer were increased and live healthy offspring born from one recipient. The overall survival of the blastocysts was very poor, but it is not clear whether it is due to the poor viability of the embryos or to the procedural difficulties experienced during the transfer project. All transfers in this dissertation took place under field conditions, which were not optimal for preventing embryo manipulation-related stresses and shocks. The encouraging aspect of this last transfer study was the fact that only 30 blastocysts were transferred to the recipients which still led to pregnancies. This is much lower than the number used for surgical transfers and is a promising indication of the applicability of our blastocyst quality scoring criteria.

Due the relative experimental nature of the non-surgical embryo transfer procedure and the cost and labor involved, an alternative method was therefore necessary to estimate or predict the overall health and wellbeing of in vitro produced porcine embryos. We subsequently developed a novel method for testing blastocyst function, namely cytochalasin-B (CytB) challenge. When blastocysts were exposed to this actin-filament depolarizing agent for a short period of time, until collapse of the blastocoel, and placed back into culture a direct correlation between blastocyst quality and its ability to re-expand after CytB removal was evident. Similar to the use of sucrose which causes shrinkage of the viable blastomeres, blastocoel collapse by CytB also allowed for more precise assessment of cells extruded in the perivitelline space and thus an accurate evaluation of blastocyst morphology. As CytB does not affect the viability of oocytes or embryos [67, 68], it is believed to be a non-invasive technique which could allow for the accurate selection of quality and function-proven blastocysts. Such embryos could then be applied for transfer or other purposes such as its use as embryonic stem cell donor.

Final remarks - ex ovo omnia -

The in vitro production of embryos is a complex assembly of procedures, based on our very limited knowledge and understanding of the intracellular processes fundamental to oocyte development. It is therefore quite remarkable that oocytes matured and embryos produced in vitro, still maintain a reasonable degree of function and developmental competence. In this dissertation we investigated a number of culture modifications, based on the conditions present in the female reproductive tract, to improve the developmental outcome of our IVP procedures. In vitro conditions cannot truly replicate in vivo conditions, and features of oocyte and embryo morphology, metabolic and biochemical properties can be altered by the in vitro environment. These alterations can become evident as errors that may be compatible with early embryonic development, but deleterious for the viability of the embryo. We have learned that treating the cause instead of the symptoms produces the most encouraging results. That is to say, the early development of the embryo appears to depend on the maturation microenvironment of the oocyte. By simply augmenting the oocyte culture environment with elements from the naturally occurring milieu in the mature sow follicle, significant improvements in the overall outcome of the IVP procedure can be obtained. As we become more sophisticated in our experience with in vitro embryo production in the pig, so will our knowledge increase and eventually improve our understanding and design of each separate phase of the IVP system for the pig.

"Keep sowing your seeds, for you never know which will grow, perhaps they all will."
Ecclesiastes 11:6

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Samenvatting

De toepassing van moderne voortplantingstechnieken bij het varken, zowel uit commercieel oogpunt als voor biomedisch onderzoek, is de laatste decennia sterk gegroeid. Daarnaast heeft de technologische vooruitgang op het terrein van de genetische modificatie de belangstelling voor het varken als donor van embryonale stamcellen en van organen voor xenotransplantatie doen toenemen. Voor het toepassen van deze technologieën zijn zowel gematureerde eicellen als embryo's nodig die zich tot een volwaardig individu kunnen ontwikkelen. De optimale kweek omgeving voor de eerste ontwikkeling van varkensembryo's is natuurlijk het geslachtsapparaat van de zeug zelf, maar door de complexe bouw van het vrouwelijke geslachtsapparaat van het varken is alleen chirurgische winning van eicellen en embryo's mogelijk. Echter, deze optie wordt ethisch als onwenselijk beschouwd. Een goed alternatief voor de winning van grote aantallen eicellen vormen ovaria die op het slachthuis kunnen worden verzameld. Dergelijke eicellen zijn echter nog immatuur en moeten verder gerijpt worden alvorens te kunnen worden gebruikt voor de hierboven vermelde doelstellingen.

Er zijn vier belangrijke factoren die de ontwikkelingscapaciteit van de eicel mede bepalen. Ten eerste een normaal groeiproces van de eicel zelf in de follikel, ten tweede adequate ondersteuning van follikelcellen gedurende de maturatie, ten derde completering van de intracellulaire programmering voorafgaand aan de bevruchting en ten vierde een goed verloop van de kritieke stappen in het controlemechanisme van de eicel. De ondersteuning van deze processen gedurende de maturatie in vitro zijn van essentieel belang voor het vermogen van de eicel om zich later, na bevruchting, tot een gezond individu te kunnen ontwikkelen. Hoewel er biggen geboren zijn afkomstig van embryo's die in vitro zijn geproduceerd, zijn de kweekomstandigheden nooit optimaal in vergelijking met de natuurlijke omstandigheden in de vrouwelijke genitaal tractus, hetgeen leidt tot een relatief hoge embryonale sterfte. Deze sterfte kan veroorzaakt worden door verschillende factoren zoals bijvoorbeeld onvoldoende intrinsieke kwaliteit van de eicel zelf en deficiënte kweekomstandigheden. De toenemende vraag naar in vitro productie (IVP) van varkensembryo's gaat derhalve gepaard met de noodzaak om de in vitro methodes voor eicelrijping (IVM), bevruchting (IVF) en embryokweek (IVC) te verbeteren. Het onderzoek dat in dit proefschrift wordt beschreven, bestudeert aanpassingen aan het standaard IVP protocol bij het varken. Er is met name aandacht besteed aan specifieke toevoegingen aan het medium gedurende de in vitro rijping van eicellen en de daaropvolgende fases van de IVP van varkensembryo's. Daarnaast is onderzoek gedaan naar fundamentele aspecten van de eicelrijping en eicelbevruchting om het inzicht in de mechanismen die essentieel zijn voor de ontwikkelingspotentie van de gevormde blastocysten te vergroten.

Bij de bestudering van de in vitro embryoproductie bij het varken moet een duidelijk onderscheid gemaakt worden tussen het gebruik van eicellen afkomstig van zeugen of van prepuberale gelten. Er zijn kenmerkende verschillen tussen eicellen afkomstig van oude en prepuberale dieren die van invloed zijn op de ontwikkelingspotentie van de

gewonnen eicellen. Deze ontwikkelingspotentie is namelijk bij een aantal diersoorten, waaronder het varken, bij prepuberale dieren lager dan bij volwassen dieren hetgeen mogelijk wordt veroorzaakt door een onvoldoende pre-maturatie. Het meest opvallende kenmerk van een dergelijke gebrekkige ontwikkelingscompetentie van prepuberale eicellen is het beperkte vermogen om tijdens het fertilisatieproces polyspermie te voorkómen. Dit vermogen is bij eicellen van zeugen wel goed ontwikkeld. Echter, bijna alle varkens-IVP laboratoria maken gebruik van ovaria van prepuberale gelten en staan voor het dilemma dan wel de uitdaging om het ogenschijnlijk onontkoombare probleem van polyspermie op te lossen. Terwijl de kernrijping bij eicellen van prepuberale gelten zonder al te veel probleem verloopt, is de cytoplasmatische differentiatie in vitro vertraagd of incompleet. Waarschijnlijk ligt hier één van de hoofdoorzaken van de polyspermie. De intrafolliculaire omgeving waaruit de eicel afkomstig is speelt een elementaire rol in de cytoplasmatische rijping en de ontwikkelingspotentie van de eicel en derhalve zouden voor een adequate in vitro ontwikkeling de in vivo condities zoveel mogelijk nagebootst moeten worden. De toevoeging van "in vivo componenten" aan de relatief eenvoudige samengestelde IVF /IVC media bij het varken, zou een manier kunnen zijn om de ontwikkelingspotentie van in vitro gematureerde eicellen te bevorderen. In Hoofdstuk 2 is het IVM medium zodanig aangepast dat het meer overeenkomt met de in vivo situatie van de volwassen zeug door toevoeging van follikelvloeistof afkomstig van zeugen in plaats van gelten. De follikelvloeistof afkomstig van zeugen bleek niet alleen meer oestradiol en progesteron te bevatten, maar ook hogere concentraties groeihormoon (GH) en LH; alle factoren waarvan bekend is dat zij een positieve bijdrage leveren aan de eicelrijping. In deze "zeugomgeving" is het vermogen van de eicellen van prepuberale gelten om het bevruchtingsproces normaal te laten verlopen verbeterd, evenals de kwaliteit van de gevormde blastocysten. Deze kwaliteit is vergelijkbaar met die van blastocysten verkregen na de bevruchting van in vivo gematureerde en geovuleerde eicellen. Echter, polyspermie blijkt drie keer vaker bij in vitro gerijpte eicellen voor te komen dan bij de in vivo gematureerde en geovuleerde eicellen. Alleen bij eicellen die worden bevrucht in aanwezigheid van een intacte cumulus is de incidentie van polyspermie verlaagd. Daarnaast kan de kwaliteit van de gevormde blastocysten niet verbeterd worden zolang eicelrijping plaatsvindt in aanwezigheid van follikelvloeistof van gelten. De kwaliteit van de blastocyst lijkt te worden bepaald door aspecten van eicelrijping die onafhankelijk zijn van het vermogen om normale bevruchting te bevorderen en polyspermie te verminderen. Toevoeging van follikelvloeistof afkomstig van zeugen aan het maturatie-medium van eicellen van prepuberale gelten blijkt een eenvoudige manier om de algehele maturatie-efficiëntie van de laatste groep te bevorderen.

De cumulus die de eicel na ovulatie bij het varken omgeeft is grotendeels intact en groepjes geovuleerde eicellen clusteren tezamen tot één massa met muceuze geëxpandeerde cumulus. Gedurende de laatste groeifase van het COC in de pre-ovulatoire follikel, synthetiseren de cumuluscellen de extracellulaire matrix die bestaat uit een muceuze, hyaluronzuurrijke cumulusmassa. Deze cumulusmassa is, tezamen met de zona pellucida, van groot belang voor het creëren van een goede bevruchtingsmogelijkheid. In de eileider komt de zaadcel eerst in contact met de extracellulaire matrix van de eicel alvorens de zona pellucida en dan de plasmamembraan van de eicel te bereiken. Het lijkt daarom logisch dat de eicel van het varken, evenals bij het rund, tijdens het fertilisatieproces in vivo nog een intacte cumulus heeft in tegenstelling tot de huidige standaard IVF procedure waarbij de cumulus

verwijderd wordt. Onze onderzoeksresultaten, beschreven in Hoofdstuk 2 en 3, tonen een afname van de spermapenetratie van eicellen wanneer de eicel worden bevrucht wanneer de cumulus nog intact is. Daarnaast neemt het percentage normaal bevruchte eicellen toe. Bij COCs gematureerd in aanwezigheid van follikelvloeistof van zeugen, komt polyspermie in minder dan 10% van de gevallen voor, hetgeen vergelijkbaar is met de situatie bij in vivo gematureerde en geovuleerde eicellen. Wanneer COC's in aanwezigheid van follikelvloeistof afkomstig van gelten worden gematureerd in vitro, treedt er ruim 50% minder polyspermie op in vergelijking met eicellen die bevrucht zijn nadat de cumulus verwijderd is. Een verhoging van de spermadosis leidt tot verhoging van de penetratiegraad bij cumulus intacte eicellen maar, helaas, ook tot een toename van de polyspermie en een afname het bevruchtingspercentage. Deze teleurstellende afname in de efficiëntie van het fertilisatieproces en de daarop volgende lage opbrengst van blastocysten beperkt de routinematige toepassing van IVF bij het varken.

Polyspermie is een algemene term die aangeeft dat twee of meer spermacellen de eicel binnendringen. Hoewel aangenomen wordt dat polyspermie niet kan leiden tot een verdere ontwikkeling van de bevruchte eicel, blijkt vooral de lokalisatie van de pronucleus voor de eerste celdeling de bepalende factor voor de ontwikkeling van de zygote. In Hoofdstuk 2 en 3 beschrijven we vier vormen van polyspermie, waarvan er twee nog niet eerder beschreven zijn. Het patroon van polyspermie kan beïnvloed worden door het rijpingsstadium van de eicel en door het type en de dosis van het sperma dat gebruikt wordt voor IVF. Wij hebben gevonden dat eicellen van prepuberale gelten, gerijpt in follikelvloeistof van gelten, een hoger percentage van het type I polyspermie vertonen dan de eicellen van prepuberale gelten die gematureerd zijn in follikelvloeistof van zeugen. Bij dit type polyspermie, waarbij alle pronucleï deelnemen in de syngamy, is het uiteindelijke resultaat minder gunstig dan bij zygoten met type II polyspermie. Bij dit type zijn de mannelijke en vrouwelijke pronucleï gelokaliseerd, tegenover elkaar, terwijl de overige pronuclei excentrisch zijn gepositioneerd. Een dergelijke zygote wordt in staat geacht de polyploidy te corrigeren en zich normaal verder te ontwikkelen na embryotransplantatie. Dit type II polyspermie is ook in onze IVF-studies het meest waargenomen. Men neemt aan dat cellen met een abnormale kerninhoud later in de embryonale ontwikkeling afsterven. Blastocysten met uitgestoten cellen (Hoofdstuk 2 en 3) zijn hier waarschijnlijk een voorbeeld van. In de behandelgroepen waarbij polyspermie in hoge percentages voorkomt, blijkt een aantal blastocysten geen waarneembare inner cell mass te hebben. Het is opmerkelijk dat alleen in deze groepen het type I polyspermie is waargenomen. De correlatie tussen polyspermie, morfologie van de blastocyst en levensvatbaarheid na embryotransplantatie vraagt uitgebreid nader onderzoek om de ontwikkelingspotentie van IVP varkensembryo's beter te kunnen voorspellen.

Hoewel er geen duidelijke moeilijkheden tijdens in vitro rijping van eicellen van zeugen zijn ondervonden, is het aantal cellen in IVP blastocysten lager dan bij in vivo geproduceerde blastocysten. Dit verschil in blastocyst kwaliteit onderstreept nogmaals de wenselijkheid om de cytoplasmatische rijping in vitro te verbeteren. Een positief effect zowel voor eicellen van prepuberale gelten als voor die van zeugen kan bereikt worden door de in vitro maturatie in twee fasen te laten verlopen, waarbij in de eerste fase hormonale stimulatie plaatsvindt. Een dergelijk eicelkweeksysteem verhoogt het percentage eicellen dat doorgroeit tot aan metafase II en verhoogt de opbrengst aan blastocysten (Hoofdstuk 4). Echter, dit systeem heeft geen effect op het aantal cellen per

blastocyst. De waarde van zo'n systeem komt dus vooral tot uitdrukking in synchronisatie van de kernrijping van de eicel binnen het cohort van eicellen die gebruikt worden voor in vitro maturatie. Het kan daarom als integraal onderdeel van het standaard IVM-systeem ingezet worden bij de in vitro maturatie van eicellen van zowel zeugen als gelten.

Bij eicellen van zeugen komen na bevruchting, in tegenstelling tot de situatie bij gelten, minder abnormaliteiten zoals polyspermie voor. Het voornaamste probleem met zeugen-eicellen in ons onderzoek is de lage sperma-penetratiegraad gedurende IVF. Om deze penetratiegraad te verbeteren hebben we een co-cultuursysteem ontwikkeld met oviduct epitheliale cellen (pOEC) (Hoofdstuk 5). Hierbij wordt een micromilieu geschapen dat meer lijkt op de in vivo situatie. Echter, dit systeem blijkt geen verbetering te geven van de spermapenetratiegraad noch van het percentage normaal bevruchte eicellen maar leidt wel tot een verhoging van het aantal cellen per blastocyst. Dit toont aan dat de invloed van de micro-omgeving van de eicel tijdens het rijpingsproces een rol speelt in de ontwikkelingsresultaten ná IVF. Aangezien de 15% toename van het aantal cellen in de blastocyst samen gaat met een 30% verminderde blastocyst-opbrengst, leidt dit pOEC-systeem niet tot een verbetering van de efficiëntie van de IVF procedure.

Hoofdstuk 6 beschrijft de effecten van de toevoeging van groeihormoon (GH) aan het IVC-medium. In tegenstelling tot de bevindingen bij het rund hebben we geen positief effect van GH op de kwaliteit noch op de opbrengst van blastocysten gevonden. Het achterwege blijven van het effect zou kunnen liggen aan een specifieke metabole behoefte van het varkensembryo. Recent is aangetoond dat glucose de ontwikkeling embryo's van prepuberale gelten remt, terwijl een significante toename in glucoseopname plaatsvindt zowel bij blastocysten geproduceerd in vitro als in vivo. GH vermindert ook de incidentie van geprogrammeerde celdood, apoptose, niet. Alhoewel apoptose bij niet meer dan één tot twee cellen per in vitro geproduceerde blastocyst blijkt voor te komen (Hoofdstuk 2 en 6), is het opvallend dat dit fenomeen bij in vivo geproduceerde blastocysten niet is waargenomen. Het meest bemoedigende resultaat uit Hoofdstuk 6 is de bevinding dat de meeste drachten tot stand komen na transplantatie van geëxpandeerde blastocysten, onafhankelijk van hun oorsprong of behandeling. Dit biedt ons het eerste duidelijke criterium voor de selectie van blastocysten voor embryotransplantatie bij het varken. De aanwezigheid van glucose in combinatie met GH in het blastocyststadium heeft een positief effect op de expansie van de blastocoel. Hoewel intrigerend, lijkt het effect van GH op deze expansie marginaal en mogelijk is de toepassing van dit hormoon bij varkens IVC dan ook overbodig. Drachtigheidspercentages 11 dagen na niet-chirurgische embryotransplantatie waren vergelijkbaar voor controle en GH behandelde IVP embryo's en voor embryo's die in vivo zijn verzameld. De uiteindelijke overleving van de in vitro geproduceerde blastocysten was echter slecht. Samenvattend, lijkt de toevoeging van GH aan het kweekmedium dus geen specifiek positief effect te hebben. Echter, aangezien GH receptor mRNA aanwezig is in het embryo (Hoofdstuk 6) en waarschijnlijk ook een elementaire functie heeft, is nadere studie gewenst.

Uiteindelijk is er maar één test die de ultieme kwaliteit van een embryo bepaalt en dat is de overleving na transplantatie in een geschikte ontvangster. In Hoofdstuk 7 zijn de embryo-evaluatiemethodes, zoals beschreven in de vijf voorgaande hoofdstukken, gecombineerd en onderzocht om essentiële factoren die van belang zijn voor de bepaling

van de embryokwaliteit te identificeren. In vivo geproduceerde embryo's vertonen zelden afwijkingen en aangezien bij IVP varkensblastocysten de aanwezigheid van één enkele afwijking al een duidelijk negatief effect heeft, worden in vitro geproduceerde varkensblastocysten alleen als kwaliteit A beoordeeld wanneer geen enkele morfologische afwijking wordt waargenomen. Dit in tegenstelling tot de situatie bij de mens en het rund, waar een geringe mate van fragmentatie of uitstoting van cellen bij kwaliteit A embryo's wordt geaccepteerd. Door toepassing van de in Hoofdstuk 7 vermelde selectiecriteria, zijn de resultaten na non-chirurgische embryotransplantatie verbeterd en zijn er gezonde biggen ter wereld gekomen. Echter de vroeg-embryonale sterfte is hoog en het is onduidelijk of dit te wijten is aan de embryo-kwaliteit of aan procedurele moeilijkheden tijdens de transplantatie. Alle in dit proefschrift vermelde transplantaties hebben namelijk onder praktijkomstandigheden plaatsgevonden, hetgeen niet de meest optimale situatie is om stress tijdens de handelingen met het embryo te voorkómen. Echter, het feit dat bij transplantatie van slechts 30 blastocysten per ontvangster toch drachten tot stand zijn gebracht, is zeer bemoedigend voor het door ons gebruikte kwaliteit-scoringssyteem, aangezien dit aanzienlijk minder embryo's zijn dan er bij chirurgische transplantatie routinematig worden overgezet.

Aangezien de non-chirurgische embryotransplantatiemethode een experimentele en kostbare methode is, is er door ons een alternatieve methode ontwikkeld om de kwaliteit van IVP embryo's te beoordelen. Bij deze methode wordt de vitaliteit van de blastocyst getest met behulp van cytochalasine-B (CytB). Deze stof depolariseert de actine-filamenten en wanneer blastocysten gedurende korte tijd aan deze stof worden blootgesteld, collabeert de blastocoel. De kwaliteit van de blastocyst wordt gecorreleerd aan het vermogen om, na terugplaatsing in controlemedium, te re-expanderen. Net zoals bij het gebruik van sucrose, dat ook tot de verschrompeling van blastocysten leidt, was ook bij toepassing van CytB een nauwkeurige beoordeling van de uitstoting van cellen in de perivitteline ruimte, en dus een meer accurate beoordeling van de morfologie van de blastocyst, mogelijk. Daar CytB de levensvatbaarheid van eicellen of embryo's niet nadelig beïnvloedt, wordt aangenomen dat deze niet-invasieve techniek een betrouwbare en accurate selectie op kwaliteit van blastocysten mogelijk maakt. Dergelijke embryo's kunnen dan geselecteerd worden voor transplantatie of voor andere doeleinden, zoals het doneren van embryonale stamcellen.

Tenslotte, de in vitro productie van embryo's is een geheel van complexe procedures waarbij nog vele intracellulaire processen van belang voor de ontwikkeling van de eicel onbegrepen zijn. Het is dan ook opmerkelijk dat in vitro gerijpte eicellen en in vitro geproduceerde embryo's toch nog een zekere mate van ontwikkelingscompetentie hebben. In deze dissertatie zijn de in vitro kweekomstandigheden aangepast, gebaseerd op de omstandigheden in vivo met het doel de uiteindelijke resultaten van de in vitro productie-procedures te verbeteren. De in vitro omstandigheden kunnen slechts deels de in vivo condities nabootsen met als gevolg dat morfologische kenmerken van de eicel en het embryo, evenals hun metabole en biochemische eigenschappen nadelig worden beïnvloed. Door negatieve invloeden weg te nemen in plaats van symptomen te bestrijden, zijn er bemoedigende resultaten geboekt. In het kort: de ontwikkeling van het jonge embryo is sterk afhankelijk van het micro-milieu waarin de eicel zich tijdens de rijping bevindt. Door het in vitro kweekmilieu te supplementeren met factoren die in vivo aanwezig zijn in de follikel van de zeug, zijn belangrijke verbeteringen in het uiteindelijke resultaat van de IVP van varkensembryo's bereikt. Als onze ervaring met

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….Soli Deo Gloria!

Curriculum Vitae

Annadie Kidson was born on 10 April 1971 in Johannesburg, Gauteng, South Africa. She graduated from high school in 1988, (Randburg, Gauteng) after which she spent a year travelling and working before commencing her university education at the University of Stellenbosch, Western Cape in 1990. She obtained the degrees Bachelor of Science (BSc) and BSc-honours in Human and Animal Physiology and Biochemistry in 1993 and 1994. In 1998 she graduated MSc *cum laude* on the subject "The application of assisted reproductive technologies for the production of cape buffalo (*Syncerus caffer*) embryos". During her MSc she was employed as an embryologist by African Embryos and Semen, an embryo export company, and later for a year (in 1996) as a Junior Scientist at the Medical Research Council, Parow, South Africa, investigating male contraceptives. She spent 6 months at the Catholic University of Leuven, Belgium, in 1997 as a research/exchange scholar. In 1998, she took a year's break from science and worked as a care assistant for disabled, stroke and elderly infirm patients in London, UK, before returning to South Africa. In 1999, she once again returned to Europe and started her PhD study as a research assistant (Assistent-in-Opleiding; AiO) at the Dept of Farm Animal Health, Utrecht University (December 1999) to investigate the influence of the oocyte maturation environment on embryo development and quality in the pig (described in this thesis). During her PhD she also spent 4 months at the Gamete and Germplasm Physiology Laboratory of the United States Department of Agriculture, in Beltsville, Maryland. She completed her PhD in March 2004 and returned to South Africa where she was appointed as Senior Scientist at the Diabetes Research Group of the Medical Research Council.

In vitro embryo development in the pig: impact of oocyte maturation milieu on blastocyst morphology and viability

Annadie Kidson