In vitro production of horse embryos: fundamental aspects

Jordi López Tremoleda

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In vitro produktie van paardenembryo's:
fundamentele aspecten
(met een samenvatting in het Nederlands)

Proefschrift

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"What does a fish know about the water in which he swims all his life?"

"¿Qué sabe el pez del agua en que nada toda su vida?"

Albert Einstein

"Penseu que el mirall de la veritat s'esmicolà a
l'origen en fragments petitíssims, i cada un dels
trossos recull tanmateix una engruna d'autèntica llum"

Salvador Espriu

A la meva família,

Als meus amics, amigos and friends,

To Mart,

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Chapter 1

General introduction

The development of techniques for assisted reproduction in horses has increased steadily over the past two decades. The desire of breeders to produce, and the consent of many studbooks to register, multiple foals from the same dam in a given year, has made it possible to apply techniques like embryo transfer (ET) commercially, and in countries like the USA, Argentina and Brazil large numbers of embryos are now collected and transferred fresh or after cooled transport. In Europe, the commercial expansion of ET has been hindered by high costs, due primarily to the need to house large numbers of recipient mares, and the unwillingness of breed associations like the Thoroughbred studbooks to allow the use of any artificial breeding techniques. Nevertheless, the increasing popularity of other equestrian sports such as show jumping, dressage-riding and harness-racing has encouraged the adoption of assisted reproductive techniques to accelerate genetic selection for performance criteria. In the Netherlands, the horse breeding industry introduced artificial insemination (AI) on a large scale some 20 years ago, partly as a means of controlling the spread of venereal diseases but also to allow more rigorous selection and widespread use of the best stallions. The success of AI has emphasized the fact that there is little selection of mares, a shortcoming that could be solved by the application of ET and related techniques.

Prime candidates for ET include older mares that have proven their worth as broodmares or in competition, and younger animals that are still competing. Of course, a proportion of the older mares are likely to be sub-fertile, often due to uterine degeneration, and may not be able to carry their own foal to term. In other cases, embryo recovery may be unrewarding and an alternative is to collect oocytes, fertilize them *in vitro* and culture the resulting embryos until they are ready for transfer to a recipient mare. Indeed, in several livestock species, *in vitro* embryo production (IVP) is preferred to multiple ovulation and ET (MOET) because it offers greater flexibility of breeding strategy (Galli *et al*., 2003; Long *et al*., 2003). In equids, however, the commercial application of IVP has been severely restricted by poor success rates (Hinrichs, 1998; Squires, 2000; Squires *et al.*, 2003), particularly with respect to conventional *in vitro* fertilization (IVF). This is unfortunate given that the horse is one of the few domestic species in which the individual is often valuable enough to warrant commercial use of these techniques.

Assisted reproduction has for varying reasons become commonplace in a number of species. For example, the cattle breeding industry has benefited greatly from the introduction of MOET and IVP, techniques that have allowed the production of large numbers of calves from high genetic merit cows and, by using pre-pubertal calves as oocyte donors, a reduction in the generation interval and corresponding acceleration in the rate of genetic progress (Galli *et al*., 2003; Merton *et al*., 2003).

In man, assisted reproduction has revolutionized the treatment of sub-fertility (recent surveys estimate that one in four European couples have difficulties in conceiving). Indeed, conventional IVF was established as an effective fertility treatment very soon after the birth of the first IVF baby (Steptoe and Edwards, 1978) and, more recently, intracytoplasmic sperm injection (ICSI: Palermo *et al*., 1992) has become accepted as the treatment of choice for male factor infertility (Campbell and Irvine, 2000).

This introductory chapter reviews the state-of-the-art in the different techniques used to produce horse embryos *in vitro*.

***In vitro* maturation of horse oocytes**

Progress in *in vitro* maturation (IVM) of equine oocytes has been hampered by the difficulty in collecting oocytes in large enough numbers to perform meaningful experiments. Although harvesting oocytes from preovulatory follicles by transvaginal ultrasound-guided follicle aspiration (ovum pick-up: OPU) is fairly successful (recovery rates of 51- 86%: Cook *et al*., 1993; Bézard *et al*., 1995; Meintjes *et al*., 1995; Goudet *et al*., 1997; Scott *et al*., 2001), oocyte yields are low because most mares only produce one preovulatory follicle per oestrus (Bézard *et al*., 1995). In addition, recovery of immature oocytes by OPU is relatively unsuccessful (18-35%: Brück *et al*., 1992; Bracher *et al*., 1993; Cook *et al*., 1993; Duchamp *et al*., 1995; Alm *et al*., 1997; Goudet *et al*., 1997) because horse oocytes are attached to the follicle wall by a broad-based cumulus hillock and are therefore difficult to detach (Hawley *et al*., 1995; Brück *et al*., 1999). The low availability of preovulatory follicles ideal for aspiration has not been helped by the failure to develop treatments to reliably stimulate multiple follicle development; although multiple ovulation has been induced with crude equine pituitary extract, initial very promising results were not so readily repeated (Alvarenga *et al*., 2001; Scoggin *et al*., 2002). For these reasons, most studies on IVM use oocytes collected from slaughtered mares either by aspirating follicular contents or by scraping the follicle wall. Comparative studies have shown that scraping yields more good quality cumulus oocyte complexes (COCs: 71-85%) than aspiration (30-48%; Okolski *et al*., 1987; Alm *et al*., 1997; Dell'Aquila *et al*., 2001) but even then the total oocyte recovery rate is low (3-5 oocytes/ovary) because the average horse ovary contains only 6 antral follicles (Hinrichs, 1991; Del Campo *et al*., 1995). To make matters worse, Hinrichs and Schmidt (2000) have suggested that oocytes should only be harvested from follicles larger than 20mm in diameter because the ability of immature oocytes to progress through maturation increases with follicle size.

In culture, immature oocytes resume meiosis and progress from the germinal vesicle (GV) stage to metaphase of the second meiotic division (MII), as they would during final follicle maturation (Fig. 1), at rates of 50 - 80 % (for review see Hinrichs, 1998; Squires, 2000; Squires *et al*., 2003). Most studies that have examined IVM in horses have, however, focused on nuclear maturation, and little attention has been paid to the accompanying cytoplasmic events that are critical to the formation of a developmentally competent oocyte. Indeed, even when the cytoplasmic changes that accompany nuclear maturation have been described (e.g. migration of cortical granules or mitochondria: Goudet *et al*., 1997; Aguilar *et al*., 2002; Carneiro *et al*., 2002), their significance to developmental competence has not been investigated. Such studies should be of considerable value given that the developmental competence of IVM oocytes transferred to the oviduct of inseminated mares is poor (Carnevale and Ginther, 1995; Scott *et al*., 2001).

The developmental competence of an oocyte depends greatly on the accumulation of mRNA and proteins to support fertilization and early embryo development, until the embryonic genome switches on at the 4 to 8-cell stage (Grøndhal *et al*., 1993). When oocytes are removed from their follicular environment and placed in culture, they spontaneously resume meiosis and condense their chromatin. This stops transcription and blocks further accumulation of proteins. It would, of course, be preferable to ensure that storage of mRNA and proteins is complete before germinal vesicle breakdown is induced (Sirard, 2001). In this respect, pre-culturing bovine oocytes under conditions that maintain meiotic but not cytoplasmic arrest appears to improve their developmental competence (Fouladi Nashta *et al*., 1998; Hashimoto *et al*., 2002). In the horse, the mechanism by which an oocyte is held in meiotic arrest is poorly understood. However, Hinrichs *et al*. (1995) reported a probable important role of the follicle wall or membrana granulosa in this process, and a better understanding of the factors involved may lead to new IVM strategies that encourage better cytoplasmic maturation and thereby production of mature oocytes better able to support embryo development.

Clearly, the ultimate criterion for the competence of an IVM oocyte is its ability to be fertilized and develop into a viable embryo. Unfortunately, the lack of an efficient equine IVP system has prevented the use of the birth of live foals, pregnancy or even blastocyst production rates as endpoints. Instead, the success of IVM has been based solely on the proportion of oocytes reaching MII. Fortunately, the recent success of intracytoplasmic sperm injection means that a better way of assessing the developmental competence of IVM oocytes is now available (Li *et al*., 2001; Galli and Lazzari, 2001; Galli *et al*., 2002; Lazzari *et al*., 2002; Choi *et al*., 2003).

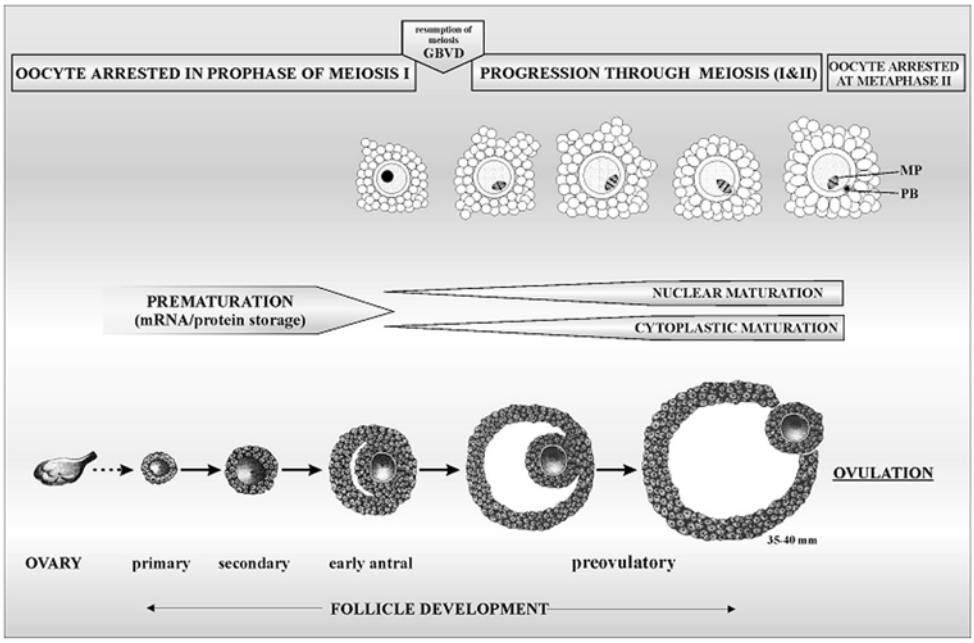

Figure 1. The different stages of equine folliculogenesis and oogenesis. Follicle development begins with formation of a primordial follicle that develops, via primary and secondary follicle stages, into an antral follicle. The process of oogenesis begins in embryonic life, with primordial germ cells that develop into oogonia via several cell pre-meiotic divisions and, finally, meiosis. Shortly after birth, meiosis proceeds to diplotene of the first meiotic division and the oocyte enters a prolonged 'resting phase' (Germinal Vesicle: GV). Subsequently, during early follicle development, oocytes build up a store of mRNA and proteins to support later development, in a process known as prematuration. The GV stage is eventually terminated only when a follicle proceeds towards ovulation in response to a dramatic rise in maternal circulating LH concentrations. During nuclear maturation, the arrested oocyte undergoes germinal vesicle breakdown (GVBD) and proceeds through the first meiotic division to extrude one set of chromosomes as the first polar body (PB). The oocyte then progresses through the second meiotic division and arrests at the metaphase stage (MII), with its chromosomes aligned along the second metaphase plate (MP); it is at this point that ovulation usually occurs. Further progression through meiosis is dependent on penetration by a spermatozoon at fertilization. Nuclear maturation is accompanied by structural changes in the cytoplasm that enable the oocyte to support subsequent fertilization and early embryonic development; these include migration of mitochondria, endoplasmic reticulum and cortical granules.

In vitro fertilization

The main hurdle to the development of a cost-efficient, high throughput system for producing horse embryos *in vitro* is conventional IVF. To date, only two foals have been produced in this way (Palmer *et al*., 1991; Bézard, 1992) and neither was

produced from an IVM oocyte, for which the fertilization rates are as low as 0-33% (e.g. Zhang *et al*., 1990; Del Campo *et al*., 1990; Li *et al*., 1995; Dell' Aquila *et al*., 1996, 1997a; Alm *et al*., 2001; Hinrichs *et al*., 2002). The principal barrier to successful IVF appears to be zona pellucida (ZP) penetration by the sperm (Fig. 2), since ZP dissection (Choi *et al*., 1994) and drilling (Li *et al*., 1995) both markedly increase fertilization rates.

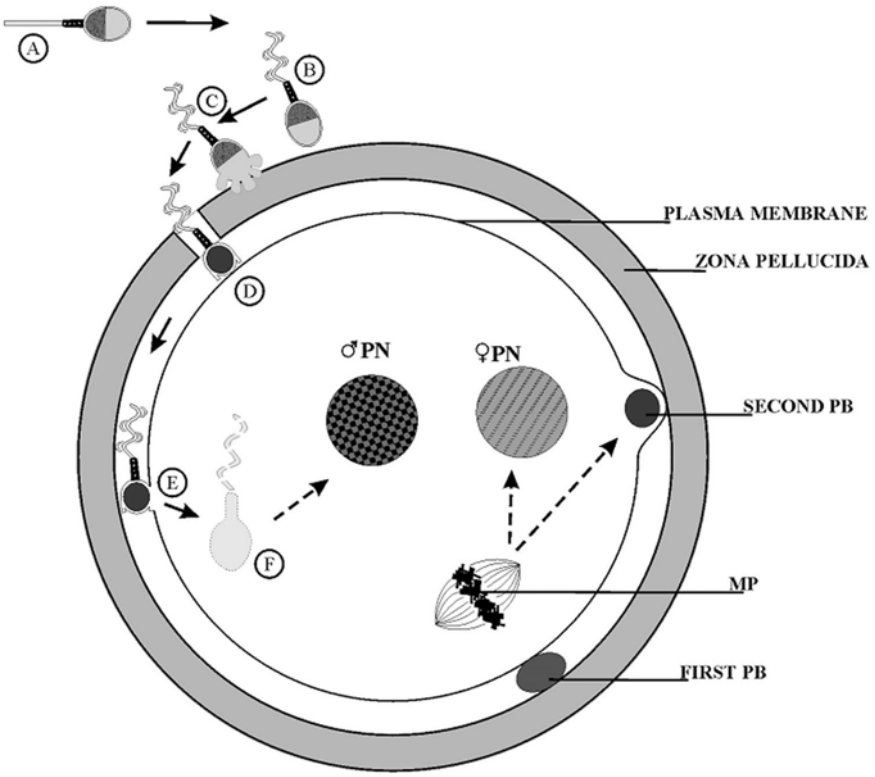

Figure 2. A schematic representation of the sequence of events that occur during mammalian fertilization. Inside the female genital tract, spermatozoa are activated during a process called capacitation (A). Capacitated sperm cells become hypermotile and able to bind to the zona pellucida (B) and thereby trigger the acrosome reaction (C). The hydrolytic enzymes thus released lyse the zona pellucida (D) and enable the hyperactive spermatozoon to enter the perivitelline space, where it can bind to the oolemma (E), fuse with and become incorporated into the oocyte. Thereafter, the sperm head begins to swell (F) and the oocyte, which was arrested at metaphase of the second meiotic division (MII) with its chromosomes arranged along the metaphase plate (MP), is activated and progresses through meiosis to extrude the second polar body. Finally, the female and male pronuclei are formed as the final prelude to syngamy.

In turn, the most commonly proposed reasons for poor ZP-penetration are changes in the oocyte investments induced during IVM, and inadequate capacitation of stallion sperm *in vitro*. Increasingly, however, the evidence suggests that IVM is not a crucial limiting factor. Indeed, although the pregnancy rates after transfer of IVM oocytes to the oviduct of inseminated mares are low (9 verus 82 % for *in vivo* matured oocytes; Scott *et al.*, 2001), the cleavage rates 40-44 h after transfer are high (77%: Hinrichs *et al*., 2002), demonstrating that failed development rather than failed fertilization is the problem. Moreover, while hardening of the ZP during IVM, for example by premature cortical granule release, has been proposed as a possible barrier to sperm penetration, preventing ZP-hardening does not improve penetration (Dell'Aquilla *et al*., 1999).

With regard to capacitation of stallion sperm *in vitro*, compounds such as heparin, equine ZP proteins, caffeine and lysophospholipids increase the percentages of capacitated and acrosome-reacted sperm, but do not facilitate sperm penetration into IVM oocytes (see Graham, 1996). By contrast, calcium ionophore A23187 induces sperm capacitation, acrosome reaction and penetration of IVM oocytes (Zhang *et al*., 1990; Li *et al*., 1995; Alm *et al*., 2001; Hinrichs *et al*., 2002) and was the sperm treatment used during production of the only two conventional-IVF foals (Palmer *et al*., 1991; Bézard *et al*., 1992). Overall, despite more than 12 years of research into conventional equine IVF, fertilization rates remain poor, there is little consistency in methodology and there is undoubtedly a need for a systematic investigation into how a sperm binds to and penetrates an oocyte.

Assisted fertilization techniques

Intracytoplasmic sperm injection (ICSI)

ICSI was developed as a means of treating male factor infertility (Fig. 3). In horses, it has been adopted as an alternative to conventional IVF. The first ICSI horse pregnancy was reported by Squires *et al*. (1996), and subsequently several foals have been produced by ICSI of both *in vivo* and *in vitro* matured oocytes (Cochran *et al*., 1998; McKinnon *et al*., 2000; Li *et al*., 2001; Galli *et al*., 2002). By bypassing critical events such as ZP-binding and penetration, ICSI has proven a valuable and repeatable means of producing equine embryos *in vitro*. Even early studies demonstrated relatively high fertilization rates after ICSI (40% versus 5% for conventional IVF: Dell'Aquila *et al*., 1997a,b; Grøndhal *et al*., 1997), although most of the resulting zygotes then arrested between the pronucleus and 4-cell stages and very few developed into blastocysts. Subsequently, several potential oocyte activators have been tested for their ability to enhance embryonic development after ICSI (calcium ionophore: Kato *et al*., 1997; thimerosal: Li *et al*., 2000), but most have lowered activation rates and only increased the proportion of

parthenogenetic embryos (up to 50%: Li *et al*., 2000). By contrast, co-culture of zygotes with either oviduct cells or fetal fibroblasts resulted in a higher percentage of 2-cell embryos developing to blastocysts, and the first reported pregnancies after transfer of IVP embryos to the uterus of recipients (Li *et al*., 2001). Blastocysts have now also been produced by culture in semi-defined media (Choi *et al*., 2003; Galli and Lazzari, 2001; Galli *et al*., 2002), and it has been shown that ICSI with frozen-thawed sperm yields embryos at similar rates to fresh sperm (Choi *et al*., 2002a), so long as a motile sperm is selected (Lazzari *et al*., 2002). Overall, ICSI has proven an efficient way to fertilize oocytes *in vitro,* although marked between-operator differences in fertilization rates persist (20-80 % cleavage rates: Dell'Aquila *et al*., 1997a,b; Grondhal *et al*., 1997; Li *et al*., 2001; Choi *et al*., 2002a; Galli *et al*., 2002).

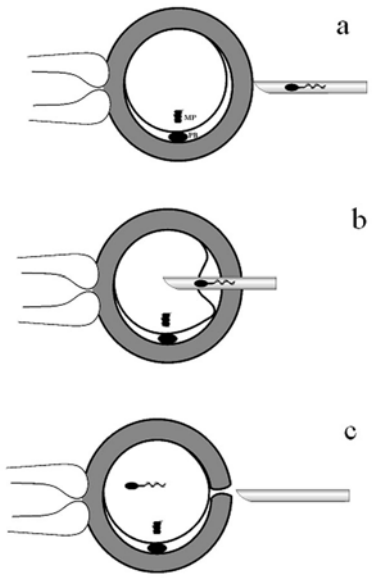

Figure 3. Schematic representation of ICSI of a metaphase II oocyte. Initially, a single, motile spermatozoon is selected and immobilized by crushing its tail against the bottom of the petri dish using the injection pipette. The sperm is then aspirated tail-first into the injection pipette. The oocyte is held on the holding pipette with its polar body (PB) orientated to 6 or 12 o'clock, to avoid damage to the meiotic spindle (MP: metaphase plate) during injection (a). When both the oocyte and the holding pipette are in focus, the needle containing the sperm is pushed through the zona pellucida and oolemma and into the ooplasm at the 3 o'clock position. Sometimes the oolemma requires slight suction to facilitate piercing, but when ooplasm enters the injection pipette, membrane rupture is complete and the sperm cell is slowly released (b). Finally the injection pipette is withdrawn gently and the injected oocyte is released from the holding pipette (c).

Oocyte Transfer

Oocyte transfer involves collection of an oocyte from the follicle of a donor mare and transfer to the oviduct of an inseminated recipient. It is essentially used only for research purposes or when the cause of sub-fertility precludes embryo transfer as a solution. The first successful equine oocyte transfer was reported by McKinnon *et al*. (1988) and subsequent studies have demonstrated that pregnancy rates are high when oocytes are collected from preovulatory follicles of young mares and transferred into the oviduct of young recipients (54-83%: Carnevale *et al*., 2000; 2001). By contrast, oocytes collected from smaller follicles and matured *in vitro* before transfer result in low pregnancy rates (9%: Scott *et al*., 2001). In a commercial program, an overall pregnancy rate of 40% can be expected, although this is affected by the quality of the oocytes, age of the donor and by semen quality (Carnevale *et al*., 2001).

Gamete intrafallopian transfer (GIFT) is a modification of oocyte transfer, in which sperm and oocyte are transferred together into the oviduct of the recipient. Since relatively few sperm are required for GIFT, it is a possible way of obtaining offspring from stallions that produce very few sperm. The first successful GIFT in a mare was reported by Carnevale *et al*. (1999). Subsequently, Countinho da Silva *et al*. (2002a) found no difference in pregnancy rates between GIFT and oocyte transfer (55% vs 65%), although pregnancy rates dropped dramatically when GIFT was performed with frozen-thawed semen (Countinho da Silva *et al*., 2002b). Within many European countries, animal welfare legislation prohibits the use of either technique for non-research applications.

Embryo culture

A critical step in any IVP system is the development of the embryo to the blastocyst stage, when it can be transferred to the uterus of a recipient. To date, only a few groups have produced equine blastocysts in culture, and success rates are still very low (1/76 Guignot *et al*., 1998; 1/79 Dell' Aquila *et al*., 1997a,b; 2/18 Maclellan *et al*., 2000; 10/33 Li *et al*., 2001; 7/47, Galli and Lazzari, 2001; 14/167 Choi *et al*., 2003). For this reason, most ICSI pregnancies have resulted from transferring zygotes to the oviduct of recipient mares (Squires *et al.*, 1996; Cochran *et al*., 1998; McKinnon *et al*., 2000). Indeed, even though several culture protocols, including co-culture (Battut *et al*., 1991; Li *et al*., 2001) and cell-free systems (Azuma *et al*., 1995; Del'Aquila *et al*., 2001; Galli *et al*., 2002), have been tested, temporary transfer of zygotes to the oviduct of progesterone-treated sheep remains the most successful *ex vivo* system for producing equine blastocysts (45% success rate: Galli and Lazzari, 2001; Galli *et al*., 2002; Lazzari *et al*., 2002). Unfortunately, both oviduct transfer techniques require surgery and commercial

use is thus prohibited within some European countries, including the Netherlands. Nevertheless, the quality and the quantity of the embryos produced justify the use of the techniques to investigate the requirements of the developing horse embryo.

IVP embryos from many species display differences to their *in vivo* derived counterparts with regard to morphology, metabolism and gene expression, and many of these differences detract from their developmental competence (cow: Kruip and den Haas, 1997; van Wagtendonk-de Leeuw *et al*., 2000; pig: Kikuchi *et al.*, 2002). Since early equine embryos exhibit a high incidence of developmental arrest during culture, it is quite likely that similar differences oocur and could be used as indicators of IVP embryo health and normality. Naturally, the existence of foals derived from IVP embryos is the best proof of their developmental potential, however such foals are still few and far between. It is also a sobering to consider that, in cattle, although the rate of blastocyst development *in vitro* has improved since the early 1990s, there has been little improvement in calving rates (Peterson and Lee, 2003). This suggests that there are further, as yet incompletely understood, limitations of IVP to be overcome.

Nuclear transfer

Somatic cell nuclear transfer has been used to produce cloned offspring in several species, including sheep (Wilmut *et al*., 1997), mice (Wakayama *et al*., 1998), cattle (Kato *et al*., 1998), goats (Baguisi *et al*., 1999) and pigs (Onishi *et al*., 2000). Briefly, enucleated recipient MII oocytes are reconstructed with somatic cell nuclei using inactivated Sendai virus, electrofusion (Fig. 4), or by direct injection of the donor nucleus into the ooplasm. Cloning of horses is currently in the developmental stages. Adult donor somatic cell nuclei have been fused to oocytes at high rates (up to 82%: Li *et al*., 2002a) using electrofusion in combination with Sendai virus and, although cleavage rates remain low, Li *et al*. (2002b) were able to produce blastocysts from these and from oocytes reconstructed with fetal fibroblasts (4 to 7% success rates). In comparison, cloning by directly injecting a somatic cell nucleus using a piezo-driven pipette, and activating the reconstructed zygote with stallion sperm cytosolic extract resulted in a 22% embryo rate after 4 days in culture (Choi *et al*., 2002b) and simple attachment of equine cumulus or adult fibroblast cells to zona pellucida free horse oocytes resulted in embryo production rates of 1.6 and 4%, respectively (Lagutina *et al*., 2003). Although a few groups have thus produced cloned horse embryos, no term pregnancies have been reported. On the other hand, Woods *et al*. (2002) have produced early several pregnancies from mule fetal fibroblasts electrofused to *in vivo* matured horse oocytes and transferred immediately into the oviduct of recently ovulated recipient mares. Indeed, seven of 195 (3.6%) transferred, cloned mule embryos developed

into ultrasonographically detectable pregnancies, but only one formed an embryo proper and all seven resorbed spontaneously between days 27 and 61 of gestation. A high rate of embryonic loss is seen for cloned pregnancies in most species, for example only 5% of transferred cloned bovine blastocysts survive to term (Galli *et al*. 2003), and pregnancy losses have been ascribed to defective placental function and epigenetic alterations presumed to result from incomplete reprogramming of the somatic nucleus (Rideout *et al*. , 2001).

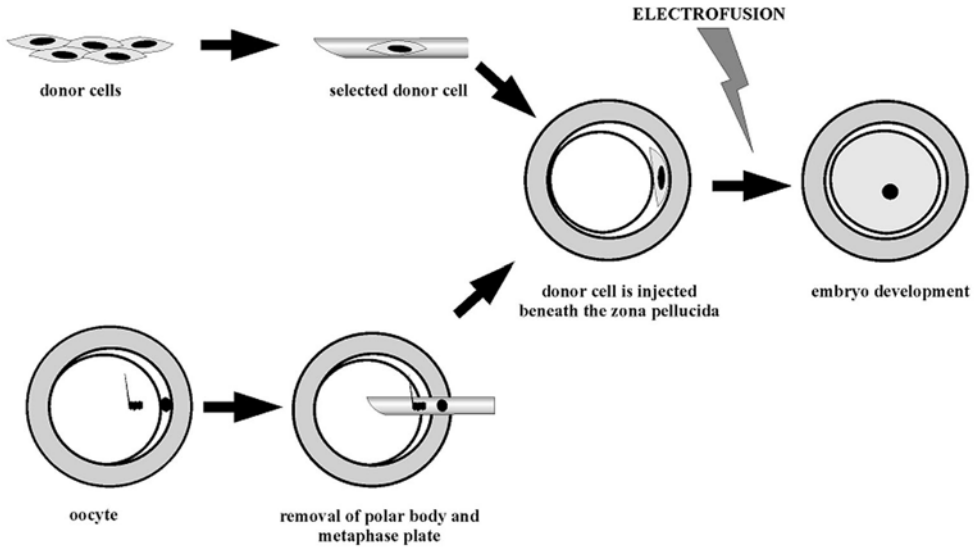

Figure 4. The stages involved in somatic cell nuclear transfer, the transfer of the nuclear DNA from a donor cell into an enucleated unfertilized oocyte. The technique involves several steps: first, donor cells are cultured under conditions that push them into the resting phase of the cell cycle. A donor cell is then selected and deposited beneath the zona pellucida of the enucleated oocyte, and the two cells are fused using an electric shock so that the donor cell's nucleus can enter the ooplasm. Within a few hours, the construct should begin to divide like a newly fertilized oocyte.

The potential use of cloning in the equine industry is unclear. It would, of course, offer a means of salvaging genetic material from rare breeds or valuable animals that die or become infertile. However, epigenetic defects and the normality of cloned offspring are serious concerns that must be addressed. On a wider scale, the use of cloning would presumably be limited by the fact that it offers no scope for genetic progress. On the other hand, the Equine Genome Sequencing Project may identify genes for factors that enhance athletic performance, and cloning combined

with genetic modification could offer a future means of producing "designer" equine athletes. Nevertheless, to justify cloning from an ethical perspective, there needs first to be a very clear demonstration that the potential benefits outweigh the risks of fetal and neonatal abnormality.

Scope of the thesis

The aim of this thesis was to investigate cellular processes critical to fertilization and embryo development *in vitro*, and to explain why IVP has met with such limited success in the horse. A better understanding of the complex cellular events that take place in the oocyte during maturation and fertilization and, later on, during early embryo development may help to define and solve the problems that have thus far hampered IVP. In this respect, advanced imaging techniques such as confocal and multiphoton laser scanning microscopy, are the perfect tools for visualizing cellular events *in situ*.

The first specific aim of the thesis was to investigate what occurs during oocyte maturation *in vitro*, why the resultant oocytes should have a lower developmental capacity than *in vivo* matured oocytes and what approaches could be used to improve the quality of cytoplasmic maturation and its synchrony with nuclear maturation. Of course, *in vivo* the control of oocyte maturation is dictated primarily by its follicular and the maternal endocrinological environment and, in Chapter 2, the role of follicle cells and FSH in the maintenance of oocyte meiotic arrest in the horse is investigated as a means of identifying conditions that allow IVM to occur in a more physiological fashion. Chapter 3 then examines how the oocyte's cytoskeleton reorganizes during IVM and how changes in microfilament and microtubule distribution relate to simultaneous changes in chromatin distribution.

It is clear that the poor fertilization rates obtained with conventional IVF remain the greatest obstacle to large-scale horse IVP. In chapter 4, the interaction between sperm and oocyte during IVF is investigated using confocal laser scanning microscopy to determine at what point fertilization fails. Of course, the problems of conventional IVF can be overcome by ICSI and, in chapter 5, the nuclear and cytoskeletal events that occur in horse oocytes fertilized by ICSI are described, with special attention to the stages at which fertilization or zygote development fails. Even after successful fertilization, however, the rate of blastocyst production *in vitro* is low, presumably because of inadequacies in culture conditions. Chapter 6 compares the structural and cellular characteristics of horse embryos produced totally *in vitro* or by temporary transfer to the oviduct of surrogate sheep, with those of *in vivo* produced embryos, with the aim of identifying the detrimental effects of culture. In a similar vein, the organization of the chromatin and

cytoskeleton of cloned embryos constructed using adult or fetal fibroblasts is described in chapter 7, to determine the success of nuclear reprogramming and the degree of subsequent developmental disturbance. Finally, the results of all of these studies and their implications for future research and for the commercial application of IVP in horses are summarized in chapter 8.

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Chapter 2

Effects of follicular cells and FSH on the resumption of meiosis in horse oocytes matured in vitro

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ABSTRACT

It has been suggested that preculturing immature oocytes in a manner that maintains them in meiotic arrest may improve cytoplasmic maturation and, thereby, the eventual developmental competence of oocytes matured *in vitro*. This study examined the ability of follicular cells to maintain meiotic arrest in horse oocytes. Cumulus oocyte complexes (COCs) recovered from dead mares were cultured for 38 h in M199 either attached to, or together with, different follicle wall components, as follows: 1) attached to the follicle wall, 2) cocultured with separated follicle wall, 3) attached to membrana granulosa (COCG), 4) COCGs cocultured with sheets of theca cells, 5) COCGs cultured in theca-cell conditioned medium, and 6) control COCs without any follicle wall components. When oocytes were cultured attached to their follicle wall, 79% remained in the germinal vesicle (GV) stage throughout the 38 h incubation. However, when oocytes were cocultured with separate pieces of follicle wall, meiosis resumed and a similar proportion of oocytes progressed to metaphase II (79%) as in control conditions (84%). Only 16% of oocytes cultured while still attached to the membrana granulosa (COCGs) maintained the GV stage, whereas when COCGs were cocultured with theca cells or in theca-cell conditioned medium, significantly more oocytes remained in the GV stage (64% and 52%, respectively), indicating that theca cells secrete a meiosis-inhibiting factor. To investigate the effect of FSH on the meiosis-inhibiting activity of follicular cells, COCs attached to the follicle wall and COCGs in the presence or absence of theca cells, were cultured in medium containing FSH. Addition of 0.05 iu recombinant human FSH ml^{-1} to the culture medium did not affect nuclear maturation and failed to overcome the suppressive effect exerted by the follicle wall or by theca cells, despite the fact that mRNA for the FSH receptor was found using RT-PCR in both cumulus and granulosa cells. These results demonstrate that the maintenance of meiotic arrest in horse oocytes during culture can be promoted by theca cells, which appear to act via a secreted inhibitory factor that can not be suppressed or counteracted by FSH.

INTRODUCTION

Within preantral and immature antral ovarian follicles, mammalian oocytes remain arrested at the dictyate stage of the first meiotic division (Edwards, 1962). Further oocyte development does not begin until the follicle matures to the preovulatory stage and is stimulated by a luteinizing hormone (LH) surge, which induces maturation of the oocyte and ripening of the follicle. Once induced, oocyte maturation encompasses a complex series of molecular and structural events,

beginning with the resumption of meiosis and germinal vesicle breakdown (GVBD) and culminating in the arrest of the oocyte at the second metaphase of meiosis (MII). However, when cumulus-oocyte complexes (COCs) are removed from their follicular environment and incubated *in vitro*, meiosis resumes spontaneously (Pincus and Enzmann, 1935), indicating that factors present in the immature follicle are responsible for holding the oocyte in meiotic arrest. This hypothesis has been strengthened considerably by evidence that follicle cells can also inhibit the resumption of meiosis during culture. Within the follicle, an oocyte is linked physically and metabolically to the surrounding follicular cells by gap junctions which permit the direct transfer of messengers from the follicle's somatic cells to the oocyte (Anderson and Albertini, 1976). However, when a COC is removed from its follicle by aspiration or scraping, these functional junctions between the COC and the follicle wall are broken down, thereby isolating the oocyte from the influence of the underlying membrana granulosa cells. Several studies have shown that contact between granulosa and cumulus cells enables the maintenance of meiotic arrest *in vitro* in both bovine (Carbonneau and Sirard, 1994; de Loos et al., 1994; Richard and Sirard, 1996a,b; VanTol et al., 1996) and porcine oocytes (Motlik et al., 1991). Furthermore, the meiotic-arresting ability of granulosa cells appears to be enhanced by coculture with theca cells, at least for bovine oocytes (Kotsuji et al., 1994; VanTol and Bevers, 1998).
Data on the role of follicular cells or FSH on the maintenance or cessation of meiotic arrest in horse oocytes are currently limited, although Hinrichs et al. (1995) reported that oocytes cultured with sections of follicle wall, sheets of membrana granulosa or within intact follicles, remained in meiotic arrest whereas oocytes cultured with follicular fluid or granulosa cells in suspension, did not.

There is also experimental evidence that FSH can modulate the meiosis-arresting activity of bovine granulosa cells. For example, Van Tol et al. (1996) found that adding FSH to oocyte culture medium prevented the meiotic arrest exerted by membrana granulosa cells. On the other hand, Carbonneau and Sirard (1994) found that FSH could not release bovine oocytes from meiotic arrest if they were cultured while still attached to their own follicle wall. Further studies suggest that theca cells can inhibit FSH-induced maturation of bovine oocytes (Kotsuji et al., 1994, Van Tol and Bevers, 1998), while a direct role for FSH in the resumption of meiosis has been supported by the demonstration of receptors for FSH in both cumulus and granulosa cells (Van Tol et al., 1996, Xu et al., 1995).

Certainly, *in vitro* maturation (IVM) of horse oocytes needs to be refined, and a better understanding of the factors that maintain an oocyte in meiotic arrest and regulate the resumption of nuclear maturation may help in the development of

strategies to improve culture conditions with particular regard to the quality of cytoplasmic maturation (Sirard et al., 1998; Sirad, 2001). The present study was designed to examine the effect of the different follicle wall cell types on the maintenance of meiotic arrest in cultured horse oocytes, and to evaluate the effect of FSH on meiosis-arresting activity. In addition, the reverse transcriptase polymerase chain reaction (RT-PCR) would be used to determine whether cumulus and granulosa cells from non-preovulatory follicles expressed mRNA for the FSH receptor.

MATERIALS AND METHODS

Collection and culture of oocytes

Approximately 250 ovaries were collected immediately after slaughter from mares of unknown reproductive history during May to August (breeding season) of two consecutive years. Recovered ovaries were transported to the laboratory in a thermoflask at 25-30ºC and, on arrival within 4 h after slaughter, ovaries were washed and immersed in 0.9% (w/v) NaCl containing 10iu penicillin ml^{-1} and 10µg streptomycin ml^{-1} (both from Gibco Life Technologies, Breda). Cumulus oocyte complexes (COCs) were recovered by aspiration from non-preovulatory follicles between 5 and 30 mm in diameter, as described by Tremoleda et al. (2001). Briefly, the contents of any visible follicle were aspirated and the follicle was flushed up to 3 times with PBS containing 50 mg BSA l^{-1} (Sigma, St Louis, MO) and 25 iu heparin ml^{-1} (Leo Pharmaceutical, Weesp). Next, the ovaries were dissected and follicles situated deeper in the ovarian stroma were similarly aspirated and flushed. The recovered follicular fluid-PBS mixture was allowed to stand for about 10 min at room temperature and, after removing the supernatant, the resulting pellet was washed twice in Hepes-buffered Tyrodes medium supplemented with 0.1% (w/v) polyvinylalcohol and 0.2% (w/v) BSA. After resedimentation, the final pellet was collected and examined under a stereomicroscope to locate the COCs. Only oocytes with a complete, compact and multilayered cumulus investment (compact COCs: Hinrichs and Williams, 1997) and oocytes connected to a piece of homogeneous, multilayered membrana granulosa at least 0.25 mm^2 in area (COCGs; Van Tol et al., 1996) were selected for culture. The selected COCs and COCGs were then washed twice with maturation medium and assigned randomly to treatment group prior to culture for 38 h in M199 medium supplemented with 100iu penicillin ml^{-1} and 100µg streptomycin ml^{-1} (both Gibco), in four-well plates (Nunc A/S, Roskilde)

maintained at 39ºC in a humidified atmosphere of 5% CO_2 in air. In total, COC recovery and preparation for culture took 1-2 h so that COCs were in their final culture conditions between 4-6 h after slaughter of the mares.

Preparation of Follicle Wall pieces with (COCsFW) or without (FW) an attached COC.

Follicles were identified within recovered ovaries and dissected free of overlying ovarian connective tissue using a scalpel and forceps. Fine forceps were then used to strip away any remaining stromal tissue without rupturing the desired follicle. Follicles were selected on the basis of their size (5-20 mm in diameter; larger follicles were technically difficult to dissect) and non-atretic appearance, as determined using the criteria of follicle wall transparency and vascularisation (Kruip and Dieleman, 1982). In the follicles selected for culture, the COC was located using a dissecting microscope and the follicle was then pierced opposite to the COC to release the follicular fluid. The wall of the collapsed follicle was then trimmed around the COC (2-3 mm^2) to obtain a COC with its attachment to the follicle wall (COCFW). Only COCsFW possessing a compact cumulus investment and a homogeneous membrana granulosa were selected for further culture. Similar sized pieces of follicle wall (2-3mm^2; FW) without a COC were prepared for co-culture with isolated COCs (COCs+FW).

Isolation of Theca Cells and preparation of Theca Cell Conditioned Medium

Non-atretic follicles between 5 and 20 mm in diameter were isolated from the ovaries as described above. The isolated follicles were then bisected with a scalpel to yield two hemifollicles from which the bulk of the granulosa cells were removed by scraping the inner wall. The removal of the granulosa cells was completed by vortexing the scraped hemifollicles for 1 min in 1ml of HEPES buffered M199 supplemented with 100iu penicillin $ml^{-1}$and 100µg streptomycin ml^{-1}, then transferred to 1 ml of fresh medium, vortexed for a further minute and finally washed twice in 2 ml of culture medium. In a preliminary experiment, histological analysis of 10 hemifollicles prepared in this way demonstrated that no granulosa cells remained on the theca interna surface and, thus, that the procedure was effective for removing granulosa cells from the theca (Fig.1).

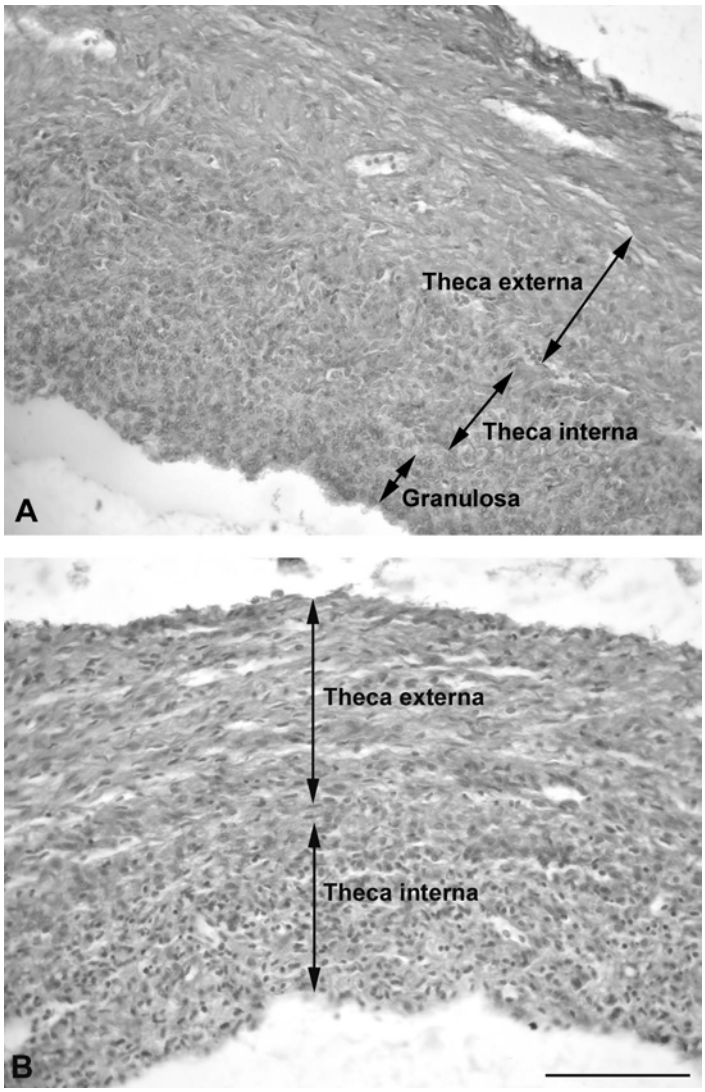

Fig.1. Photomicrographs of haemotoxylin and eosin stained histological sections of hemifollicles before and after removal of the granulosa cells. A) Intact follicle wall, displaying the theca externa and interna, with the granulosa cell layer on the luminal side of the follicle. B) Follicle wall after treatment, displaying only the theca externa and interna without any visible granulosa cells. Scale bar represents 50μm.

Theca cell conditioned medium (CM_{theca}) was prepared by culturing three 3-5mm^2 sheets of theca cells (the three pieces covered roughly half of the floor of the 1.9cm^2 culture well) in 500 µl of M199 medium supplemented with 100iu penicillin ml^{-1} and 100µg streptomycin ml^{-1}, in 4-well plates (Nunc A/S) maintained at 39ºC in a humidified atmosphere of 5% CO_2 in air. After 38 h of culture, the conditioned medium was collected in Eppendorf tubes and centrifuged for 3 min at 11600 g. The supernatant was collected and stored at -20ºC until subsequently used for oocyte maturation.

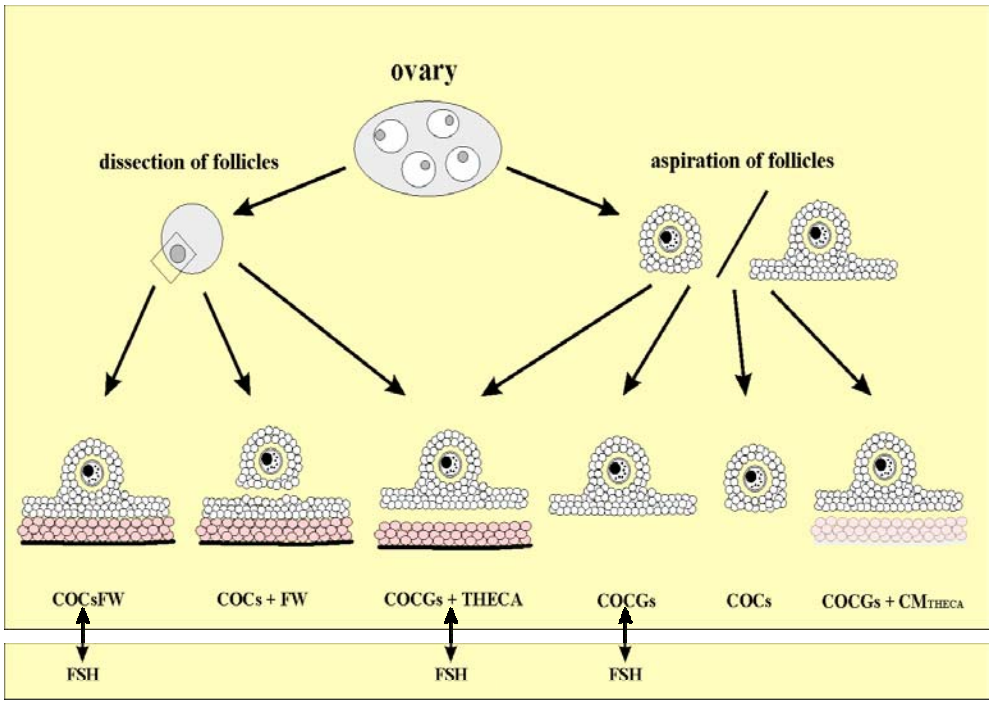

Fig. 2. Diagrammatic representation of the recovery of oocytes and the various follicle components to produce the culture conditions used in this study; that is, isolated cumulus-oocyte complexes (COCs) attached to their follicle wall (COCsFW); COCs + FW: COCs co-cultured with trimmed pieces of follicle wall; COCGs: COCs attached to a piece of membrana granulosa; COCGs + theca: COCGs co-cultured with sheets of theca cell; COCGs + CM_{theca} : COCGs cultured in theca-cell conditioned medium. FSH: supplementation of the culture medium with 0.05 iu recombinant human FSH ml^{-1}.

Experimental Design

Effect of follicular cells on oocyte nucleus maturation

The effect of follicle wall components and theca cell conditioned medium on the progression of meiosis in horse oocytes during IVM was examined by culturing COCs in the following conditions (Fig 2):

Table 1. **The number (and percentage) of horse oocytes at given stages of nuclear maturation after in vitro culture with different follicular components.**

Nuclear stage of the oocyte	Maturation conditions (*)					
	COCsFW	COCs+FW	COCGs	COCGs+theca	COCGs+CM $_{theca}$	COCs
GV	41 (61)	11 (14)	11 (12)	59 (50)	40 (38)	19 (11)
Metaphase I	7 (10.5)	7 (8.5)	8 (9)	6 (5)	4 (4)	11 (7)
Metaphase II	4 (6)	34 (42.5)	37 (41)	29 (24)	27 (26)	95 (55)
Degenerate	15 (22.5)	28 (35)	34 (38)	25 (21)	34 (32)	46 (27)
Number of oocytes	67	80	90	119	105	171

* COCsFW= Individual COCs attached to their follicle wall; COCs+FW= COCs cocultured with trimmed pieces of follicle wall; COCGs= COCs attached to a piece of membrana granulosa; COCGs+theca= COCGs cocultured with sheets of theca cell; COCGs+ CM $_{theca}$ = COCGs cultured in theca-cell conditioned medium. Total number of oocytes analyzed=632. Average rate of oocyte degeneration across all culture conditions: 30 ± 6.8% (mean ± SD).

1) Individual COCs attached to their follicle wall in 500 µl of culture medium (COCsFW: nine replicates, n= 67)

2) Groups of 10-15 COCs cocultured with 3-5 pieces of trimmed follicle wall (COCs+FW: FW covered approx. 1/3 of the culture well floor) in 500µl culture medium (three replicates, n= 80)

3) COCGs cultured in groups at a ratio of 1 per 100µl medium (six replicates, n= 90)

4) COCGs cocultured with three 3-5mm^2 theca cell sheets (covering approx. half of the culture well floor; COCGs+theca) in 100µl medium per COCG (four replicates, n= 119)

5) COCGs in groups of 5 in 500µl of CM_{theca} (three replicates, n= 105)

6) COCs in groups of 25 in 500µl of culture medium as a "normal" IVM control (ten replicates, n= 171)

The preparation of the various follicle components (Fig. 2) and the assignment of oocytes to treatments (Table 1) are summarized.

Effect of FSH on the meiosis arresting activity of follicular cells

The following co-cultures were performed using culture medium (M199 containing 100iu penicillin ml^{-1} and 100µg streptomycin ml^{-1}) supplemented with 0.05iu recombinant human FSH ml^{-1} (rh-FSH-Org 32489: Organon, Oss), to determine whether FSH could alter the effects of follicular cells on cultured equine oocytes:

1) COCs attached to their follicle wall (COCsFW) and cultured individually in 500 µl of FSH-supplemented medium (six replicates, n= 36)

2) COCGs in FSH-supplemented medium at a ratio of one COCG to100 µl medium (six replicates, n= 89)

3) COCGs cocultured with three theca cell sheets (COCGs+Theca) in 100 µl FSH-supplemented medium per COCG (four replicates; n= 141)

These incubations were performed contemporaneously with those in Expt 1 and, for the purposes of analysis, the latter were used as FSH-free controls. The allocation of oocytes to experimental groups is summarized (Table 2).

Assessment of stage of oocyte nucleus maturation

At the end of the 38 h culture period, COCs were denuded by vortexing for 3 min in a 0.25% (v/v) solution of trypsin in $CaCl_2$- and $MgCl_2$-free Earle's Balanced Salt

Table 2. The number (and percentage) of horse oocytes at the different stages of nuclear maturation after in vitro culture with different follicle wall components in medium supplemented with 0.05 IU/ ml recombinant human FSH.

	Maturation conditions (*)		
Nuclear stage of the oocyte	COCsFW+FSH	COCGs +FSH	COCGs+theca+FSH
GV	22 (61)	9 (10)	68 (48)
Metaphase I	1 (3)	2 (2)	11 (8)
Metaphase II	5 (14)	51 (57.5)	37 (26)
Degenerate	8 (22)	27 (30.5)	25 (18)
Number of oocytes	36	89	141

* COCsFW= Individual COCs attached to their follicle wall; COCGs= COCs attached to membrana granulosa; COCGs+theca= COCGs cocultured with sheets of theca cell.
For the purpose of analysis, the equivalent FSH-free treatments carried out contemporaneously in experiment 1 were used as controls. The total number of oocytes analysed for this experiment was thus 572 (266 with FSH and 276 in control conditions). The average rate of oocyte degeneration for all oocytes cultured in the presence of FSH was 25 ± 6.3% (mean ± SD).

Solution (EBBS: Gibco) containing 1mmol EDTA l^{-1}. The denuded oocytes were then washed three times in PBS before being fixed for 30 min at room temperature in 2% (w/v) paraformaldehyde in PBS, and stored at 4ºC until staining. Their nuclear status was assessed by first washing fixed oocytes twice in PBS and then incubated them in 0.1 μg 4,6-diamino-2-phenyl-indole (DAPI) ml^{-1} in PBS for 5 min, as described by Mori et al. (1988). Once stained, the oocytes were mounted on glass microscope slides and examined using a BH2-RFCA Olympus (Olympus, Tokyo, Japan) epifluorescence microscope equipped with a set of dichronic mirror unit (DMU) filters. Oocytes were classified as follows: a) germinal vesicle (GV) stage, if their chromatin appeared either as a single condensed mass associated to the nucleolus (condensed chromatin) or diffusely or patchily throughout the

nucleus (fluorescent nucleus), as described by Hinrichs et al. (1993); b) metaphase I (MI), encompassed all stages from germinal vesicle breakdown to formation of the first metaphase plate; c) metaphase II (MII), included all stages from anaphase I to formation of the second metaphase plate; d) degenerate oocytes, included oocytes with abnormal chromatin, chromatin spread throughout the ooplasm or with no visible chromatin, as described by Hinrichs et al. (1993).

Collection of tissue for total RNA extraction and reverse transcription

COCs with a compact multilayered cumulus investment, and compact pieces of membrana granulosa at least 0.25mm^2 in area, were selected from follicular contents harvested by aspiration. Cumulus cells were then harvested from the COCs by repeated aspiration through a narrow-bore Pasteur pipette. Next, the collected granulosa or cumulus cells were washed four times in PBS, loaded into Eppendorf tubes and stored at -80˚C until RNA extraction. Each tube contained one piece of membrana granulosa or the cumulus cells from ten COCs.
Isolation of total RNA was performed using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia). After elution in 30µl RNAse-free water, the concentration of extracted total RNA was determined using a Gene Quant 2 (Pharmacia Biotech, serial no. 61708) and adjusted to 0.3µg per 10µl. Before the reverse transcription reaction, the RNA sample was incubated for 5 min at 70^{0}C, vortexed for 5 s and chilled on ice. Reverse transcription was performed in a total volume of 20 µl, made up of 10 µl sample RNA, 4 µl of 5 x reverse transcriptase buffer (Gibco BRL, Breda), 8 U RNAsin (Promega), 150 U Super-script II reverse transcriptase (BRL), 0.036 U random primers (Life Technologies BV, Leiden), 10 mmol dithiothreitol l^{-1} and each dNTP at final concentrations and 0.5 mmol l^{-1}, respectively. The mixture was incubated for 1 h at 42°C and then for 5 min at 95°C before being stored at -20°C. RT-free blanks were prepared using the same conditions but excluding the reverse transcriptase.

Amplification of FSH receptor cDNA by PCR

The oligonucleotide primers used for amplifying the FSH receptor (FSHR) mRNA were based on a previously published donkey FSHR mRNA sequence (Richard *et al*., 1997). Amplification of RNA from membrana granulosa and cumulus cells was performed in two stages. For the first round of amplification, the FSHR-primers used were: FSHRL2 (5'-CTTGCCAGCTGTTCACAAGA-3'; sense, position 402-421) and FSHRR1 (5'-TCAGGATCACCAGCACTATG-3'; antisense, position 1119-1138). A second round of PCR using nested primers was then performed to

increase the specificity of the final product. The second round primers were: FSHRL4 (5'-GTTGCACTCATGGAGGCCAG-3'; sense, position 784-803) and FSHRR4 (5'-CCAGTGATGGCTAGGATGCT-3'; antisense, position 1093-1112). Reactions were carried out in 200 µl tubes (Eurogentec, Seraing), using 1 µl cDNA as a template for 25 µl of the PCR mixture, which contained 2 mmol $MgCl_2$ l^{-1}, 100 µmol $l^{-1}$of each dNTP, 0.5 µmol $l^{-1}$of each primer and 0.625 units Taq DNA polymerase (HotStarTaq, Qiagen, Valencia) in 1xPCR buffer. The thermal cycling profile for the first round was: initial denaturation and activation of the polymerase for 15 minutes at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 55°C and 45 s at 72°C. Final extension was for 10 min at 72^0C. For nesting, 1 µl of the first - round product was transferred to another 200 µl tube containing 24 µl PCR buffer and amplified for 30 cycles using the same thermal profile. All PCRs were performed in a 24-well thermocycler (Perkin-Elmer, Gouda). The PCR products were identified by resolving 10 µl of the second round product on a 1% Agarose gel containing 0.4µg ethidium bromide ml^{-1}. A 100 bp ladder (Gibco BRL) was included as a reference for fragment size and an image of the gel was taken using a CCD camera (Appligene, B & L Systems, Zoetermeer) and stored in digital form. The second round product from the granulosa cell-PCR was purified using the QIAquick® PCR purification kit (Qiagen, Valencia, USA) and sequenced (Eurogentec, Seraing).

Statistical analysis

For the purpose of analysis, oocytes were first classified either as GV (that is remaining in meiotic arrest) or GVBD (that is, oocytes that resumed meiosis, irrespective of how far they then progressed). GVBD oocytes were then further subdivided into how far they had progressed through nuclear maturation (MI or MII). The effects of the different culture conditions and the addition of FSH on the proportion of oocytes that underwent GVBD and the proportion of GVBD oocytes that reached metaphase II, were analysed using SPSS software (SPSS, Chicago, Illinois) and logistic regression analysis (McCullagh and Nelder, 1989) for binomially distributed data. Culture conditions and the presence of FSH were considered as independent categorical variables in this model and $P< 0.05$ was taken to indicate a statistically significant difference. Where differences existed, further comparison of groups was performed by chi-square analysis to indicate the source of the differences ($P< 0.05$).

RESULTS

In Expt 1, approximately 30% of the 632 oocytes cultured were categorized as degenerate after culture and were excluded from further calculations (Table 1). The majority (79%) of oocytes from COCs cultured while still attached to a piece of their follicle wall (COCsFW), were still in the GV stage at the end of culture (Fig. 3).

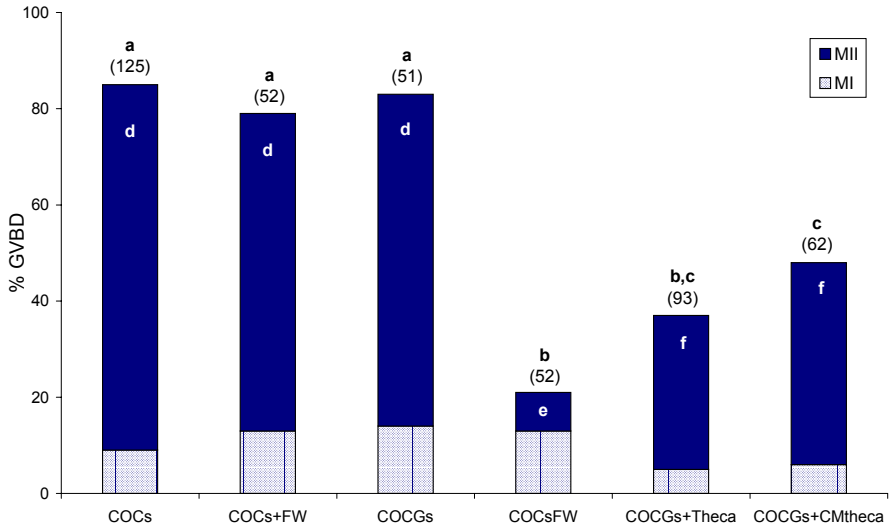

Fig. 3. The effect of follicle components on the progression of meiosis by horse oocytes during *in vitro* maturation. Oocytes were cultured for 38h in M199 as cumulus-oocyte complexes (COCs), COCs attached to the follicle wall (COCsFW), COCs coincubated with pieces of follicle wall (COCs+FW), COCs connected to a piece of membrana granulosa (COCGs), COCGs in the presence of theca cells (COCGs+theca) and COCGs in theca-cell conditioned medium (CM_{theca}). The number of oocytes analysed is indicated above each column in parentheses, at MI (■) or MII (□) stages. [a, b, c] Bars with different letters differ significantly with regard to the percentage of oocytes that underwent germinal vesicle breakdown (GVBD) ($P<0.05$, Logistic regression). [d, e, f] Different letters indicate a significant difference in the percentage of GVBD oocytes reaching the MII stage during culture ($P<0.05$, Logistic regression). Degenerate oocytes were excluded from these analyses.

By contrast, only 21% ($P<0.005$) of oocytes remained in meiotic arrest when COCs were cocultured with separate pieces of follicle wall. Indeed, the percentage of oocytes that underwent GVBD did not differ between COCs+FW and control, isolated COCs (79% versus 85%; Fig. 3), and in both conditions the majority of GVBD oocytes reached the MII stage (83% and 89% of GVBD-oocytes, respectively). COCs cultured while adhered to just the membrana granulosa (COCGs) also underwent GVBD and nuclear maturation at a similar rate to control COCs (80% GVBD of which 82% reached MII). However, when COCGs were cultured in the presence of thecal tissue (COCGs + theca), the percentage of oocytes that remained arrested at the GV stage was significantly higher than when COCGs were cultured alone (63% versus 20%) and similar to that observed for the COCsFW group (79%: Fig.3). Furthermore, oocytes cultured as COCGs in CM_{theca} maintained meiotic arrest at a similar rate to COCGs cultured with thecal tissue (56% versus 63%). Nevertheless, the percentage of oocytes that underwent GVBD during culture as COCGs in CM_{theca} was significantly higher than that for COCsFW (44% versus 21%; $P<0.005$: Fig. 3) and, of the oocytes that underwent GVBD, significantly more reached the MII stage after culture of COCGs in CM_{theca} than after culture attached to the follicle wall (87% versus 36%; $P<0.005$: Fig.3).

In Expt 2, a total of 572 oocytes were cultured with different follicle components in the presence or absence of FSH (Table 2). Similarly to Expt 1, approximately 25% of the oocytes were categorized as degenerate after culture and were excluded from further analysis. In general, addition of 0.05 iu recombinant human FSH failed to influence the number of oocytes that resumed meiosis. For example, the percentage of GVBD oocytes after culture of COCGs in medium supplemented with FSH was similar to that for COCGs cultured without FSH (86% versus 80%: Fig. 4). However, the percentage of GVBD oocytes that reached the MII stage was, in many cases, increased significantly by FSH (e.g. 82% for COCGs vs. 96% for COCGs plus FSH; $P<0.05$: Fig.4). Similarly, for oocytes cultured as COCsFW or COCGs+theca, addition of FSH did not increase the proportion of oocytes achieving GVBD (COCsFW: 79% versus 79%; COCGs+theca: 59% versus 63% for plus and minus FSH incubations, respectively; Fig. 4) but did tend to increase the proportion of oocytes from COCsFW that reached the MII stage (83% versus 36% for plus and minus FSH groups). However, addition of FSH to COCGs cocultured with theca cells did not affect the number of GVBD oocytes reaching MII.

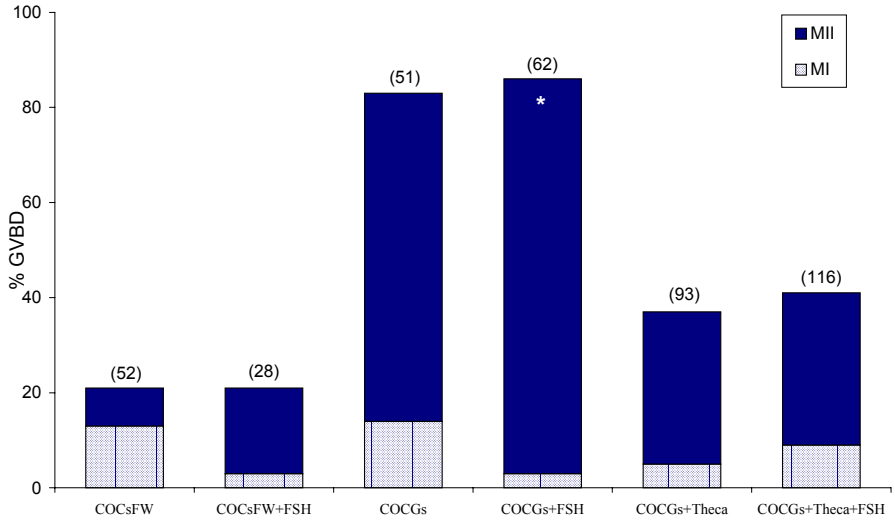

Fig.4.The effect of FSH on the resumption of meiosis by equine oocytes during *in vitro* maturation. Oocytes were cultured in M199 with or without 0.05 iu recombinant human FSH for 38h, in the form of cumulus-oocyte complexes (COCs) attached to their follicle wall (COCsFW) or COCs connected to a piece of membrana granulosa (COCGs) either in the presence or absence of theca cells. The number of oocytes in each experimental group is indicated above each column in parentheses, at MI (■) or MII (□) stages. FSH treatment did not significantly affect the proportion of oocytes that underwent germinal vesicle breakdown (GVBD) ($P>0.05$: Logistic regression). * Indicates the FSH treated group that differed with the corresponding FSH-free control in terms of the percentage of GVBD oocytes that reached MII ($P<0.05$, Chi-square test). Degenerate oocytes were excluded from these analyses.

Amplification of cDNA from granulosa and cumulus cells using FSHR-specific primers, resulted in one abundant PCR product after the two rounds of amplification, whereas amplification of RT blanks and the water control yielded no specific products (Fig. 5). The PCR product was 350bp and sequence analysis of the FSHR band amplified from granulosa cells demonstrated 90.0% identity to donkey FSHR mRNA. The major difference was the presence of 21 nucleotides in positions 155-175 that were absent from the donkey sequence described by Richard et al. (1997). This difference explained the difference in sequence length between the obtained

350 bp product and the 329 bp donkey sequence. Comparison of the sequenced horse FSHR product with FSHR mRNA from cattle, rats and mice showed 91.6, 83.2 and 83.5% similarity, respectively, with no deletions in the section corresponding to positions 155-175 in the amplified product (Fig. 6).

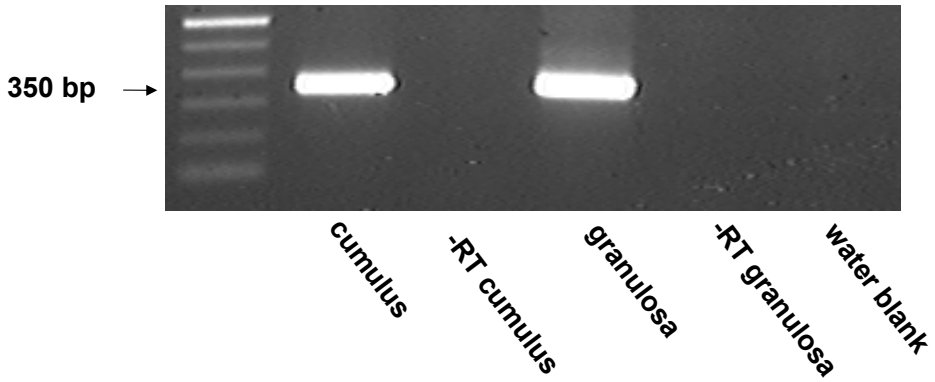

Fig.5. Expression of equine FSH receptor as detected by RT-PCR. The PCR products of 350 bp were produced using FSH receptor- specific primers in two rounds of amplification. The source of samples subjected to PCR are indicated at the bottom and a 100bp ladder is included in the far left-hand lane as a marker for fragment size.

Fig. 6. Sequence of the amplified equine FSH receptor product (SEQhorse) and its homology with corresponding sequences in cow, donkey, rat and mouse. The donkey FSHR cDNA lacks 21 nucleotides corresponding to positions 156-176 of the sequenced horse product.
* Denotes nucleotides in the sequence that are conserved across all species compared.

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FSHRbovine      CCTCACCTACCCCAGCCACTGCTGTGCCTTTGCAAACTGGAGGCGGC   850
FSHRdonkey      CCTCACCTACCCCAGCCATTGCTGTGCCTTTGCAAACTGGAGACAAC   850
FSHRrat         CCTCACCTACCCCAGCCACTGCTGTGCTTTTGCAAACTTGAAGCGGC   850
FSHRmouse       CCTTACCTACCCCAGTCACTGCTGTGCCTTTGCAAACTGGAGGCGGC   850
SEQhorse        TTCCACCTACCCCAGCCATTGCTGTGCCTTTGCAAACTGGAGACGAC    47
                    *********** ** ******** **********.**..*..*

FSHRbovine   AAACCTCTGACCTTCATCCAATTTGCAACAAATCTATTTTAAGGCAAGAA   900
FSHRdonkey   AAACCTCTGAGCTTCAGACAACTTGCAACAAATCTATTTTAAGGCAAGAA   900
FSHRrat      AAATCTCTGAACTTCATCCAATTTGCAACAAGTCTATTTTAAGGCAAGAT   900
FSHRmouse    AAACCTCTGAACTTCATCCAATTTGCAACAAGTCTATTTCAAGGCAAGAT   900
SEQhorse     AAACCTCTGAGCTTCAGACAACTTGCAACAAATCTATTTTAAGGCAAGAA    97
             *** ****** *****. *** *********.******* *********.

FSHRbovine   GTTGATGACATGACTCAGGCTAGGGGTCAGAGAGTCTCTTTGGCAGAAGA   950
FSHRdonkey   GTTGAT---ATGACTCAGGCTAGGGGTGAGAGAGTCTCTTTGGCAGAGGA   947
FSHRrat      ATTGATGATATGACTCAAATTGGGGATCAGAGAGTCTCTCTGA---TAGA   947
FSHRmouse    ATTGATGATATGACTCAGCCTGGGGATCAGAGAGTCTCTCTCG---TAGA   947
SEQhorse     GTTGAT---ATGACTCAGGCTAGGGGTGAGAGAGTCTCTTTGGCAGAAGA   144
             .*****   ********.  *.***.* *********** * .   ..**

FSHRbovine   TGATGAGCCCAGCTATGCCAAAGGATTTGACGTGATGTACAGTGAATTTG  1000
FSHRdonkey   CGATGAGTCCA--------------------TGATGTACAGTGAATTTG   976
FSHRrat      TGATGAACCCAGTTATGGAAAAGGATCTGACATGATGTACAATGAATTTG   997
FSHRmouse    TGATGAACCCAGTTATGGAAAAGGATCTGACATGTTGTATAGTGAATTTG   997
SEQhorse     TGATGAGTCCAGTTACCCCAAAGGATTTGACATGATGTACAGTGAATTTG   194
              *****. ***                     **.**** *.********

FSHRbovine   ACTATGACTTATGCAATGAAGTGGTTGATGTGACTTGCTCCCCTGAGCCA  1050
FSHRdonkey   ACTATGACTTATGCAACGAAGTCGTTGATGTGACTTGCTCCCCCAAGCCA  1026
FSHRrat      ATTATGACTTATGTAATGAAGTTGTTGATGTGACCTGCTCACCAAAGCCA  1047
FSHRmouse    ACTATGACTTATGCAATGAATTTGTTGATGTGACCTGCTCGCCAAAGCCA  1047
SEQhorse     AGTATGACTTATGCAACGAAGTCGTTGATGTGACTTGCTCCCCCAAGCCA   244
             * *********** ** ***.* *********** ***** ** .*****

FSHRbovine   GACGCATTTAATCCATGTGAAGATATCATGGGGGATGATATTCTCAGAGT  1100
FSHRdonkey   GATGCATTTAATCCATGTGAAGATATCATGGGGTATGATATTCTCAGAGT  1076
FSHRrat      GATGCATTTAATCCATGTGAAGATATCATGGGGTACAACATCCTCAGGGT  1097
FSHRmouse    GATGCATTTAATCCATGTGAAGACATCATGGGGTACAACATCCTCAGAGT  1097
SEQhorse     GATGCATTCAATCCATGTGAAGATATCATGGGGTATGATATTCTCAGAGT   294
             ** ***** ************** *********.* .* ** *****.**

FSHRbovine   CTTGATATGGTTTATT                                    1116
FSHRdonkey   CTTGATATGGTTTATA                                    1092
FSHRrat      CTTGATATGGTTTATT                                    1113
FSHRmouse    CTTGATATGGTTTATC                                    1113
SEQhorse     CTTGATATGGTTTATT                                     3
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DISCUSSION

The major finding of this study was that the spontaneous resumption of meiosis by equine oocytes cultured *in vitro* was inhibited when cumulus oocyte complexes were cultured while still connected to their follicle wall, or when COCs connected to a piece of membrana granulosa were cocultured with theca cells or in theca cell-conditioned medium. This finding supports strongly the hypothesis that some components of the follicle wall or, more specifically, some product(s) of the theca cells are essential for maintaining the oocyte in meiotic arrest in horses, as in other mammalian species (Leibfried and First, 1980; Sirard and Coenen, 1993; De Loos et al., 1994). Moreover, in this study FSH was not able to overcome the meiosis-arresting effect exerted by follicle wall or theca cells. Interestingly, the inhibitory effect which was apparently a function of the theca cells, clearly depended on the maintenance of cell-to-cell contact between the cumulus-oophorus and the granulosa cells since, when COCs were cultured with separate pieces of follicle wall the majority of oocytes underwent GVBD (79%), whereas when COCs were cultured attached to their follicle wall, most oocytes remained arrested in GV (79%). Similarly, when whole hamster follicles are cultured, close contact between the COC and granulosa cells appears to be central to the maintenance of oocyte meiotic arrest (Racowsky and Badwin, 1989), and bovine oocytes can be maintained in the GV stage in vitro by culturing COCs with undisrupted contact to the follicle wall (Carbonneau and Sirard, 1994; De Loos et al., 1994). In the horse, Hinrichs et al. (1995) previously reported that meiotic arrest was maintained when isolated COCs were cultured with sections of follicle wall, or when they were injected into and cultured within intact isolated follicles. By contrast, in the current study, meiotic arrest was not maintained when isolated COCs were cocultured with pieces of follicle wall. The dramatic difference in outcome presumably relates to the different culture conditions used. In the study of Hinrichs et al., groups of at least 30 COCs were cocultured with three to four 5 mm^2 sheets of follicle wall in 50 µl of culture medium (M199) supplemented with 10% neonatal calf serum. In the present study, groups of 10-15 COCs were cocultured with three to five 2-3 mm^2 sheets of follicle wall in 500 µl of medium (M199). The difference in the medium volume: amount of follicle wall tissue ratio could conceivably have compromised the metabolic activity of the oocytes and their ability to progress through meiotic maturation. More likely, the different tissue: medium ratio would have significantly altered the concentration of follicle wall inhibitory factors to which oocytes were exposed during coculture (Sirard and Coenen, 1993). Of course, the fact that the proportion of degenerate oocytes after coculture with pieces of follicle wall did not differ from that after culture of isolated COCs

(22.5% for COCsFW group vs. 27% for the COCs group; P> 0.05) indicates that the tissue: culture medium ratio used did not markedly impair either the viability of cultured oocytes or their ability to progress through meiosis. Nevertheless, whether the failure of separated follicle wall to prevent meiotic resumption in the current study was due to dilution of inhibitory factors in the culture conditions used or failure of signal transmission due to loss of the intercellular junctions between the COCs and the adjacent follicle wall (Kotsuji et al., 1994), requires further investigation.

This study revealed that spontaneous resumption of meiosis by horse oocytes in vitro is effectively inhibited when cumulus oocyte complexes attached to membrana granulosa are cultured in the presence of theca cells, whereas granulosa alone failed to induce this suppression. The critical role of theca cells in the maintenance of meiotic arrest in bovine oocytes has been studied extensively (Kotsuji et al., 1994; Richard and Sirard, 1996a,b; Van Tol and Bevers, 1998, 2001) and, in cattle, it is clear that there is a synergistic effect of theca cells on the meiosis modulating activity of granulosa cells, such that the inhibitory action of theca cells is much more effective on COCGs than on COCs. Unfortunately, information on the effect of theca cells on horse oocytes cultured in vitro is scarce. Okólski et al. (1993) examined the rate of nuclear maturation in equine oocytes with a compact cumulus that were cocultured with theca interna and/or granulosa cells, and found no effect of either on the number of oocytes reaching metaphase II, although follicular cells did influence cumulus expansion. Choi et al. (2002) similarly reported that coculture with theca cells did not increase the likelihood of successful nuclear maturation for compact COCs. However, in both of these latter studies, FSH, LH and/or estradiol were added to the culture medium, and it is therefore difficult to definitively interpret whether follicular cells had any modulatory effect on oocyte maturation. In short, the data presented in the current study is the first experimental evidence that theca cells are important to the maintenance of meiotic arrest in horse COCs connected to membrana granulosa.
Bovine (Van Tol et al., 1996) and porcine (Motlik et al., 1991) COCs cultured with an attached piece of membrana granulosa do not resume meiosis. In the horse, Hinrichs et al. (1995) reported that while cells in suspension could not maintain meiotic arrest, dissected sheets of mural granulosa cells layers could. It must be presumed that this difference in response was due either to the relative number of granulosa cells included in the cultures, or to the method of granulosa cell preparation, and it is possible that remaining theca cells or other follicle components on the dissected granulosa cell layers could have been responsible for the observed suppression. In a similar vein, the fact that the cumulus oophorus is more firmly attached to the follicle wall in horses than in other species (Ginther,

1992), presumably affects not only the oocyte recovery rate after follicle aspiration but also the quality and quantity of follicle components recovered with the COCs. For example, the cumulus granulosa cells surrounding a horse oocyte are broadly distributed and are connected to the outermost layer of mural granulosa cells via a loose network of intercellular spaces (Brück et al., 1999). This particular oocyte-follicle connection may compromise the recovery of large pieces of mural granulosa cell tissue when COCGs are isolated from the follicle and this may, in turn, lower the overall meiosis-inhibiting ability of attached granulosa cells. Thus, it is possible that in the current study, the failure of pieces of membrana granulosa attached to the COCs to suppress nuclear maturation was due to an insufficient quantity of mural granulosa cells. This would also help to explain why meiotic arrest was not maintained in cultured horse COCGs, but is in bovine or porcine COCGs. Further examination of the function of granulosa and cumulus cells in the horse may help to elucidate their specific functions in modulating meiotic activity of the oocyte. Despite the apparent lack of a direct meiosis inhibiting effect, granulosa cells did appear to play an important modulating role in the meiotic arresting activity of other follicular cells, namely the theca cells. A synergism between granulosa and theca cells has been reported in other species (Tsafiri and Channing, 1975; Van Tol and Bevers, 1998) and it appears that cellular contact between granulosa and cumulus cells are important for maintaining an oocyte in the GV stage during culture (Sato et al., 1982).

In the present study, oocytes cultured as COCGs in CM_{Theca} maintained meiotic arrest at a similar rate to those cultured as COCGs with pieces of theca, thereby indicating that the inhibitory effect is exerted via a factor produced and secreted by the theca cells. In this respect, Van Tol and Bevers (1998; 2001) characterised the meiosis inhibiting factor (s) produced by bovine theca cells as being a small, stable, polar, non-peptide molecule that acts via the cumulus cells (Richard and Sirard, 1996b) but whose identity is still to be elucidated. The current results suggest that equine theca cells also produce a meiosis inhibiting factor, although the efficacy of meiosis suppression by theca cells or theca-cell conditioned medium was lower than that by attached follicle wall (21% GVBD for COCsFW vs. 37% and 45% for COCGs+Theca group and COCGs+ CM_{theca}, respectively). Furthermore, of the oocytes that did resume meiosis, fewer from the COCsFW group reached MII within the 38 h culture period than from the other two groups (36% for COCsFW versus 86% for COCGs+Theca and 87% for COCGs+ CM_{theca}). These differences in the rate of maintenance of meiotic arrest may, as previously discussed, be associated to the relative paucity of membrana granulosa tissue in COCGs as compared to COCs dissected with their follicle wall attachment. If so, this would

suggest that inhibition can only properly be achieved by the theca cell meiosis-inhibiting factor(s) when large quantities of viable granulosa cells are present.

With regard to the effect of FSH on the meiosis-inhibiting activity of follicular cells during culture, this study demonstrated that FSH can not reverse the inhibitory action of contiguous follicle wall or of theca cells acting via a granulosa layer attached to the COC. However, FSH did significantly increase the proportion of reactivating oocytes that reached the MII-stage during the 38 h culture period. Previous studies on bovine oocytes showed similarly that treatment with gonadotrophic hormones could not reverse the inhibitory action of follicle wall on meiotic resumption when oocytes were cultured within their own half-follicles (Carbonneau and Sirard, 1994) or as COCGs cocultured with theca cells. However, FSH did stimulate the resumption of meiosis in oocytes cultured as COCGs in the absence of thecal tissue (VanTol et al., 1996). In the present study, when horse COCGs were cultured in the presence of FSH, the percentage of GVBD oocytes that reached MII was increased and there appeared to be an increase in the degree of cumulus expansion, when compared to COCs cultured without FSH. That FSH might be expected to affect horse oocyte maturation and cumulus expansion was supported by the detection of mRNA for the FSH receptor in cumulus and membrana granulosa cells recovered from 10-20mm follicles. Together with previous studies that used ligand binding and immunocytochemistry to demonstrate that specific FSH receptors are present within equine follicles (Stewart and Allen, 1979; Fay and Douglas, 1987), this confirms the production and expression of the FSH-receptor in small equine follicles.

In addition to its inability to overcome the meiosis-inhibiting effect of theca cells during IVM, FSH also failed to improve the MII rate of those oocytes that did undergo GVBD in the presence of theca cells. By contrast, FSH treatment resulted in a better progression of GVBD oocytes to MII for COCGs, and a similar tendency was observed for COCsFW, all of which suggests that theca cells may also interfere with the progression of meiosis in oocytes already committed to meiotic resumption. Similarly during IVM of bovine oocytes, theca cells antagonize the effect of FSH on COCGs and suppress the number of oocytes resuming meiosis and halt the majority that do resume GVBD at MI (Van Tol and Bevers, 1998, 2001). The inhibitory effects of theca cell secreted factor(s), may include interfering with activation of the Maturation Promoting Factor (MPF), a complex of cyclin B and $p34^{cdc2}$ kinase; activation depends on specific phosphorylation and dephosphorylation of the $p34^{cdc2}$ kinase (Coleman and Dunphy, 1994). For GVBD to occur, MPF has to be initially inactivated to regulate the G2 to M transition of oocytes and then reactivated to regulate the M phase

(Epping et al., 1996). Arrest of oocytes at MI may be related to failure of MPF activity to recover after the first metaphase, since this is necessary for progression from MI to MII. Indeed, insufficient MPF activity has been associated with failure of partially competent oocytes recovered from juvenile females to proceed through MI; in these oocytes the amount of $p34^{cdc2}$ was lower than in completely competent oocytes (De Vantery et al., 1997; Dedieu et al., 1998). In addition, Motlik et al. (1996) have reported that pig granulosa cells can modify the activity of the $p34^{cdc2}$ kinase in bovine oocytes and it is possible that the role of the granulosa cells in modulating MPF activation might by further regulated by theca cell secreted factors.

To conclude, "preculture" of oocytes in conditions that maintain meiotic arrest has been proposed as a means of improving their developmental competence, by allowing more complete cytoplasmic maturation and molecular reprogramming (Sirard, 2001). Indeed, immature bovine oocytes maintained in the GV stage in vitro, by adding butyrolactone to the culture medium and reducing the oxygen tension (Hashimoto et al., 2002) or by culturing them in intact antral follicles (Fouladi Nashta et al., 1998), attain higher levels of developmental competence. Unfortunately, due to the low efficiency of in vitro production of equine embryos, it is difficult to undertake similar studies in the horse. Indeed, while studies have shown that culturing horse oocytes in the presence of the protein synthesis inhibitor cycloheximide (Alm and Hinrichs, 1996) or protein kinase inhibitors such as roscovitine (Pimentel et al., 2002; Franz et al., 2002; Hinrichs et al., 2002), butyrolactone (Hinrichs et al., 2002) or 6-dimethylaminopurine (Hinrichs et al., 2002), can maintain meiotic arrest, it is unclear whether these treatments actually improve the developmental potential of the treated oocytes. In addition, the meiosis-inhibiting effect of theca cells presumably more closely resembles the in vivo situation within a non-preovulatory follicle than the inhibitors listed, and may thus provide a more physiological system for investigating the prematuration conditions that best allow immature horse oocytes to acquire developmental competence during maturation in vitro.

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Chapter 3

Organisation of the cytoskeleton during in vitro maturation of horse oocytes

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ABSTRACT

Meiotic maturation of mammalian oocytes is a complex process during which microfilaments and microtubules provide the framework for chromosomal reorganisation and cell division. The aim of this study was to use fluorescence and confocal laser scanning microscopy to examine changes in the distribution of these important cytoskeletal elements and their relationship to chromatin configuration during the maturation of horse oocytes in vitro. Oocytes were cultured in M199 supplemented with pFSH and eLH and, at 0, 12, 24 and 36 hr after the onset of culture, they were fixed for immunocytochemistry and stained with markers for microtubules (a monoclonal anti-α-tubulin antibody), microfilaments (AlexaFluor™488 Phalloidin) and DNA (TO-PRO_3). At the germinal vesicle stage, oocyte chromatin was amorphous and poorly condensed and the microfilaments and microtubules were distributed relatively evenly throughout the ooplasm. After germinal vesicle breakdown, the microtubules were aggregated around the now condensed chromosomes and the microfilaments had become concentrated within the oocyte cortex. During metaphase I, microtubules were detected only in the meiotic spindle, as elongated asters encompassing the aligned chromosomes, and, as maturation progressed through anaphase-I and telophase-I, the spindle assumed a more eccentric position and gradually rotated to assist in the separation of the homologous chromosomes and in the subsequent formation of the first polar body. During metaphase-II, the meiotic spindle was a symmetrical, barrel-shaped structure with two poles and with the chromosomes aligned along its midline. At this stage, microtubules were found intermingled with chromatin within the polar body and although the bulk of the microfilaments remained within the oocyte cortex, a rich domain was found overlying the spindle. Thus, during the in vitro maturation of horse oocytes both the microfilament and microtubular elements of the cytoskeleton were seen to reorganise dramatically in a fashion that appeared to enable chromosomal alignment and segregation.

Key words: Microtubules, Microfilaments, Chromatin, Equine, Oocyte, Meiotic Maturation

INTRODUCTION

To date, in vitro production of horse embryos has met with only limited success (Grøndahl et al., 1995a; Squires, 1996a; Hinrichs, 1998), largely because of difficulties in ensuring oocyte maturation, sperm capacitation and zona penetration in conventional fertilisation systems. Research into in vitro maturation of horse oocytes has lagged behind that in other species, principally because of difficulties in obtaining enough material for experiments to establish the optimal conditions for in vitro development. Not only is the supply of equine ovaries from slaughterhouses limited, but the number of oocytes retrieved per ovary remains low (about 4 oocytes per ovary), compared to other domestic species (Hinrichs et al., 1991; Choi et al., 1993; Alm et al., 1997; Guignot et al., 1999). With regard to oocyte maturation in vitro, several culture systems have been adapted from those used successfully in other species but most have yielded disappointing rates of maturation to metaphase of the second meiotic division (40-70%: Willis et al., 1991; Dell'Aquilla et al., 1997, 1999; Goudet et al., 1998, 2000; Brück et al., 2000). Furthermore, since in vitro matured equine oocytes show poor rates of fertilisation in vitro it seems likely that their developmental competence is also poor. In all, and despite several years of research, only two foals have been born via conventional IVF (Palmer et al., 1991).

Oocyte maturation is a complex process during which the oocyte must undergo a series of nuclear and cytoplasmic changes in order to become viable, fertilisable and developmentally competent (Albertini et al, 1993). The process of nuclear maturation involves the breakdown of the germinal vesicle (GVBD) and reorganisation and segregation of the chromosomes to form the meiotic structures following extrusion of the first polar body. Most studies of horse oocyte maturation have focussed on describing changes in chromatin configuration (Zhang et al., 1989; Willis et al., 1991; Hinrichs et al.,1993a; Shabparebh et al., 1993) and there are relatively few descriptions of the cytoplasmic changes that accompany during this maturation (Vogelsang et al., 1987; Grøndahl et al., 1995b; Neumann et al., 1995; Goudet et al., 1997). However, Grøndahl et al. (1995b) described the migration of the, initially evenly distributed, cortical granules (CG) to form a distinct layer just beneath the oolema and a coincident change in the distribution of mitochondria during in vivo maturation of equine oocytes and Goudet et al (1997) examined the location of CG in relation to follicular size and observed that CG migration during maturation was more likely to fail in oocytes aspirated from small follicles (<10mm).

It is generally accepted that for an oocyte to achieve developmental competence, nuclear and cytoplasmic maturation must proceed in a highly integrated fashion (Albertini et al., 1993). Furthermore, the ability of the ovum to undergo nuclear division along with the reorganisation of the cytoplasmic organelles depends greatly on the cytoskeleton, a complex network of protein filaments that extends throughout the oocyte cytoplasm and comprises two major elements, namely the microtubules and the microfilaments. The microfilaments are, in essence, a network of actin filaments that make up the cell cortex, thereby giving strength to the surface of the cell and determining its shape and polarity. The microtubules are distinct supporting structures which, in some species, have been shown to play a fundamental role in the formation of the meiotic structures, reorganisation of the chromosomes and migration of cytoplasmic organelles (Alberts et al., 1994). Indeed, in mouse oocytes the distribution of microtubules changes dramatically during the course of meiosis. While the microtubules are distributed throughout the cytoplasm and organised in numerous centrosomal foci during interphase, in metaphase, apart from many discrete foci around the cytoplasm near the cell cortex, they are mostly found in association with the spindle-associated centrosomes (Maro et al, 1985, Messinger and Albertini, 1991; Verlhac et al., 1994). In oocytes of many other mammalian species, the cytoplasmic microtubules are not readily detectable and it is only after treatment of oocytes with taxol, a drug that promotes microtubule assembly, that a number of groups have been able to demonstrate microtubule aster formation soon after GVBD (rabbit: Yllera-Fernandez et al., 1985; sheep: Le Guen and Crozet., 1989; pig: Kim et al., 1996a; man: Kim et al., 1998). The distribution of microfilaments during mammalian oocyte maturation has also been investigated. In short, in matured oocytes recovered from mice (Maro et al., 1984; Schatten et al., 1986), rats (Zernicka-Goetz et al., 1993), pigs (Kim et al., 1996a) and women (Kim et al., 1998), the microfilaments are concentrated in the cell cortex, although a secondary aggregation has been described overlying the meiotic spindle and has been proposed to maintain the spindle and the chromosomes in a peripheral position (Kim et al., 1996a).

Despite the critical role of microtubules and microfilaments during oocyte development, little is known about the changes that take place in the distribution of these cytoskeletal elements during the meiotic maturation of equine oocytes, either in vivo or in vitro. The aim of the current study was, therefore, to examine and describe the changes in the distribution of microtubules and microfilaments and to relate these changes to the simultaneous changes in chromatin configuration that occur during in vitro maturation of horse oocytes.

MATERIALS AND METHODS

Experimental design

In order to describe the cytoskeletal changes that accompany nuclear reorganisation during the in vitro maturation of horse oocytes, a triple-staining technique was used to simultaneously localise chromatin, microfilaments and microtubules. In addition, oocytes were analysed at 4 different incubation times (0, 12, 24 and 36 hr) selected to ensure that oocytes at all of the important stages of meiotic maturation were included, as described previously by Hinrichs et al. (1993a). In total, 464 oocytes were analysed either using conventional fluorescence microscopy (IFM; n=263) or confocal laser scanning microscopy (CLSM; n=201).

Collection and culture of cumulus oocyte complexes

Ovaries were recovered from mares immediately after slaughter and transported to the laboratory at 25-30 ºC within 4 h. Upon arrival, the ovaries were washed in 0.9% (w/v) NaCl and any extraneous tissue and most of the tunica albuginea was removed. Thereafter, the ovaries were maintained at 30 ºC in 0.9% (w/v) NaCl containing 1% (v/v) penicillin/streptomycin (Gibco BRL, Life Technologies, Paisley, UK), until they were processed. Cumulus oocytes complexes (COCs) were recovered by aspirating the contents from follicles smaller than 30 mm in diameter using a 16 gauge short-bevel needle attached, via an infusion set, to a 250 ml collection tube which was, in turn, connected to a vacuum pump. First, the contents of any visible follicles were aspirated and the follicle lumen was flushed 2-3 times in rapid succession with PBS supplemented with 50 mg/l bovine serum albumin (BSA; Sigma, St Louis, MO, USA) and 25 IU/ml heparin (Leo Pharmaceutical, Weesp, the Netherlands). When all the readily detected follicles had been punctured, the ovary was bisected along its midline, from ovulation fossa to greater curvature, and any newly exposed follicles were aspirated and flushed as before. Subsequent incisions were made at 5-10 mm intervals to expose all of the aspiratable follicles and, finally, the recovered follicular fluid-PBS mix was allowed to stand for approximately 10 min at room temperature to allow the COCs to settle to the bottom of the tube. COCs were then isolated by searching the lower layers of the fluid with a stereomicroscope. All recovered COCs were evaluated for their quality and washed twice in HEPES-buffered Tyrodes medium supplemented with 0.1% polyvinylalcohol and 0.2% BSA (HEPES-TL-PVA), as described previously (Funahashi et al., 1994). Only oocytes with a complete, compact, multilayered cumulus investment were selected for culture and these were passed through two changes of maturation medium prior to actual culture. Finally,

oocytes were allocated randomly to groups of 25 which were incubated in 500µl aliquots of M199 medium, supplemented with 10% FCS, 0.01 units/ml porcine FSH and 0.01 units/ml equine LH (Sigma, St Louis, MO, USA), in 4-well plates (Nunc A/S, Roskilde, Denmark) maintained at 39 ºC in a humidified atmosphere of 5% CO2 in air.

Fixation and examination of oocytes

At either 0, 12, 24 or 36 hr of culture, COCs were removed from incubation and denuded by vortexing them in a calcium-free 0.25% (v/v) solution of trypsin (Trypsin-EDTA) in EBSS (both Gibco BRL). The oocytes were then washed three times in PBS and permeabilised, by treating them for 1 h at 39 ºC with medium M (Simerly and Schatten, 1993), a glycerol-based microtubule-stabilising solution that contained 25% (v/v) glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 50 mM imidazol, pH 6.7, 4% Triton-X-100 and 25µM phenylmethylsulphonyl fluoride (all from Sigma). Next, the oocytes were fixed by immersing them for 30 min at room temperature in a 2% solution of paraformaldehyde in PBS and, after fixation, they were maintained at 4ºC for 2-5 days prior to staining. Depending on which type of microscope was to be used, that is the confocal laser scanning microscope (CLSM) or a conventional immunofluorescence microscope (IFM), different fluorescent probes were used to demonstrate the cellular structures of interest. However, in both cases the microtubules were labelled first by incubating fixed oocytes for 90 min at 37 ºC in a 1:250 solution of a monoclonal anti-α-tubulin antibody (Sigma) in PBS. After this incubation, the oocytes were washed 3 times in PBS containing 0.1% BSA and then incubated for 1 hr in a blocking solution containing 0.1M glycine, 1% goat serum, 0.01 % Triton X-100, 0.5% BSA and 0.02 % sodium azide (all from Sigma), as described by Albertini et al.(1984). Next, the oocytes were exposed for 1 hr at 37ºC to a goat anti-mouse antibody, diluted 1:250 in PBS containing 0.5% Triton X-100 and 0.5% BSA, and conjugated to either fluorescein isothiocyanate (FITC; for IFM) or tetramethylrhodamine isothiocyanate (TRITC; for CLSM). Once the microtubules had been thus labelled, the oocytes were washed twice in PBS with 0.1% BSA and 0.1% Triton X-100 and then twice more in PBS alone. Next, the oocytes to be examined by CLSM were incubated for 1 hr with Alexa Fluor™ 488 phalloidin (15 IU/ml: Molecular Probes, Inc. Eugene, OR, USA) to enable detection of the microfilaments. Oocytes destined for IFM were not stained with a microfilament label. Finally, the oocytes were washed twice more in PBS containing 0.1% BSA and, to enable visualisation of the DNA, oocytes for CLSM analysis were incubated with TO-PRO_3 (1µM in PBS: Molecular Probes) for 15 min, while oocytes intended for IFM analysis were incubated with 0.1µg/ml of 4,6-

diamino-2-phenyl-indole (DAPI) in PBS for 5 min, according to the method described by Mori et al. (1988).
For control purposes, further oocytes were treated with unlabelled phalloidin (25 IU/ml) before incubation with labelled microfilament marker and, to demonstrate the specificity of microtubule labelling, oocytes were incubated directly with labelled goat anti-mouse secondary antibody, without pre-treatment with primary anti-tubulin antibody.

Fluorescence and confocal laser scanning microscopy

Once stained, oocytes for IFM were mounted with an antifade medium (to retard photobleaching; Vectashield, Vector Lab. Burlingame, CA, USA) on glass microscope slides and sealed under the coverslip with nail polish. Conventional immunofluorescence microscopy was performed using a BH2-RFCA Olympus (Olympus, Tokyo, Japan) epifluorescence microscope equipped with a set of DMU filters. Oocytes for CLSM analysis were mounted as above except that, to avoid excess pressure being exerted on the oocytes, the coverslips were supported with thick droplets of a Vaseline-wax mixture placed in each corner. These oocytes were examined using a laser scanning confocal microscope (Leica TCS MP, Heidelberg, Germany) mounted on an inverted microscope (Leica DM IRBE) equipped with x40 and x100 oil immersion objectives. The CLSM was equipped with a Krypto-argon ion lasers for simultaneous excitation of Alexa Fluor™ 488 (microfilaments), TRITC (microtubules) and TO-PRO_3 (DNA) using 488/568/650nm excitation/barrier filter combinations. The images were recorded digitally and archived on an erasable optical diskette.

RESULTS

Chromatin configuration

The various chromatin configurations were clearly visualised using either TO-PRO_3 and CLSM or DAPI and IFM. At the onset of culture, the majority of oocytes were at the germinal vesicle stage (Table 1; 70% of CLSM and 78% of IFM oocytes), as demonstrated by the diffuse appearance of their chromatin which was contained within an organelle free area of the ooplasm (Fig. 1A, 2a). Despite this superficial uniformity, GV oocytes showed a variety of different chromatin configurations, ranging from very diffuse to various degrees of condensation. However, the chromatin was always confined to a defined area (the germinal

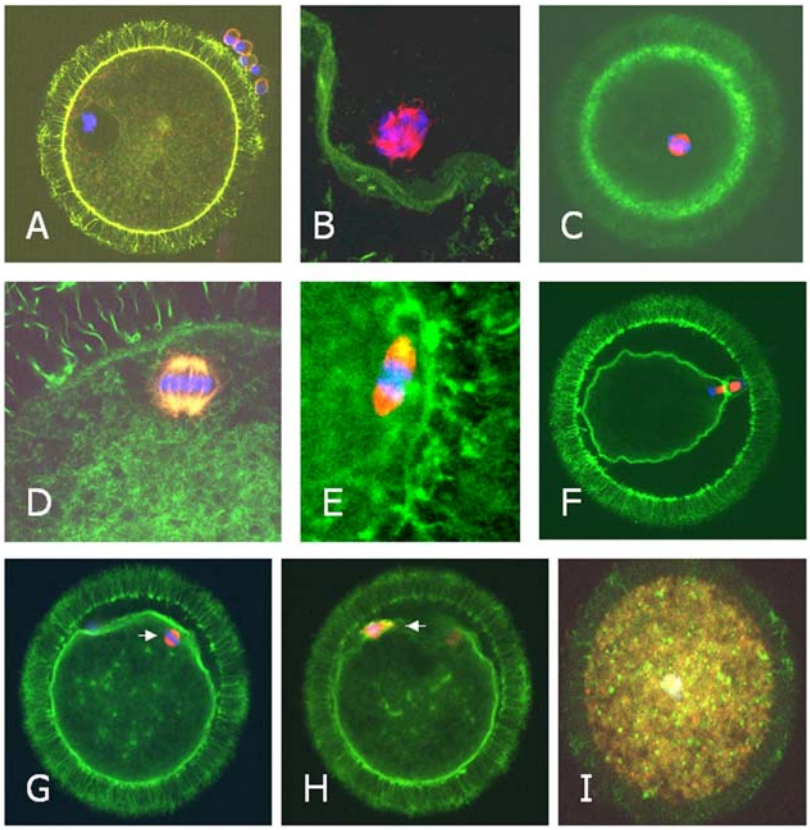

FIGURE 1. Confocal laser scanning photomicrographs of horse oocytes at different stages of maturation in vitro. The nuclear material and cytoskeletal elements have been labelled such that microtubules are displayed in red, microfilaments in green and the chromatin in blue. A) A germinal vesicle stage oocyte: the chromatin has yet to condense and the microtubules and microfilaments are seen to form a network throughout the ooplasm. B) The nuclear region of a prometaphase oocyte; Microtubule asters can be seen in association with the now condensed chromatin strands while the microfilaments are clearly concentrated within the oocyte cortex. C,D) Metaphase I: The microtubules have now formed a clear meiotic spindle with the chromosomes aligned at its equator. E) Meiotic spindle during anaphase I: The microtubular spindle is considerably longer than during metaphase I and the 2 sets of chromosomes have begun to separate. F) Telophase I: A rich microtubular domain is now seen at the midpoint of the meiotic spindle. G,H) A Metaphase II oocyte: This oocyte has a well-organised second meiotic spindle (g) and a polar body(h). Microtubules can be seen within both the spindle and the polar body and a further microfilament rich domain has formed over the second meiotic spindle. I) A Degenerate oocyte: The scattered and disorganised aggregations of chromatin, microtubules and microfilaments clearly demonstrated aberrant development or degeneration. Fig's A, C, F, G, H and I were taken at a magnification of x400 and fig's B,D and E at x1000.

vesicle) and remained as a single mass without any visible organisation into distinct chromosomes. After 12 hr in culture, most oocytes had resumed meiotic progression and had passed through the latter phases of diakinesis. As a result of the resumption of meiosis, the germinal vesicle had broken down and the chromatin had condensed into short, thick, distinct chromosomes. A large proportion of oocytes examined after a 12 hr incubation were in prometaphase (Table 1) and individual chromosomes were now visible as aggregated dots of condensed chromatin (Fig. 1B, 2b).

TABLE 1.The number (and percentage) of oocytes at various stages of meiotic progression

Time (h) in culture	Nm of oocytes	GV (%)	Prometaphase (%)	M-I (%)	M-II (%)	Degenerate (%)
0	104	77 (74)	5 (5)	-	-	22 (21)
12	125	36 (29)	53 (42.5)	3 (2)	1 (1)	32 (25.5)
24	111	3 (3)	9 (8)	28 (25)	28 (25)	43 (39)
36	124	6 (5)	4 (3)	9 (7)	52 (42)	53 (43)

* GV= germinal vesicle; M-I= metaphase –I; MII= metaphase –II.

After 24 hr in culture, approximately 25% of the oocytes had progressed to metaphase of the first meiotic division (Table 1; M-I; 28% of CLSM oocytes and 22.5% of IFM oocytes). At this stage, the highly condensed chromosomes were aligned along the centre of the spindle (the “metaphase plate”: Fig. 1C, 1D, 2c). A further 25% of oocytes (Table 1; 35% of CLSM and 17% of IFM oocytes) had, by the 24 hr time point, reached metaphase of the second meiotic division (M-II) and further oocytes were observed at all of the intervening stages of first meiotic division. In anaphase I, the homologous chromosomes had moved from their initial position at the centre of the meiotic spindle to opposite ends of that spindle (Fig. 1E) and by telophase the separation of the two sets of chromosomes had been completed and the unequal division of the egg then culminated with the extrusion of the first polar body (PB: Fig. 1F, 2d). After 36 hr of culture, the majority of viable oocytes (NB:>40% were degenerate) were in metaphase II (Table 1; 43% for CLSM and 41 % for IFM), with their chromosomes aligned along the second metaphase plate and with the first PB detectable as an amorphous chromatin mass (Fig. 1G, 1H, 2e).

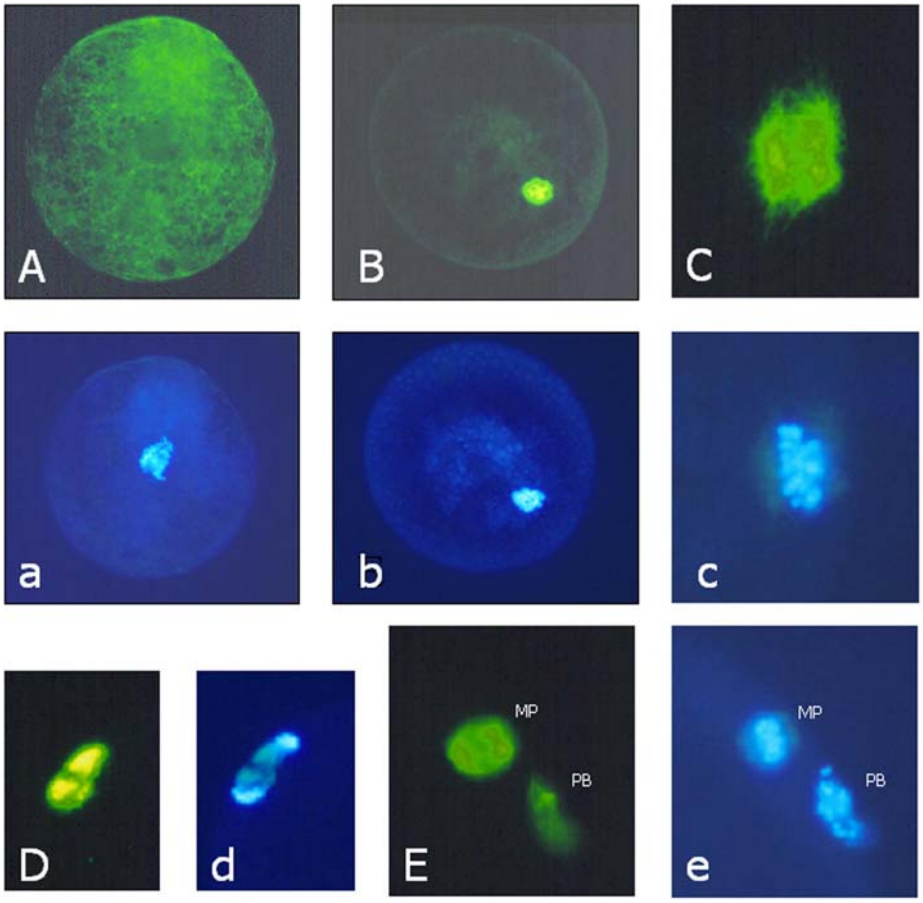

FIGURE 2. Immunofluorescence photomicrographs of horse oocytes stained to demonstrate the presence of microtubules (A, B, C, D, E) and chromatin (a,b,c,d,e) at different stages during maturation in vitro. A,a) A Germinal vesicle stage oocyte: A network of microtubules can be seen extending throughout the cytoplasm (A), while the chromatin is partly condensed and contained within the nuclear envelope (a). B,b) A prometaphase oocyte: In this early post germinal vesicle breakdown stage, the microtubules (B) have started to concentrated around the chromatin which has now condensed to form more distinguishable chromosomes (b). C,c) A Metaphase I oocyte: the microtubules are now seen exclusively within the asters of the first meiotic spindle (C) and chromosomes have aligned along the equator of this spindle to form the metaphase plate (c). D,d) A telophase I oocyte: the microtubules are now seen in the central part of the spindle (D) separating the two sets of chromosomes (d). E,e) A metaphase II oocyte: In this section both the metaphase plate (MP) and polar body(PB) are visible. Microtubules can be seen forming a well-organised second meiotic spindle and in a more diffuse arrangement in the polar body (E). Chromosomal material can be seen at the centre of the metaphase II plate and within the polar body (e). Fig's A,B,a and b were taken at a magnification of x400 and fig's C,c,D,d,E and e at x1000.

Organisation of the microtubules

The microtubule components of the cytoskeleton were visualised using an anti-tubulin antibody and secondary antibody system and the patterns observed were related to the chromatin configurations, as described above. Oocytes in the GV stage displayed a complex network of thread-like, weakly-labelled microtubules, distributed uniformly throughout the ooplasm. This pattern was observed clearly in oocytes analysed by both CLSM (Fig. 1A) and IFM (Fig. 2A). At this stage, no clear cytoplasmic domains of microtubules were detected and therefore microtubule organising centers (MTOC) were not evident. Indeed, no marked differences in microtubule organisation were evident between oocytes displaying different chromatin configurations but yet to undergo germinal vesicle breakdown (GVBD). Soon after GVBD, however, a complete rearrangement of the microtubules was observed. In prometaphase oocytes displayed densely stained radial-like microtubules in close association with the recently condensed and distinguishable chromosomes (Fig 1B, 2B) and, at this stage, no other microtubular foci were detected within the ooplasm. By metaphase I, the microtubules were radially elongated forming the meiotic spindle and thus supporting and holding in alignment the soon to be segregated chromosomes (Fig. 1C, 1D, 2C). As maturation progressed through anaphase I, the spindle assumed a more eccentric position within the ooplasm and its major axis rotated gradually until the spindle was orientated radially. By telophase, when the separation of the two sets of chromosomes was complete, microtubules were detected only within the spindle that was now elongated (Fig. 1F). In metaphase II oocytes, microtubules were detected within both the second meiotic spindle and the polar body (Fig. 1G, 1H, 2E). This second meiotic spindle was also located peripherally in the ooplasm and orientated radially and it was essentially a symmetrical, barrel-shaped structure with two anastral poles and with the chromosomes aligned along its centre. The microtubules within the polar body had no discernable organisation and instead appeared as an amorphous mass intertwined with chromatin and lying within the perivitelline space.

Distribution of the microfilaments

At the germinal vesicle stage, microfilaments, like the microtubules, were distributed throughout the ooplasm, albeit with a slightly greater concentration evident just beneath the plasma membrane of the oocyte (Fig. 1A). By prometaphase, however, the microfilaments were clearly concentrated within the cortical region of the oocyte (Fig. 1B). This peripheral concentration of microfilaments was retained throughout the first meiotic division, during which

further microfilament-rich domains developed over the meiotic spindle and around the first polar body. During polar body formation, the microfilaments formed a "furrow" between the two separated sets of chromosomes, in the region of the elongated spindle and the approximate location of subsequent cytoplasmic cleavage (Fig. 1F). Metaphase II oocytes, showed similar patterns of microfilament distribution with a concentration in the cortex of the oocyte and a rich domain overlying the second meiotic spindle (Fig. 1G, 1H). Interestingly, strands of intensive phalloidin staining were seen to transverse the zona pellucida and these presumably represented the actin-containing transzonal cumulus processes described by Plancha et al (1994). However, the intensity of this intrazonal staining varied considerably between oocytes and did not change in any consistent manner during the progression of oocytes through meiotic maturation.

Degenerate oocytes

At the onset of culture (0 hr), a high proportion of oocytes were found to be degenerate (Table 1; 30% of CLSM and 13% of IFM oocytes). This proportion increased markedly during maturation such that by 36 hr 43% of the oocytes (46% of CLSM and 40% of IFM oocytes; FIG 1I) were degenerate, as evidenced by their aberrant chromatin and cytoskeletal patterns. In these degenerate oocytes, the DNA was either not visible or was visible only as hairlike strands or scattered small drops. Similarly, the microtubules and microfilaments were aggregated in small clusters and scattered throughout the ooplasm (Fig. 1I).

DISCUSSION

The present study describes the cytoskeletal reorganisations that accompany and facilitate nuclear and cytoplasmic segregation during the maturation of horse oocytes in vitro. This was enabled by the combination of multiple fluorescent labelling and CLSM, which proved an effective means of simultaneously visualising chromatin configuration and microfilament and microtubular distribution. In addition, the removal of COCs from culture at 12 hr intervals during IVM ensured that we were able to characterise the cytoskeletal appearance at all of the significant stages of meiotic maturation. Furthermore, because an adequate number of oocytes was examined (464), we were able to get a good idea of the time course of the various changes.

At the onset of incubation, the majority of oocytes (74%) were in the GV-stage, as reported by Hinrichs et al (1993a). The observed gradation in the chromatin

configuration of GV horse oocytes has been described previously (Hinrichs et al., 1993b) and the changes are thought to be a prerequisite for enabling the prophase-arrested oocyte to successfully resume meiosis. In this paper, we describe an extensive, relatively uniform complex of microtubules distributed throughout the ooplasm of GV-stage oocytes. A similar pattern has been described in GV mouse oocytes at the early transitional stages of meiotic maturation (Mattson and Albertini, 1990; Messinger and Albertini et a., 1991). However, the same authors recorded that with the progression of mouse oocytes towards GV breakdown, the cytoplasmic microtubules began to concentrate at the margins of the GV and, from there, to extend into the subcortical regions of the ooplasm. Such a transition, and indeed the latter microtubular pattern, was not evident in the horse oocytes studied. On the other hand, in species such as the pig (Kim et al., 1996a), microtubules were not detected at all in GV stage oocytes.

Classically, GVBD and chromosome condensation are accompanied by a complete reorganisation of the microtubule network. In the present study, by prometaphase I of in vitro maturation the cytoplasmic microtubules of horse oocytes had moved from the diffuse distribution characteristic of the GV stage and concentrated around the strands of chromatin. Although a similarly timed aggregation of microtubules around the chromosomes occurs in the oocytes of other mammalian species, and is associated with the development of meiotic competence (Kim et al., 1996a,1998), the stimulus for this recruitment is not known. However, the aggregation of microtubules does correlate with the phosphorylation of centrosomes (Wickramansinghe et al., 1991) and further studies on the distribution and function of these latter microtubule organising structures may help to clarify events surrounding the resumption of meiosis in horse oocytes. Indeed, a better understanding of the mechanisms that release a horse oocyte from its arrested phase may help in the development of strategies to improve the quality of oocyte maturation and, in particular, to optimise the efficiency of cytoplasmic as well as nuclear maturation in vitro (Sirard et al., 1992).

During metaphase of both meiotic divisions, microtubules were concentrated in the meiotic spindle surrounding the aligned chromosomes. Our description of the horse meiotic spindle as a symmetrical barrel-shaped structure with two anastral poles echoed that of Goudet et al. (1997) and was very similar to that described for other species (mouse: Messinger and Albertini, 1991; man: Pickering et al., 1988; Kim et al., 1998; cow: Aman et al., 1994; pig: Kim et al., 1996a). Goudet et al. (1997) also reported the occurrence in horse oocytes matured in vivo of abnormal spindles, in which the microtubules between the two poles were disorganised. Similar abnormal spindles have been reported previously in oocytes from women

(Pickering et al., 1988; Baka et al., 1995) and mice (Eichenlaub-Ritter et al., 1986) but, while we did not specifically measure any structural parameters of the meiotic spindles, we did not observe any obvious spindle or chromosome desegregation abnormalities the current study.

Maro et al. (1985) reported that in metaphase stage mouse oocytes, in addition to the spindle-associated centrosomes, there were numerous other centrosomal foci within the ooplasm. By contrast, the spindle was the sole microtubule-containing structure in IVM horse oocytes and we did not detected any cytoplasmic MTOC; MTOCs are similarly absent from the oocytes of a number of other mammalian species. (pig: Kim et al., 1996a; man: Kim et al., 1998; sheep: LeGuen and Crozet., 1989). In the mouse, it is proposed that MTOCs are important for pronuclear migration, after fertilisation, and for mitotic division during the initial cell cycles (Maro et al., 1985; Schatten et al., 1986). Thus, the presence of MTOCs in mice and not in other species almost certainly relates to the fact that while in mice the migration of the female and male pronuclei after fertilisation is controlled by maternal centrosomal material (in the MTOCs: Schatten et al., 1986), in most other species this mechanism depends on the paternal centrosome (from the sperm).

The changes that we observed in microfilament organisation during oocyte maturation are similar to those reported in other species (rat: Zernicka-Goetz et al., 1993; pig: Kim at al., 1996a; man: Kim et al., 1998; mouse: Webb et al., 1986). That is, from an even distribution of microfilaments throughout the ooplasm during the GV stage to a concentration in the subcortical region of the oocyte immediately after GVBD, and with subsequent development of secondary rich domains over the meiotic spindle and around the polar body. With regard to the role of microfilaments during oocyte maturation, Longo and Chen (1985) demonstrated that treating mouse oocytes with the microfilament disrupting agent, cytochalasin B, prevented the normal migration of chromosomes to a peripheral position in the oocyte and interfered with polar body formation. Thus it appears that actin filaments play important roles in ensuring that the spindle maintains a peripheral location appropriate for extrusion of the polar body beyond the oolemma. Microfilaments have also been shown to play an active role in the migration of cortical granules from deep within the oocyte to their classical peripheral or cortical location in the mature oocyte (Kim et al., 1996b; DiMaggio et al., 1997;Terada et al., 2000).

A further interesting observation from the current study, was the presence of strands of microfilamentar material within the zona pellucida. A similar pattern has been described in other species, where the material is thought to consist of

transzonal processes extending from cumulus cells (Allworth and Albertini, 1993; Plancha and Albertini, 1994). Moreover, these intercellular connections between cumulus cells and the oocyte are thought to be essential to the production of a developmentally competent female gamete (Albertini et al., 1993). Indeed, hormonal regulation of oocyte maturation appears to involve remodelling of the actin cytoskeleton at the level of the cumulus cell transzonal processes and within the oocyte cortex (Plancha and Albertini, 1994).

During this study we found that a large proportion of oocytes were either degenerate at the onset of culture (21%) or became so during maturation in vitro (43% after 36 hr). This is not entirely surprising when we consider the heterogeneous population of healthy and atretic follicles from which the oocytes were harvested. Nevertheless, this degeneration rate compares extremely unfavourably to that observed, for example, for bovine oocytes harvested and matured under similar conditions (Bevers, unpublished data). However, average rates of oocyte degeneration reported for in vitro matured horse oocytes vary greatly between laboratories (Willis et al., 1991; Alm and Torner, 1994; Brinsko et al., 1995; Del Campo et al., 1995) and it seems that this, at least in part, relates to the selection of follicles from which oocytes are collected. For example, Hinrichs and Schmidt (2000) reported recently that initial chromatin configuration at the GV stage was strongly correlated with the meiotic competence of horse oocytes, and that the former was influenced positively by increasing follicle size. For this reason, they suggested that if horse oocytes were to be selected for maximum potential meiotic competence, then only follicles ≥ 20 mm should be selected for puncture. In a similar vein, Pedersen et al. (2000) reported that follicles <16 mm in diameter had a higher proportion of apoptotic granulosa cells than larger follicles and this might affect oocyte quality. In the present study, only follicles ≤ 30 mm were punctured and aspiration of only of follicles >20 mm in diameter would have restricted greatly the number of oocytes available. Although this strategy may have contributed to the high rates of degeneration recorded, it is noted that the above studies have not yet led to definitive criteria for follicle selection and there is still no repeatable, reliable and objective method for assessing the developmental competence of an horse oocyte.

In summary, this study has provided a basic description of the cytoskeleton of horse oocytes and has demonstrated that changes in the distribution of both the microtubules and the microfilaments are highly integrated with the reorganisation of genetic material during oocyte maturation. This emphasizes the importance of the structural elements of an oocyte's cytoplasm, particularly when we consider

that the cytoskeleton almost certainly play similarly important roles in the redistribution of other cytoplasmic organelles in a manner that may be critical to fertilising capacity and subsequent developmental competence. Furthermore, analysis of the effects of various oocyte handling procedures, including IVM, IVF and cryopreservation, on the structure of the cytoskeleton may help to explain why such techniques have, thus far, yielded such poor results in equids.

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Chapter 4

Evaluation of sperm-oocyte interaction during *in vitro* fertilization in the horse

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ABSTRACT

Conventional *in vitro* fertilization (IVF) has proven an unsuccessful means of producing equine embryos. However, the reasons for this failure are unclear and the aim of the current study was to assess sperm-oocyte interaction during IVF. In an initial experiment, oocytes categorized by cumulus morphology (compact or expanded) were matured *in vitro* and then denuded of their cumulus investment. Those with a visible polar body were incubated for 6 or 20 h with stallion sperm in the presence or absence of 150 ng/ml progesterone to stimulate the acrosome reaction. In a second experiment, *in vitro* matured oocytes with or without their cumulus layer were incubated for 6 h and, in the third experiment *in vivo* matured oocytes recovered from preovulatory follicles were incubated for 20 h, with sperm in the presence of progesterone. Most incubations were performed with frozen/thawed sperm from a single stallion. However, to determine whether stallion or semen cryopreservation critically influenced the outcome of IVF, further oocytes were incubated with fresh or frozen-thawed sperm from the same and one other stallion. The resulting sperm-oocyte complexes were labeled with calcein-AM and ethidium homodimer-1 to assess sperm viability or, mostly, fluorescein-conjugated peanut agglutinin and ethidium homodimer-1 to assess acrosome status and localize sperm position.
Irrespective of the duration of IVF, the presence of progesterone, initial cumulus morphology or denudation, fresh and frozen-thawed sperm from both stallions failed to undergo the AR or penetrate the oocytes, despite binding to the zona pellucida. Higher numbers of sperm bound to the ZP of *in vivo* matured oocytes and cumulus free *in vitro* matured oocytes, but still failed to acrosome react or fertilize. In summary, fertilization failed because sperm did not acrosome react after binding to the ZP. For conventional IVF to become a practical proposition in equids, reliable methods for inducing 'physiological' sperm activation *in vitro* must be developed.

INTRODUCTION

In vitro fertilization (IVF) procedures are well established for most farm animal species. However, only two foals have been produced via conventional IVF, and both were derived from *in vivo* matured oocytes recovered from preovulatory follicles (Palmer *et al*., 1991; Bézard, 1992). Indeed, despite numerous attempts to mature and fertilize equine oocytes *in vitro*, fertilization rates have remained poor (0-30%; where fertilization was taken as sperm decondensation or pronucleus formation) and no offspring have been produced (Del Campo *et al*., 1990; Zhang *et*

al., 1990; Grøndhal *et al*., 1995; Dell'Aquila *et al*., 1997; Hinrichs *et al*., 2002). These poor equine IVF results have been proposed to relate to inadequate sperm activation *in vitro* and poor developmental competence of oocytes after *in vitro* maturation (IVM) (Alm *et al*., 2001; Hinrichs *et al*., 2002; Squires *et al*., 2003). To acquire fertilizing capacity, sperm need first to capacitate, a process that involves dramatic reorganization of the sperm plasma membrane (Flesch and Gadella, 2000). Capacitated sperm become hypermotile (Yanagimachi, 1989; Rathi *et al*., 2001) and acquire the ability to bind to the oocyte's extracellular matrix (the cumulus matrix and ZP: Brewis and Wong, 1999). ZP-binding in turn triggers the acrosome reaction (AR), an exocytotic event involving the release of enzymes (Töpfer-Petersen *et al*, 2000) to lyse the zona pellucida (ZP) and enable the hypermotile sperm to burrow through into the perivitelline space, fuse with the oolemma and initiate fertilization *per se* (for review see: Yanagimachi, 1994). During conventional IVF, these specific physiological events need to be stimulated in the appropriate order.

Several compounds have been tested for their ability to induce capacitation and the AR in stallion sperm *in vitro*. Heparin (Farlin *et al*., 1993; Varner *et al*., 1993; Alm *et al*., 2001), equine ZP proteins (Arns *et al*., 1991) caffeine and lysophospholipids (Blue *et al*., 1989, Hochi *et al*., 1996), all increase the percentage of sperm that capacitate and acrosome react, but none significantly improve oocyte penetration rates. On the other hand, calcium ionophore A23187 induces AR-like changes in stallion sperm and facilitates oocyte penetration (Del Campo *et al*., 1990; Zhang *et al*., 1990; Li *et al*., 1995: Alm *et al.*, 2001). Indeed, calcium ionophore was the sperm activator used during the production of the only two documented IVF foals (Palmer *et al*., 1991; Bézard, 1992). Another compound that induces the AR is progesterone. P4: Meyers *et al*., 1995, Cheng *et al*., 1998a), which is present in follicular fluid (Linford *et al*., 1992) and therefore postulated to be involved in sperm activation *in vivo*. Indeed, binding of stallion sperm to homologous ZP and subsequent AR are dose-dependently enhanced by progesterone (Cheng *et al*., 1998a).

Assisted reproductive techniques such as ZP dissection (Choi *et al*., 1994), drilling (Li *et al*., 1995) and partial cumulus removal (Dell'Aquila *et al*., 1996) markedly improve fertilization rates by facilitating ZP penetration. It is, however, not entirely clear whether poor ZP penetration is primarily a sperm problem or an oocyte problem arising as a consequence of IVM. In the latter respect, mouse and rat oocytes undergo ZP-hardening during culture, which hampers sperm penetration (Zhang *et al*., 1991) and is associated with premature cortical granule exocytosis; this does not appear to occur in equine oocytes (Dell'Aquila *et al*., 1999).

The aim of this study was to investigate the cause of the low rates of oocyte penetration during conventional IVF. To this end, we examined whether sperm-

binding, oocyte penetration and fertilization of both *in vitro* and *in vivo* matured oocytes was influenced by cumulus cells and/or progesterone. The interaction between sperm and the cumulus oocyte complex (COC) was assessed using confocal laser scanning microscopy (CLSM) combined with stains to demonstrate sperm viability and acrosome status. In addition, incubated sperm were stained with Merocyanine 540 (Rathi *et al*., 2001), fluorescein-conjugated peanut agglutinin (FITC-PNA: Cheng *et al*., 1996), Mitotracker Red (Gadella and Harrison, 2002) and Yo-Pro-1 or propidium iodide (PI) to evaluate the effects of IVF conditions on capacitation, acrosome status, mitochondrial function and viability, respectively. Alterations in staining patterns were monitored over time using a flow-cytometer.

MATERIALS AND METHODS

Collection and culture of cumulus oocyte complexes

Oocytes were collected and cultured as described previously (Tremoleda *et al*., 2001). In brief, COCs were collected from the ovaries of slaughtered mares by aspirating follicles < 30mm in diameter. Recovered COCs were washed in Hepes buffered Tyrodes medium containing 0.1% (w/v) polyvinylalcohol and matured in M199 culture medium supplemented with 10% FCS (Gibco BRL™, Breda, the Netherlands; # 40F6414K), 0.01 units/ml porcine FSH and equine LH (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) at 39 °C in a humidified atmosphere of 5% CO_2 in air. After 36 h in culture, oocytes with a fully expanded cumulus investment were denuded by incubation in PBS containing 0.1% (w/v) hyaluronidase (Hyaluronidase type I-S; Sigma-Aldrich) and subsequent vortexing. The denuded oocytes were examined using an Olympus SZX9 (Olympus, Tokyo, Japan) inverted microscope and those with an intact oolemma and visible first polar body (PB) were selected for IVF. The selected oocytes were washed twice in Earle's Balanced Salt Solution (EBBS) without calcium and magnesium (Gibco BRL™), supplemented with 0.1% BSA (Sigma-Aldrich), before transfer to IVF medium.

Sperm treatment for IVF

Fresh and frozen-thawed sperm from two fertile stallions was prepared for IVF by centrifugation for 5 min at 200g and 15 min at 700g through a discontinuous (45%:90%) Percoll density-gradient made up in modified Tyrodes medium (Sp-TALP: Parrish *et al*., 1988). The resulting sperm pellet was resuspended in 100 µl

of Sp-TALP and subsequently diluted to a concentration of $25x10^6$ spermatozoa/ml with IVF medium.
Oocytes were incubated in groups of 10-15 in 480 µl IVF medium (fert-TALP: Parrish *et al*. 1998) modified by addition of 1% (v/v) penicillin/streptomycin (Gibco BRL™) and omission of gentamycin, hypotaurine and epinephrine. Twenty µl of sperm suspension was added to each group of oocytes (4-well plates: Nunc A/S, Roskilde, Denmark) such that the final sperm concentration was $1x10^6$ /ml. Fert-TALP medium was supplemented with progesterone (Sigma-Aldrich) dissolved in ethanol to final concentrations of 150 ng/ml progesterone and 0.1% v/v ethanol. Control medium was supplemented with a similar concentration of ethanol. IVF was performed at 39 ºC in a humidified atmosphere of 5% CO_2 in air.

IVF treatments

Effect of progesterone, initial cumulus morphology and denudation on IVF
For these initial experiments, frozen-thawed semen from a single stallion was used.

Effect of cumulus expansion and progesterone on IVF of *in vitro* matured COCs
COCs were classified by their cumulus morphology as compact (C-COCs) or expanded (E-COCs: Hinrichs and Schmidt, 2000) and cultured in groups of a single cumulus category. After IVM, oocytes were denuded of their cumulus cells and those with a visible polar body were incubated with sperm in IVF medium with or without 150 ng/ml P4 for 6 or 20 h. In total 236 oocytes were used; the division into morphological or treatment groups is shown in table 1.
Effect on IVF of denuding *in vitro* matured COCs
Oocytes with a complete, compact and multilayered cumulus investment were matured *in vitro*. After culture, one group of oocytes were stripped of their cumulus cells (denuded) and washed twice in EBBS containing 0.1% BSA to remove any residual hyaluronidase before transfer to IVF medium. The second group of oocytes was transferred directly into IVF medium using a large bore Pasteur pipette, to avoid mechanical disruption of the cumulus (non-denuded oocytes). Both groups were incubated in IVF medium supplemented with 150 ng/ml P4 for 6 h. Only oocytes displaying a first PB after denudation (n=52) or, for non-denuded oocytes (n=67), when later visualized by CLSM, were included in the final analyses.
Collection and IVF of *in vivo* matured COCs
To determine whether IVM affects sperm-ZP binding or oocyte penetrability, *in vivo* matured oocytes were also subjected to IVF. *In vivo* matured COCs (n=15) were recovered from Dutch Warmblood mares by transvaginal ultrasound-guided aspiration (Bracher *et al*., 1993) and flushing of preovulatory follicles 32 h after

intravenous injection of 2250 iu human chorionic gonadotrophin (hCG: Chorulon®, Intervet BV, Boxmeer, The Netherlands). After recovery, COCs were washed and held at 30°C in Hepes-buffered M199 (Gibco BRL) until transfer to the IVF well, where they were co-incubated with sperm for 20 h in IVF medium supplemented with 150 ng/ml of P4.

IVF with fresh versus frozen-thawed sperm

To examine whether there was an influence of stallion or cryopreservation on sperm-penetration during IVF, denuded IVM oocytes (from C-COCs or E-COCs) were incubated with either fresh or frozen-thawed sperm from one of two fertile stallions. Groups of 10-15 oocytes were incubated with sperm for 20 h in IVF medium supplemented with 150 ng/ml of P4. The number of sperm that had bound to or penetrated the ZP was assessed.

Assessment of sperm membrane status and ZP penetration

The position (bound to the cumulus oophorus, ZP or within the oocyte), viability and acrosome status of sperm were analyzed using confocal laser scanning microscopy and a combination of fluorescent probes.

Acrosome status: After IVF, sperm-oocytes complexes were rinsed gently several times with PBS containing 0.1% BSA via a narrow-bore Pasteur pipette, to remove loosely attached spermatozoa. The complexes were then transferred to 50 µl droplets of 0.05% (v/v) Nonidet-P40 (Fluka, Buchs, Germany) for 1 min to permeabilize the cellular membranes and, thereafter, fixed for 30 min at room temperature in 3% paraformaldehyde. Sperm-oocyte complexes were stored in fixative at 4 ºC for 2-5 days. Before staining, the complexes were incubated for 1 h at room temperature in 0.02 M glycine (Sigma) in PBS to block free aldehyde groups. Next, complexes were washed three times in PBS containing 0.1% BSA before incubation for 30 min in 100 µl droplets containing 20 µg/ml FITC-PNA (an outer acrosomal membrane marker: EY Laboratories, San Mateo, CA) and 2 µM ethidium homodimer-1 (EthD-1: a DNA stain; Molecular Probes Europe, Leiden, The Netherlands). The complexes were washed a further three times in PBS, mounted with an antifade medium (Vectashield, Vector Lab. Burlingame, CA, USA) on glass microscope slides and sealed under a coverslip with nail polish.

Sperm viability was assessed using calcein-AM and EthD-1 (Althouse and Hopkins, 1995) in the form of the LIVE/DEAD® kit (Viability/Cytotoxicity; Molecular Probes Europe). Sperm-oocytes complexes were removed from the IVF medium, rinsed gently to remove loosely attached sperm and, without any permeabilization treatment, they were incubated for 15 min at 37 ºC in 100 µl PBS droplets containing 2 µM EthD-1 and 1 µM calcein-AM. The complexes were then immediately fixed in 3% paraformaldehyde for 30 min at room temperature. After

fixation, the stained sperm-oocyte complexes were kept in the dark to retard photo bleaching and, within 24 h, mounted on glass microscope slides with an antifade medium as described previously.

Confocal laser scanning microscopy
Stained sperm-oocyte complexes were examined using a confocal laser-scanning microscope (CLSM: Leica TCS-SP, Heidelberg, Germany) mounted on an inverted microscope (Leica DM IRBE) with x40 and x100 oil immersion objectives. The CLSM was equipped with krypton and argon ion lasers. The 488 nm argon laser was used to excite FITC-PNA or calcein-AM (emission range 498-530nm) while the 568 nm krypton laser was used simultaneously to excite EthD-1 (emission spectrum 590-640 nm). Examination was performed as a continuous series of digital images to enable precise localization of sperm within the oocyte and its investments. For a proportion of oocytes selected randomly from each experimental group, the total number of spermatozoa bound to each COC/oocyte was counted.

Effect of in vitro incubation on sperm parameters
To assess the adequacy of sperm activation *in vitro*, changes in capacitation status, acrosome integrity, mitochondrial activity and viability over time in culture were analyzed flow cytometrically. For this, 3 samples of the frozen-thawed semen used in most of the IVF experiments were divided into two portions, one of which was diluted with fert-TALP medium and the other with fert-TALP medium supplemented with 150 ng/ml progesterone. Both samples were incubated at 39ºC in a humidified atmosphere of 5% CO_2 in air for 6 h. At 0, 3 and 6 h, an aliquot was recovered and stained (unfixed) for flow cytometric assessment of the parameters listed above. Between measurements, samples were maintained in a water bath at 37 ºC in air-tight 5 ml tubes which, after removal of an aliquot, were flushed with air containing 5% CO_2.

Flow cytometry
Sperm cell analysis was performed using a flow-cytometer equipped with an Argon laser (FACScan; Becton Dickinson, San Jose, CA) and PBS as sheath fluid. Sperm were analyzed at a rate of 8000 - 10000 per sec and, for each sample, 10000 sperm-specific events were analyzed and stored for further analysis with Cell-Quest software (Becton Dickinson, San Jose, CA).

Evaluation of sperm viability and acrosome status: For simultaneous evaluation of viability and acrosome status, sperm were stained for 10 minutes with 5 µg/ml FITC-PNA and 1 µM PI (Propidium iodide: Molecular Probes Europe),

respectively. FITC-PNA emission was recorded via the FL-1 detector (495/530-nm band-pass filter) and PI via the FL-3 detector (620-nm long-pass filter) as described by Rathi *et al*. (2001).

Evaluation of capacitation status: For the analysis of capacitation status, sperm were stained for 10 minutes with 2.7 μM merocyanine 540 (MC540: Molecular Probes Europe), a red fluorescent probe reported to sense stallion sperm capacitation (Rathi *et al*., 2001). Viability staining was performed simultaneously with 25nM Yo-Pro-1 (a green fluorescent membrane-impermeable nucleic acid stain; Molecular Probes Europe). Yo-Pro-1 and MC540 emission were recorded using the FL-1 and FL-3 detectors, respectively.

Evaluation of mitochondrial activity: Damage to the mitochondria can be detected with Mitotracker Red (Molecular Probes Europe) which senses the potential of the inner mitochondrial membrane. Aerobic production of ATP is blocked in mitochondria with depolarized inner mitochondrial membranes. The red fluorescent emission is only present in mid-pieces where mitochondria still produce ATP (i.e. with normal potential over the inner mitochondrial membrane, a functional electron transport chain and proton gradient). The sperm suspension was simultaneously stained with Mitotracker Red and Yo-Pro-1 as a viability counterstain according to Gadella and Harrison (2002) and their emissions were recorded via FL-3 and FL-1 detectors, respectively.

Data Analysis

The total number of sperm bound to oocytes of different experimental groups was compared using one–way ANOVA followed by pair-wise multiple comparisons (Bonferroni *t*-test) or unpaired Student's t tests. Analysis of sperm data was based on identifying the population capable of fertilization. Thus, percentages of non-capacitated or acrosome-intact sperm or those with functional mitochondria were tracked over time. Statistical analysis was carried out using repeated measures ANOVA to examine the effects of progesterone and incubation time. The statistics package used was SPSS 8.0 (SPSS for Windows 1996; SPSS Inc., Chicago, IL). Differences were taken to be statistically significant when $P<0.05$.

RESULTS

Effect of cumulus morphology, denudation and progesterone on sperm-oocyte interaction

Effect of initial cumulus morphology:

Two hundred and thirty-six oocytes were categorized at recovery as having a compact (C-COCs, n=136) or an expanded (E-COCs, n=100) cumulus, before being subjected to IVM, IVF and analysis by CLSM. For purposes of analysis, these oocytes were further divided by the duration of IVF (6 or 20 h), and whether or not progesterone was included in the IVF medium (Table 1). Irrespective of any of these 3 variables, sperm did not penetrate the ZP of IVM oocytes and appear in either the perivitelline space or ooplasm, even though large numbers bound to the ZP (Figs 1 A-D). This absolute failure of sperm penetration was, of course, accompanied by failure of any of the oocytes to show signs of fertilization (e.g. resumption of meiosis, pronucleus formation); all oocytes instead remained in metaphase of the second meiotic division (M-II), with their chromatin divided between an arrested second metaphase plate (MP) and a first polar body (PB: Figs 1A and 1B).

TABLE 1. Number of sperm-oocyte complexes analysed by CLSM to determine whether cumulus morphology at the onset of IVM (compact or expanded), duration of IVF or addition of 150 ng/ml progesterone to the IVF medium affected the viability or acrosome status of ZP-bound sperm.

Initial cumulus morphology	Duration of IVF (h)	No. oocytes	No. sperm-oocyte complexes used for acrosome status analysis[a]		No. sperm-oocyte complexes used for viability assessment[b]	
			Progesterone[c]	Control[d]	Progesterone[c]	Control[d]
Compact	**6**	50	20	15	7	8
	20	86	28	16	26	16
Expanded	**6**	45	17	6	17	5
	20	55	19	13	19	4

[a] The acrosome status of sperm was evaluated using FITC-PNA, with EthD-1 as a viability counter stain.
[b] Sperm viability was assessed using LIVE/DEAD® (Calcein-AM and EthD-1).
[c]Incubation in fert-TALP medium supplemented with 150ng/ml progesterone
[d]Incubation in fert-TALP without progesterone: control IVF medium (0.1% ethanol)

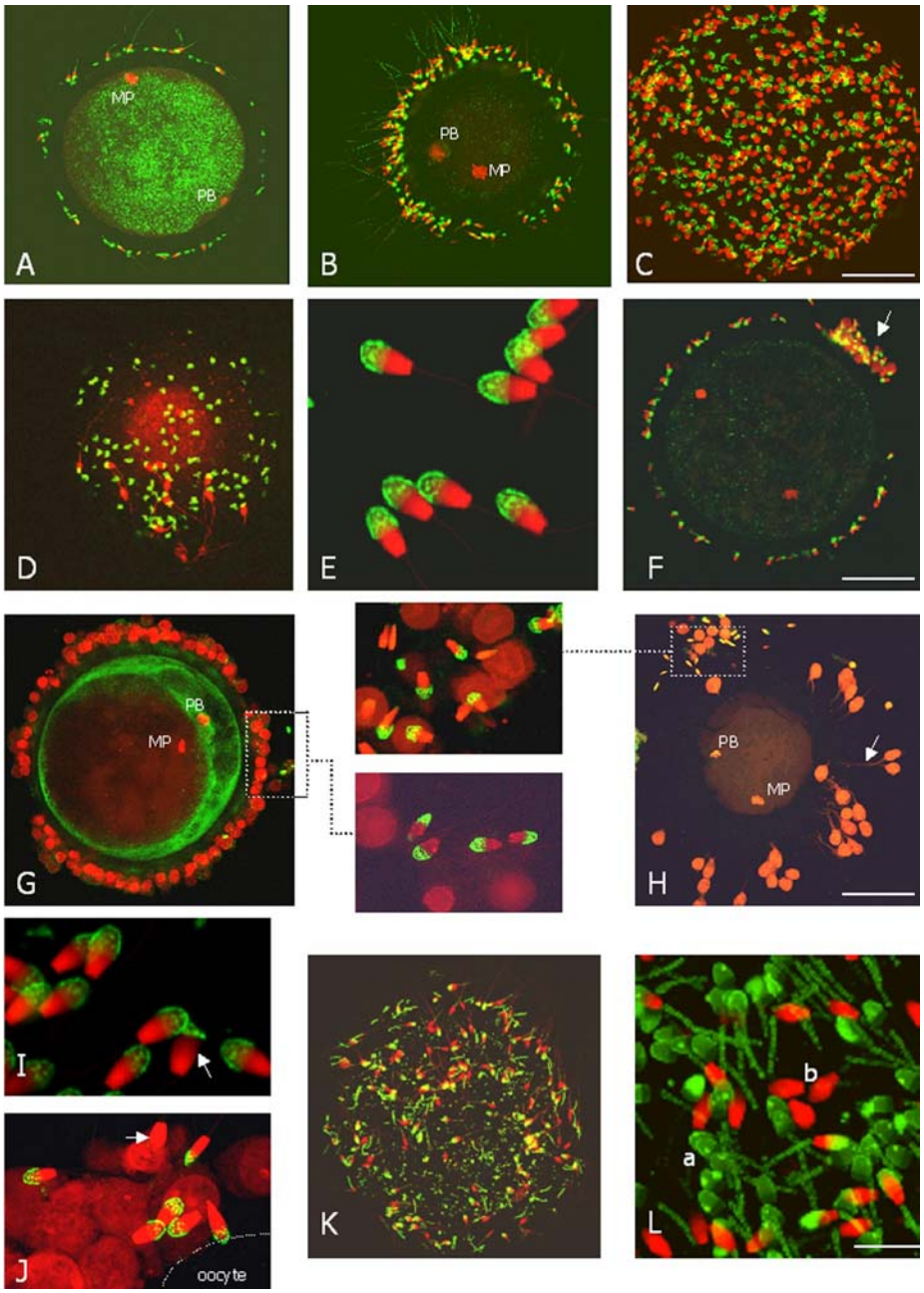

MP
PB
A
PB
MP
B
C
D
E
F
PB
MP
G
PB
MP
H
I
J
oocyte
K
b
a
L

Fig 1. Confocal laser scanning photomicrographs of equine sperm-oocyte complexes resulting after IVF. FITC-PNA (green) was used to determine the acrosome status of bound sperm. Acrosome-intact sperm would have a uniformly (green) stained cap, whereas acrosome reacted sperm would have either a residual, intensely stained equatorial band or would have lost the stained cap altogether. Chromatin of both oocyte and sperm was stained red with EthD-1. (A, B) Sperm bound to cumulus denuded oocytes. The condensed chromatin of the oocytes is clearly visible within the metaphase plate (MP) and polar body (PB). (C) Large numbers of sperm bound to a denuded oocyte. The green acrosomal caps and red sperm nuclei are clear. (D) Sperm and detached acrosomal caps bound to a denuded oocyte. (E, I) The acrosomal caps of ZP-bound sperm were characteristically mottled, slightly disrupted and swollen, but had not acrosome reacted. Occasionally, a sperm was seen lying adjacent to its detaching but intact acrosome (I: arrow). (F) On denuded oocytes, sperm tended to concentrate and bind close to any remaining cumulus cells. (G) Sperm bound to a cumulus intact oocyte. The corona radiata of the cumulus is clearly visible, and sperm bound to or interacting with the cumulus cells have a mottled but non-reacted acrosome (magnified part of G). (H) In cumulus-intact *in vivo* matured oocytes, transzonal processes extending from the cumulus cells towards the oocyte were readily distinguished (arrow). Again bound sperm had not acrosome reacted (magnified area of H). (J) Sperm bound to or interacting with cumulus cells or ZP displayed similar mottled, disrupted, swollen acrosomal caps. (K, L) Sperm viability was assessed using the LIVE/DEAD ® kit; live cells are stained green and dead cells red. The ZP-bound population therefore included both dead (b) and live (a) sperm.
Magnification bars: Figures A, B, E, G, H, K = 50 μm; C, D = 40 μm; L and magnified sections from G and H = 15 μm; F, I, J: bar = 10 μm.

Sperm bound to the ZP of IVM oocytes in large numbers independent of initial cumulus morphology, duration of IVF or addition of progesterone (65 ± 32 and 62 ± 28, mean ± SD for C- and E-COCs, respectively: Table 2; Figs 1B,C). However, after FITC-PNA labeling, bound sperm did not display classical signs of the AR i.e. fluorescence confined to the equatorial segment or absent due to total loss of the acrosome (Cheng *et al*., 1998). Instead, most bound sperm had a mottled, slightly disrupted and swollen acrosomal cap (Figs 1E,I). Furthermore, numerous similarly stained acrosomal caps minus the rest of the sperm cell were found attached to the ZP (Fig 1D). Examination of sperm viability was performed for 102 sperm-oocyte complexes (see Table 1) and demonstrated that the bound sperm population included both viable and dead sperm (see Figs 1 K,L) at a ratio (2:1) that was not affected by cumulus morphology, denudation, duration of IVF or presence of progesterone.

Effect of removing the cumulus prior to IVF:

Cumulus removal from IVM oocytes significantly increased the number of sperm that bound to the ZP (69±40 versus 5±4 sperm/oocyte for denuded and cumulus intact oocytes, respectively: $P<0.0001$: Table 2; Figs 1F,G), but did not enable sperm to penetrate or fertilize the oocyte. In cumulus intact COCs, additional sperm were detected within the cumulus investment (Figs 1G, H, J). As before,

most ZP-bound sperm had a mottled, slightly disrupted, swollen acrosomal cap but had not undergone a normal AR (Fig 1G, J). The number of ZP-bound detached acrosomal caps was lower for cumulus-intact than denuded oocytes.

TABLE 2. The effect of mode of horse oocyte maturation (*in vitro* versus *in vivo*), initial cumulus morphology, cumulus removal prior to IVF and duration of IVF on the number of stallion sperm binding to the ZP during IVF.

Ooocyte maturation*, cumulus morphology and fate	Duration of IVF (h)	No. of oocytes	Mean ± SD ZP-bound sperm** (no. of oocytes analysed)	Fertilization events
Compact cumulus	6	50	-	Sperm failed to penetrate the ZP and oocytes remained arrested at MII
(Denuded)	20	86	65 ± 32 (19) [a]	
Expanded cumulus	6	45	-	
(Denuded)	20	55	62 ± 28 (19) [a]	
Compact cumulus (Not denuded)	6	67	5 ± 4 (20) [b]	
Compact cumulus (denuded)	6	52	69 ± 40 (20) [a]	
In vivo matured oocytes (not denuded)	20	15	23 ± 17 (15) [c]	

*Except when stated, oocytes were matured *in vitro*

**ZP-bound sperm were counted from sequential digital CLSM images. [a,b,c] Values with different superscripts differ significantly ($P<0.001$).

IVF of *in vivo* matured oocytes:
Fifteen oocytes were recovered by transvaginal ultrasound-guided aspiration of 38 preovulatory follicles (40% collection rate). The oocytes were used immediately for IVF without disruption of their cumulus investment. As with IVM oocytes, sperm did not penetrate the ZP of *in vivo* matured oocytes during 20h of IVF in medium supplemented with progesterone (Fig 1H). On the other hand, the number of sperm bound to the ZP (23±17 sperm/oocyte) was higher than that for cumulus intact IVM oocytes (5±4 sperm/oocyte; $P<0.001$: Table 1).
Once again, the bound sperm did not undergo the AR but displayed the mottled, swollen, acrosomal cap described above, although only few detached acrosomal caps were detected adhered to the ZP (Fig 1H and its inset). As before, all oocytes remained in M-II and did not display signs of activation or fertilization. One marked difference between the *in vivo* and *in vitro* matured oocytes was the dramatic expansion of the cumulus investment in the former, which enabled clear visualization of the transzonal processes extending from the cumulus cells (Fig 1H).

Effects of stallion and sperm cryopreservation on sperm-oocyte interaction during IVF
There was no significant effect of stallion or fresh versus cryopreserved sperm on ZP-binding or penetration when oocytes with a compact COC at recovery were used for IVF. When oocytes were derived from expanded COCs there was a between-stallion difference in the number of bound sperm for fresh (57±26 vs 43±24 sperm/oocyte; $P<0.05$) but not frozen/thawed sperm(Fig 2). In all cases, bound sperm failed to penetrate or fertilize the oocytes and were found to have not undergone the AR but to have an abnormal looking acrosome cap, as described above.

Flow Cytometric detection of the effects of incubation in the absence of COCs on sperm integrity
FITC-PNA Staining: FITC-PNA staining differentiate the viable sperm (PI negative) into two distinct groups: sperm not labeled with FITC-PNA, thereby demonstrating that their acrosomes were intact, or showed acrosomal FITC-PNA staining, which indicates that they had embarked on the AR (Note that these sperm cells had not undergone any permeabilization treatment, differently to those permeabilized and stained for CLSM analysis). The addition of progesterone to the fert-TALP medium did not affect the acrosome status of sperm during the 6 h incubation. Indeed, the percentage of viable acrosome–intact sperm at the end of the incubation (15.7±4.9% and 19.1±2.4 % for without and with P4, respectively) did not differ to that at the onset of incubation (19.1±1.9 % and 18±1.9% for

without and with P4). On the other hand, the percentage of acrosome-reacting live sperm decreased markedly during incubation (17.8±1.8 % and 17.6±1.5% at 0 h versus 8.6±1.2% and 9.7±1.8% at 6 h for without and with P4, respectively; P=0.0001) and, presumably as a consequence, the percentage of dead sperm (PI-positive) increased over time (63.9±3.7% and 62.9±2.8% at 0 h vs 78.6%±6.0% and 70.8±3.8 % at 6 h, without and with P4, respectively; P=0.01).

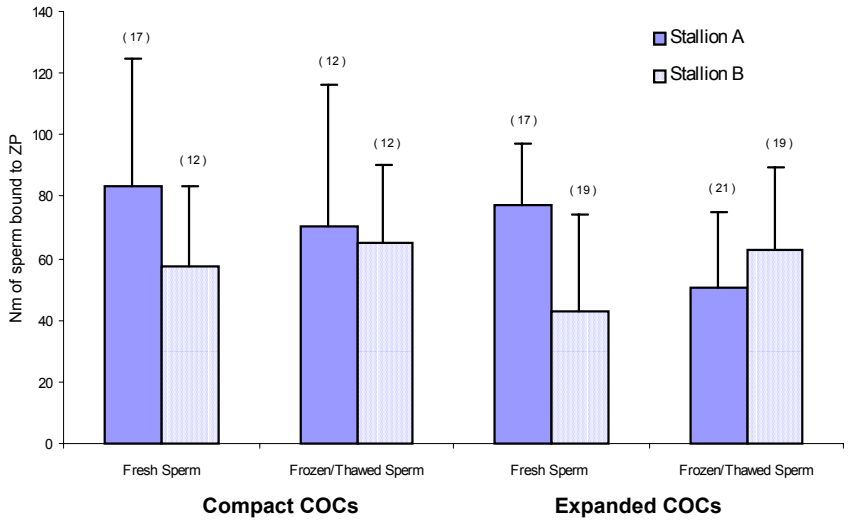


Fig 2. The mean (± S.D.) number of stallion sperm bound to the zona pellucida of *in vitro* matured oocytes incubated with fresh or frozen/thawed sperm from one of two fertile stallions for 20 h in the presence of 150 ng/ml of progesterone. Oocytes were classified as being from compact or expanded cumulus oocyte complexes (COCs) depending on their cumulus morphology at the onset of IVM. No between-stallion or sperm preparation method differences were observed in the number of sperm binding to C-COC oocytes, but the number of bound sperm did differ between stallions when E-COC oocytes were co-incubated with fresh sperm (*P<0.05). In all cases, sperm failed to fertilize the oocytes. The number of sperm-oocyte complexes analyzed is indicated in parenthesis.

Merocyanine Staining*:* Merocyanine 540 differentiated two populations of viable (i.e. Yo-Pro-1 negative) sperm. The first was characterized by poor MC540 uptake and was considered to represent non-capacitated sperm (with a well ordered plasma lipid bilayer). The second consisted of sperm with high levels of fluorescence in the head region, and therefore a more disordered lipid bilayer, and were considered to be capacitated sperm (Rathi *et al*., 2001). Progesterone did not affect the rate of

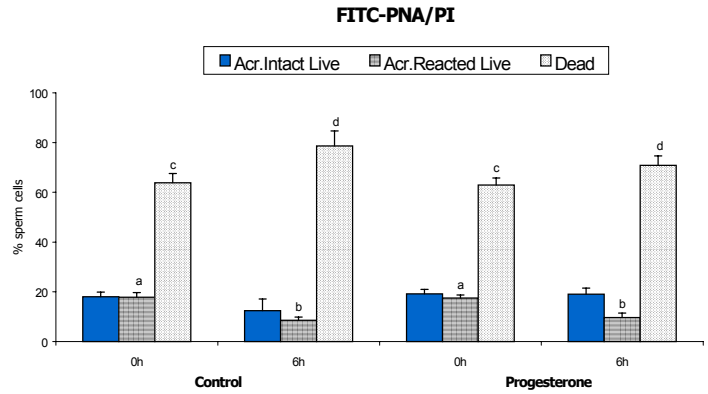
sperm capacitation since no significant differences in MC540 staining patterns were detected between sperm incubated in fert-TALP with or without 150ng/ml progesterone (Fig. 3B). There was, however, an effect of time in culture on capacitation status (P=0.013); the percentages of non-capacitated live sperm decreased from 20.1±0.5 and 23±0.9 at 0 h to 18±1.5 and 20.2±0.5 at 6 h, in medium without or with progesterone, respectively. There was no corresponding increase in the proportion of capacitated live sperm, instead this population also decreased with time in culture (7.1±1.0% and 6.1±1.6 at 0 h vs 3.1±1.2% and 3.3±1.6 % at 6 h, without or with P4, respectively; P= 0.06). Finally, the proportions of dead sperm (Yo-Pro-1 positive) increased during the course of the incubation from 72.6±1.5% and 71.2±0.7 at 0 h to 77.2±2.3% and 75.8±2.2 % at 6 h without or with P4, respectively (P=0.01).

Mitotracker Staining*:* Mitotracker Red® differentiated two populations of viable (Yo-Pro-1 negative) sperm, those that had bright fluorescent mid-pieces, indicating the presence of functional mitochondria, and those with non-stained midpieces and thus no ability to generate energy. Fluorescent microscopic analysis of sperm motility confirmed that only those with functional mitochondria (mitotracker red positive) and an intact plasma membrane (Yo-Pro negative) were motile. Not surprisingly, nearly all sperm with dysfunctional mitochondria were dead and *vice versa.* The presence of P4 in the IVF medium did not significantly affect the proportion of live sperm with functional mitochondria. However, the proportion of sperm with brightly fluorescent mid-pieces decreased from 20.1±1.7 % and 20.5±1.6 % at 0 h to 13.9±1.4% and 14.7±0.4 % after 6 h without or with P4, respectively (P=0.0001: Fig.3C). Interestingly, while the percentage of dead sperm increased during incubation (75.9±2.2% and 74.9±0.9 at 0 h vs 81.2±3.3% and 78.6±2.5% at 6 h, without and with P4, respectively; P=0.03) there was no time-related change in the (small) proportion of live sperm with non-functional mitochondria (4.0±0.5% and 4.5±0.2 % at 0 h vs 4.1±0.7% and 4.2±1.0% at 6 h, without and with P4 respectively).

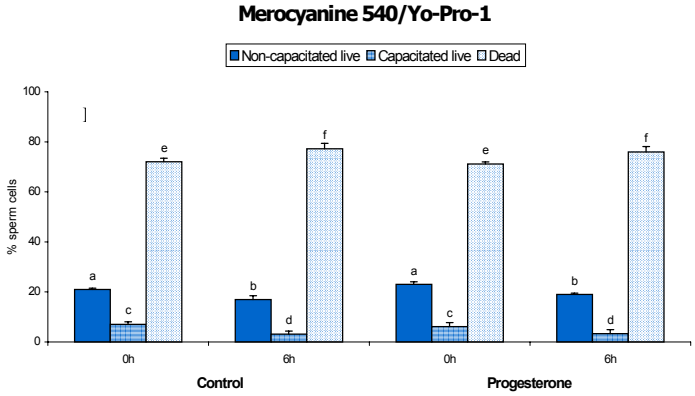
DISCUSSION

In this study, stallion sperm failed to penetrate the ZP of either *in vivo* or *in vitro* matured oocytes during IVF, thereby demonstrating that limited zona penetration *in vitro* is not solely a factor of changes to the oocyte investments during IVM. A similar conclusion was suggested by the finding that, while spontaneous ZP-hardening occurred in horse oocytes cultured in serum-free medium (Dell'Aquila *et al*., 1998), the addition of fetuin, a protease inhibitor that suppresses ZP-hardening

A


FITC-PNA/PI
Acr.Intact Live
Acr.Reacted Live
Dead
% sperm cells
0h
6h
0h
6h
Control
Progesterone

B


Merocyanine 540/Yo-Pro-1
Non-capacitated live
Capacitated live
Dead
% sperm cells
0h
6h
0h
6h
Control
Progesterone

C

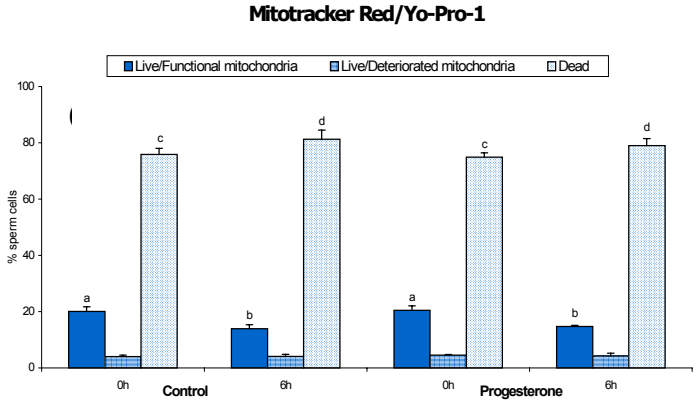

Mitotracker Red/Yo-Pro-1
Live/Functional mitochondria
Live/Deteriorated mitochondria
Dead
% sperm cells
0h
6h
0h
6h
Control
Progesterone

Fig 3. The mean (± SD) percentages of viable stallion sperm with specific membrane/organelle states at the onset and after a 6 h incubation in fert-TALP (control) or fert-TALP supplemented with 150 ng/ml progesterone (progesterone) in the absence of oocytes. Three aliquots of frozen-thawed sperm from a single stallion were analyzed.
A) Percentage of viable sperm (PI negative) that had either an intact or a reacted acrosome as determined by FITC-PNA staining. There was no significant effect of P4, but the proportion of viable acrosome-reacted sperm decreased, and the proportion of dead sperm increased, significantly over time. [a-d] Columns with the same superscripts did not differ significantly (P>0.05).
B) Percentages of noncapacitated (Merocyanine 540 negative) or capacitated (Merocyanine negative) viable sperm (Yo-Pro-1 negative). The presence of P4 in the incubation medium did not affect capacitation status but the percentages of both capacitated and non-capacitated live sperm decreased, and the percentage of dead sperm increased, over time. [a-f] Columns with the same superscript did not differ significantly (P>0.05).
C) Percentages of viable sperm (Yo-Pro-1 negative) with either functional or deteriorated mitochondria as detected by the uptake or not of Mitotracker Red, respectively. Progesterone did not affect the number of sperm with functional mitochondria, but the percentage of viable sperm with functional mitochondria decreased over time in incubation. Columns with the same superscript did not differ significantly (P>0.05).

(Schroeder *et al*., 1990), did not enhance penetration of IVM oocytes by stallion sperm (Dell'Aquila *et al*., 1999). Another physical parameter that has been proposed to influence oocyte penetrability is ZP thickness. Bertrand *et al*. (1995) reported that human oocytes that became fertilized *in vitro* had a significantly thinner ZP than those that did not, although ZP thickness of individual oocytes did not change during or after fertilization. IVM horse oocytes also exhibit differences in ZP thickness but until an IVM system that allows reasonable penetration/fertilization rates is developed, these inter-oocyte differences will not be open to investigation. Furthermore, the fact that IVM oocytes are fertilized at a high rate *in vivo* (after transfer to the oviduct of a synchronized recipient: Hinrichs *et al*., 2002) indicates clearly that they can be penetrated and that the deleterious the effects of IVM are either few, or overcome by short term exposure to the oviductal environment (Ellington *et al*., 1993; Boatman and Magnoni, 1995).
Previous studies of equine IVF have also reported low levels of fertilization (0-30%: Del Campo *et al*., 1990; Zhang *et al*., 1990; Palmer *et al*., 1991; Bézard, 1992; Grøndhal *et al*., 1995; Dell'Aquila *et al*., 1997; Alm *et al*., 2001; Hinrichs *et al*., 2002), although comparison of rates between reports is difficult because many different criteria have been used to indicate fertilization, e.g. presence of a sperm head within the ooplasm, formation of a paternal pronucleus, and cytoplasmic cleavage with or without nuclear division. Moreover, various methods have been used to collect, categorize and culture oocytes and to induce capacitation of semen stored or prepared in different ways. In addition, the techniques used to visualize

the structural changes associated with fertilization, namely removal of the cumulus cells before fixing the oocyte in an alcohol based solution that dissolves the ZP, have precluded examination of sperm interactions with the ZP or cumulus cells. In the current study, the use of CLSM coupled with specific labels for acrosome status (FITC-PNA), DNA (EthD-1) and plasma membrane integrity (Calcein-AM) provided novel information about sperm interactions with the cumulus cells and ZP while still allowing assessment of intra-ooplasmic events. Moreover, the optical serial sectioning enabled 3-D visualization of how sperm had interacted with oocyte and cumulus.

The time course of sperm penetration *in vitro* it is not well described for equine gametes, therefore the incubation times used in the current study were based on data from both *in vivo* and *in vitro* fertilization. *In* v*ivo*, pronuclear stage oocytes have been collected as early as 12 h after ovulation (Grøndhal *et al*., 1993) or 12-21 h after mating (Enders *et al*., 1987), while the same stage was reached *in vitro* within 8-16 h of incubation with sperm (Blue *et al*., 1989; Zhang *et al*., 1990). And while there is no information about the time needed for stallion sperm to pass between the cumulus cells, bind to and then penetrate the ZP, in other species sperm passage through the ZP appears to be very rapid, e.g. 30 min in rodents (Yanagimachi, 1994), but influenced greatly by the physiological state of the spermatozoa. In any case, the incubation times used in the current study were appropriate for a study of sperm binding, oocyte penetration and fertilization *in vitro*, even if the 6 h period would probably have been too short to detect pronucleus formation if sperm penetration had been successful.

The effect of cumulus cells on fertilization of equine oocytes *in vitro* remains unclear since some groups have reported increased fertilization rates after partial cumulus removal (Dell'Aquila *et al*., 1996) while others have suggested a beneficial effect of the cumulus (Marcos *et al*., 1996). It is, however, difficult to compare these two studies because the numbers of oocytes tested were low and the types of semen (frozen-thawed vs fresh semen) and capacitation treatments used were different. In other species, cumulus cells appear to be beneficial to IVF (cow: Fatehi *et al*., 2002; pig: Kikuchi *et al*., 1993), presumably because some cumulus cell products stimulate sperm motility and prepare the sperm for ZP binding (Tesarik *et al*., 1988; Fatehi *et al*., 2002). Studies in the hamster have demonstrated that only capacitated sperm with an intact acrosome are able to pass through the cumulus (Yanagimachi, 1994), which suggests that the cumulus acts as a 'filter' for sperm capable of fertilization. Meyers and Rosenberger (1999) reported the presence of a potent hyaluronidase in the plasma membrane of stallion sperm that they proposed may facilitate sperm transit through the cumulus, essentially a network of cells embedded in an extracellular matrix composed primarily of hyaluronic acid. The same authors proposed that the sperm hyaluronidase plays an

important role in capacitation, rendering the sperm competent for ZP penetration even before ZP-binding or the AR have taken place. The fact that sperm did not penetrate either denuded or cumulus intact IVM oocytes in the current study prevented any clarification of the importance of the cumulus, although considerably fewer sperm managed to access and bind to the ZP of cumulus intact than denuded oocytes. Of course, the degree of cumulus expansion achieved during IVM is considerably less than that occurring during maturation *in vivo* (Goudet *et al*., 1997), with the major difference appearing to lie in the extracellular matrix. The presence of an abnormally compact cumulus may thus impose a physical barrier to sperm penetration of IVM oocytes, a hypothesis supported by the greater numbers of sperm that reached and bound to the ZP of *in vivo* than *in vitro* matured cumulus-intact oocytes.

In the current study, progesterone did not significantly affect sperm capacitation, AR or ZP penetration. By contrast, previous studies suggested that progesterone stimulates the AR of fresh (Percoll-washed) stallion sperm *in vitro* in a dose-dependent fashion (Meyers *et al*., 1995; Cheng *et al*., 1998a). Cheng *et al*. (1998b) further proposed that the action of progesterone was exerted via a receptor located on the plasma membrane of the sperm and that only sperm with exposed receptors, where exposure was induced by capacitation (Cheng *et al*., 1998a, 1998b), could acrosome react in response to progesterone. In the current study, most IVF attempts used frozen-thawed rather than fresh semen because it was easier to tailor its preparation into the IVF programme. On the other hand, it is possible that alterations in the surface organization of the sperm after cryo-preservation (Watson, 2000) may have resulted in insufficient P4-receptor exposure to allow the AR in response to P4. In fact, in the current study, merocyanine 540 analysis demonstrated that frozen-thawed stallion sperm failed to capacitate *in vitro* in Sp-TALP whether or not P4 was present. This suggests that freezing and thawing may affect the ability of sperm to capacitate and expose their P4 receptors during subsequent IVF incubation and thereby compromise their ability to acrosome react. Alm *et al*. (2001) reported similarly that calcium ionophore was unable to induce frozen/thawed sperm to penetrate or fertilize IVM horse oocytes, despite the fact that most of the studies that have reported successful IVF have used calcium ionophore to induce capacitation/AR of fresh stallion sperm (Del Campo *et al*., 1990; Palmer *et al*., 1991; Li *et al*., 1995; Hinrichs *et al*., 2002). Disappointingly, this better response, in terms of oocyte penetration, of fresh semen to calcium ionophore treatment was not replicated for P4 treatment in the current study even though P4, like calcium ionophore, induces the AR by prompting a massive influx of Ca^{2+} into the sperm (Flesch and Gadella, 2000). P4-mediated sperm activation is transduced via a protein tyrosine kinase pathway (Rathi *et al*., 2003) and tyrosine phosphorylation of specific plasma membrane proteins appears to be crucial to

sperm-zona binding. However, cryopreservation and/or Percoll-treatment have been proposed to induce early capacitation-like changes that destabilize the sperm plasma membrane such that primary sperm binding to the ZP no longer activates the cellular signaling pathway required for the AR and subsequent ZP penetration (Gadella *et al*., 2001). This membrane destabilization could explain the high proportion of apparently dead sperm bound to the ZP (Fig.1L) together with the mottled, swollen, detached acrosomal caps (Figs. 1E, I). Although analysis of the functional characteristics of frozen-thawed sperm during incubation *in vitro* identified 70% of the sperm as dead (in any case with a permeable plasma membrane) at the onset of incubation, approximately 20% were viable, non-capacitated, with functional mitochondria and intact plasma and acrosomal membranes. This subpopulation of sperm should have been able to undergo the appropriate changes, bind to the ZP, penetrate and fertilize the oocytes but for some reason primary sperm-zona binding was not followed by induction of a normal AR. In this respect, zona pellucida glycoprotein ZP3 is generally accepted to be the primary oocyte protein involved in initial sperm-ZP binding in most species (Wassarman and Albertini, 1994). Spermadhesins (Fayrer-Hosken *et al*., 1991) and galactosyltransferases (Töpfer-Petersen *et al*., 1998) found on the plasma membrane of stallion sperm are believed to be the complementary receptors for ZP3, but their role during fertilization has yet to be proven. A better understanding of the sperm plasma membrane proteins involved in ZP recognition and adhesion is needed to explain why stallion sperm readily binds to but does not AR and penetrate the ZP *in vitro*.

In summary, in the IVF conditions used, stallion sperm were able to bind to the ZP but failed to undergo the AR and penetrate either *in vitro* or *in vivo* matured oocytes. Neither initial cumulus morphology, cumulus removal nor addition of progesterone to the IVF medium affected AR induction or ZP penetration, although the cumulus of IVM oocytes did restrict sperm access to the ZP. Nevertheless, poor ZP penetration by stallion sperm *in vitro* seems not to be a factor of suboptimal oocyte maturation *in vitro* but rather to reflect a general failure of stallion sperm to capacitate and to AR after binding to the ZP. Unfortunately, until a repeatable method for IVF is developed, studying the factors that affect functional activation of horse sperm *in vitro* will remain difficult. For this reason, there may be more immediate mileage in trying to examine the chain of functional changes that the gametes undergo during interaction in the specific microenvironments of the reproductive tract of live mares.

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Chapter 5

Cytoskeleton and chromatin reorganization in horse oocytes following intracytoplasmic sperm injection: patterns associated with normal and defective fertilization

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ABSTRACT

Intracytoplasmic sperm injection (ICSI) is the method of choice for fertilizing horse oocytes in vitro. Nevertheless, embryo development rates are low, for reasons that are not yet clear. The aims of this study were to examine cytoskeletal and chromatin reorganization in horse oocytes fertilized by ICSI or activated parthenogenetically. Additional oocytes were injected with a sperm labeled with a mitochondrion-specific vital dye to help identify the contribution of the sperm to zygotic structures, in particular the centrosome. Oocytes were fixed at set intervals after sperm injection and examined by confocal laser scanning microscopy. In unfertilized oocytes, microtubules were present only in the metaphase-arrested second meiotic spindle and the first polar body. After sperm injection, an aster of microtubules formed adjacent to the sperm head and subsequently enlarged such that, at the time of pronucleus migration and apposition, it filled the entire cytoplasm. During syngamy, the microtubule matrix reorganized to form a mitotic spindle upon which the chromatin of both parents aligned. Finally, after nuclear and cellular cleavage were complete, the microtubule asters dispersed into the interphase daughter cells. Sham injection induced parthenogenetic activation of 76% of oocytes, marked by the formation of multiple cytoplasmic microtubular foci that later developed into a dense microtubule network surrounding the female pronucleus. The finding that a parthenote alone can produce a microtubule aster whereas the aster invariably forms at the base of the sperm head during normal fertilization, indicates that both gametes contribute to the formation of the zygotic centrosome in the horse. Finally, 25% of sperm injected oocytes failed to complete fertilization, mostly due to absence of oocyte activation (65%), which was often accompanied by failure of sperm decondensation. In conclusion, this study demonstrated that union of the parental genomes in horse zygotes is accompanied by a series of integrated cytoskeleton-mediated events, failure of which results in developmental arrest.

INTRODUCTION

The commercial application of in vitro embryo production (IVP) to horses has been limited by the poor rates of fertilization obtained after conventional in vitro fertilization (IVF) [1, 2, 3], which appears to be primarily due to the poor capacity of stallion spermatozoa to penetrate the zona pellucida in vitro [4, 5]. Recently, intracytoplasmic sperm injection (ICSI) was introduced as an alternative to conventional IVF and has resulted in the birth of several foals [6-10]. By bypassing

the critical events of oocyte penetration, ICSI has proven to be a promising means of producing equine embryos in vitro. There are, however, large differences in the rates of successful fertilization reported by different groups, with the rate of male pronucleus formation in horse oocytes fertilized by ICSI ranging from 21-71% [11-14] and cleavage rates ranging from 20-80% [9-12, 14]. These differences indicate that significant failures in zygote development still occur despite injection of a motile sperm into a mature oocyte. In addition, because in vitro culture conditions are still not optimal, only a low percentage of cultured zygotes will develop to the blastocyst stage [1-30% of cleaved oocytes: 11, 13, 15, 16]. At present, transferring ICSI-derived horse zygotes into the oviduct of a ewe remains the most successful method for producing horse blastocysts ex vivo, with rates reaching 50% of cleaved oocytes [10]. Moreover, the majority of equine ICSI pregnancies have resulted from the transfer of early zygotes to the oviduct of a recipient mare [6, 7, 8], which underlines the negative effects of in vitro culture on the developmental capacity of equine ICSI-produced zygotes.

Although ICSI can be applied successfully to horses, the disparity in fertilization rates and the inefficiency of current culture conditions remain obstacles to the development of a reliable and reproducible system for producing horse embryos in vitro. To improve the efficiency of equine IVP, it is of utmost importance to gain more insight into the processes of fertilization and embryo development after sperm penetration in vivo. Since in vivo embryos are retained within the fallopian tube until day 5-6 after ovulation, from where they can only be recovered by surgery or slaughter of the mare, only limited information is available on the sequence of events that occurs during fertilization and early equine embryonic development in vivo. Enders et al. [17] reported clear signs of fertilization as early as 10 h post mating in oviductal zygotes, when the incorporated sperm had acquired its pronuclear envelope and the oocyte had progressed to telophase of the second meiotic division. By 12 h post mating, horse zygotes had reached the pronuclear stage [1, 17, 18]. Similarly, because of difficulties of producing equine zygotes in vitro and since most zygotes are transferred to the oviduct of a recipient as soon as possible after cleavage, only a few studies on embryo development in culture have been documented. These studies described the development in culture of horse oocytes fertilized in vivo, and recorded the first cell division to occur between 22 and 24 h after ovulation [1]. More recently, sperm chromatin decondensation has been reported to occur between 2 and 4 h after IVF [19] or ICSI [13, 14], with pronucleus formation following at around 16 to 24 h after sperm incorporation.

In general, for fertilization to proceed, a series of cytoplasmic and nuclear changes must occur in a precisely orchestrated fashion. Changes in nuclear structure include

the formation of the male and female pronuclei, migration and apposition of these pronuclei and mixing of the maternal and paternal genomes. Finally, initiation of a mitotic division heralds the onset of embryonic development (reviewed by Yanagimachi [20]). These nuclear events are, however, highly dependent upon reorganization of the microtubular and microfilamentar elements of the fertilizing gametes. An oocyte loses its centrosome, the organelle that acts as a microtubule organizing center, early in gametogenesis. During fertilization it is the sperm centrosome that acts as the zygotic microtubule organizing center and induces formation of the radial microtubule-containing structure, the sperm aster, that co-ordinates migration and union of the two pronuclei and formation of the mitotic spindle (reviewed by Schatten [21]). This paternal inheritance of the centrosome is seen in most mammalian species studied, including man [22], nonhuman primates [23] and many domestic animal species (e.g.cow [24]; pig [25]; sheep [26]). In contrast, the centrosome in rodent zygotes has a maternal origin [27, 28]. Little is known about the pattern of centrosomal inheritance and subsequent cytoskeletal dynamics in the horse zygote during fertilization.
The aim of this study was to characterized the nuclear and cytoskeletal events that occur in horse oocytes during fertilization after ICSI by examining chromatin, microtubule and microfilament organization in oocytes cultured for up to 48 hrs after sperm injection. In addition, we investigated the stages in which the fertilization process deviates in cases of arrest or delay of zygote development.

MATERIALS AND METHODS

Collection and culture of cumulus oocyte complexes

The procedures for collecting and culturing horse oocytes have been described previously [29]. In brief, cumulus-oocyte complexes (COCs) were recovered by aspiration from the ovaries of slaughtered mares within 3-5 h after slaughter, during the breeding season (March-September). All visible follicles less than 30mm in diameter were punctured and the follicular lumenae were flushed with PBS supplemented with 50mg/L bovine serum albumin (BSA; Sigma, St Louis, MO, USA) and 25 IU/ml heparin (Leo Pharmaceutical, Weesp, the Netherlands). The ovary was then bisected and any exposed follicles were similarly aspirated and flushed. Next, COCs were isolated by examining the collected fluid with a stereomicroscope. Recovered COCs were evaluated for their quality and washed twice in HEPES-buffered Tyrodes medium containing 0.1% (w/v) polyvinylalcohol and 0.2% BSA (HEPES-TL-PVA). Only oocytes with a complete, compact, multilayered cumulus were selected and were washed twice

with maturation medium before being placed in the culture medium proper. In our experimental protocol we use only compact COCs as we consider them to be a more homogeneous population than the expanded COCs, which displayed a different degrees of expansion within the granulosa, cumulus and corona cells. Finally, oocytes were allocated randomly into groups of 20-25 which were incubated for 36 h in 500 µl aliquots of M199 supplemented with 10% FCS and 0.01 units/ml of both porcine FSH and equine LH (both Sigma), at 39 °C in a humidified atmosphere of 5% CO_2 in air.

Preparation of spermatozoa for ICSI

Ejaculated sperm from a stallion of proven fertility was used for ICSI after freezing and thawing in the manner described by Parlevliet et al. [30]. The straws were thawed at 37 °C for 45 s and the spermatozoa were rinsed free of cryoprotectant by centrifugation at 700g for 15 min in HEPES buffered modified Tyrodes medium (Sp-TALP; Parrish et al. [31]). The resuspended sperm pellet (approx 200 µl) was then placed at the bottom of a 10 ml tube containing 2 ml of HEPES-Sp-TALP and incubated at 39 °C in an atmosphere of 5% CO_2 in air for "swim-up". After 40 min, the uppermost 1.5 ml of medium was collected and centrifuged at 400g for 5 min in a 2 ml polypropylene tube. The sperm pellet was again resuspended in 1 ml of HEPES-Sp-TALP and the suspension was maintained at 38 °C until ICSI. No attempt was made to induce sperm capacitation, although the bicarbonate in Sp-TALP medium should have stimulated this process [32].

Preparation of oocytes and ICSI

After 36 h of in vitro maturation, the COCs were incubated for 5 min at 37 °C in calcium and magnesium free Earles Balanced Salt Solution (EBBS: Gibco BRL, Paisley, UK) containing 0.1% (w/v) hyaluronidase (Type I-S; Sigma H-3506). Next, the cumulus cells were removed by aspirating the COCs several times through a fine pipette or, if necessary, by vortexing. Cumulus-free oocytes were examined with an Olympus SZX9 (Olympus, Tokyo, Japan) inverted microscope (X400), and those with an intact oolema and an extruded first polar body were selected for ICSI. The selected oocytes were washed twice in HEPES-buffered synthetic human tubal fluid (Q-HTF HEPES; BioWhittaker, Verviers, Belgium) supplemented with 0.4% BSA and maintained in synthetic human tubal fluid (Q-HTF; BioWhittaker) containing 0.4% BSA, until injection. Oocyte injection was performed in a 5 µl microdrop of Q-HTF HEPES at 37 °C on a heated stage (Linkam Scientific Intruments, Tadworth, UK) mounted on an Olympus-CK40 inverted microscope equipped with Narishige micromanipulators (Narishige CO., LTD, Tokyo, Japan). Sperm injection was performed essentially in the same

manner as described by Palermo et al. [33]. Just prior to injection, 2µl of the motile sperm suspension was mixed with 5 µl of Clinical Grade PVP (10% polyvinylpyrrolidone in HEPES buffered salt solution; Lucron Bioproducts B.V. Gennep, The Netherlands) to slow sperm movement and aid capture. A motile spermatozoon was then immobilized by swiping the injection pipette (10-MIC-Angled 30°; Gynotec, Malden, The Netherlands) across its tail and it was then moved, with a minimal volume of medium, to the microdrop containing the oocyte. For sperm injection, an oocyte was held stationary by suction via the holding pipette (10-MPH-120-Angled 40°; Gynotec) with the polar body positioned at 6 or 12 o'clock. The injection pipette containing the spermatozoon was advanced through the zona pellucida and plasma membrane at the 3 o'clock position and the spermatozoon was injected into the ooplasm with a minimal volume of accompanying medium. Further oocytes were "sham" injected with PVP solution only. After injection, the oocytes were returned to the Q-HTF medium and within 15-30 min they were transferred to 20 µl drops of fresh Q-HTF medium containing 0.4% BSA, and cultured at 39 °C in 5% CO_2 in humidified air.

Experimental Design

In order to describe the chronology of cytoskeleton and chromatin rearrangements in horse oocytes during fertilization induced by ICSI, injected oocytes were cultured for 24 or 48 h and then labeled simultaneously with stains for chromatin, microfilaments and microtubules. To further characterize the events associated with sperm reorganization within the activated oocyte and the changes in microtubule distribution that accompany pronucleus formation, additional injected oocytes were cultured for 6, 12 or 18 h before being stained for the visualization of microtubules and chromatin. In order to track sperm incorporation and the conversion of sperm-derived structures into zygotic structures, oocytes were injected with a sperm labeled with a mitochondrion-specific vital dye [34]. Finally, the organization of microtubules and chromatin in sham-injected oocytes was examined to elucidate the role of oocyte-derived structures in maternal pronucleus formation and oocyte activation.

Immunocytochemistry and confocal laser scanning microscopy

Visualization of microtubules, microfilaments and chromatin in horse zygotes

After 6, 12, 18, 24 or 48 h, the presumptive zygotes were removed from culture and permeabilized by incubating them for 15 min in buffer M, a glycerol-based microtubule-stabilizing solution at 37 °C [35]. The zygotes were then fixed in 3% paraformaldehyde in PBS and subsequently maintained in the fixative at 4 °C for 4-6 days prior to staining. Following fixation, the zygotes were washed twice in

PBS containing 150 mM glycine and 0.1% (w:v) BSA (both Sigma) for 15-30 min, to reduce free aldehydes and to block non-specific reactions. Depending on the duration of the incubation, injected oocytes were double (6, 12 and 18 h incubations) or triple (24 and 48 h incubations) stained using different combinations of fluorescent probes. In all cases, microtubules were labeled first by incubating the zygotes with a monoclonal anti-α-tubulin antibody (Sigma; T-5168) diluted 1:250 in PBS containing 0.5% (v:v) Triton X-100 and 0.1% BSA (PBS-TX100-BSA) for 90 min at 37 °C. Next, the zygotes were washed three times in PBS-TX100-BSA before being incubated for 1 h in a blocking solution (0.1M glycine, 1% goat serum, 0.01% Triton X-100, 0.5% BSA and 0.02% sodium azide; all from Sigma). The zygotes were then incubated for 1 h at 37 °C in goat anti-mouse antibody diluted 1:100 in PBS-TX100-BSA and conjugated to either AlexaFluor™ 488 (Molecular Probes Europe BV, Leiden, The Netherlands; A-11029) or tetramethylrhodamine isothiocyanate (TRITC; Sigma; T-5393), for the dual and triple stained zygotes respectively. Once their microtubules had been labeled, the zygotes were washed once with PBS-TX100-BSA and twice with PBS alone. Next, the presumptive zygotes from the 24 and 48 h cultures were incubated for 1 h with AlexaFluor™ 488 Phalloidin (15 IU/ml: Molecular Probes; A-12379) to enable microfilament detection. Injected oocytes from 6, 12 and 18 h cultures were not stained for microfilament detection. Finally, to enable visualization of the DNA, the presumptive zygotes from 24 and 48 h cultures were incubated with TO-PRO_3 (5μM in PBS; Molecular Probes; T-3605) for 15 min, while those from 6, 12 or 18 h cultures were stained for 15 min with Ethidium homodimer (EthD-1: 2μM in PBS; Molecular Probes; E-1169).

Tracking sperm chromatin within the zygote

To differentiate the paternal and maternal chromatin and to describe their contribution to zygotic structures during ICSI-induced fertilization, injected sperm cells were tagged with a mitochondrion-specific vital dye. For this, frozen/thawed ejaculated stallion sperm were selected by swim-up in Sp-TALP medium, as described previously. The motile sperm fraction was recovered and centrifuged at 400 g for 5 min, and the resulting sperm pellet was resuspended in a 500 μM solution of Mitotracker Red (CMH $_2$XROS; Molecular Probes; M-7512) in Sp-TALP, in which it was incubated for 30 min at 39 °C in 5% CO_2 in air. The labeled sperm were then washed by two cycles of centrifugation and resuspension in Sp-TALP medium. ICSI with tagged sperm was performed as described previously and within 10 min after ICSI, the injected oocytes were transferred to the incubation droplets. After a 6 h incubation, the developing zygotes were removed from culture, fixed in 3% paraformaldehyde in PBS and maintained in the dark at

4°C for 1-2 days. Fixed zygotes were permeabilized by incubation for 30 min at room temperature in PBS containing 0.1% Triton X-100 and 0.1% BSA and possible non-specific labeling was prevented by incubation for 1 h with the blocking solution described above. Localization of labeled microtubules was performed using a mouse monoclonal anti-α-tubulin antibody (Sigma) diluted 1:250 in PBS-TX100-BSA and the goat anti-mouse secondary antibody, this time conjugated to AlexaFluor™ 633 (1:100 in PBS-TX100-BSA; Molecular Probes; A-22284). DNA was stained with SYTOX® Green (1 μM in PBS; Molecular Probes; S-7020).

Confocal laser scanning microscopy (CLSM)

Stained presumptive zygotes were mounted on glass slides with an antifade-containing mounting medium (Vectashield, Vector Lab. Burlingame, CA, USA). To avoid excessive pressure being exerted on the mounted oocytes, coverslips were supported by thick droplets of a Vaseline-wax mixture placed at each corner, and sealed with nail polish. These zygotes were examined using a laser scanning confocal microscope (Leica TCS MP, Heidelberg, Germany) attached to an inverted microscope (Leica DM IRBE) equipped with 40x and 100x oil immersion objectives. The CLSM was equipped with three lasers (Krypton 563nm, Argon 514 nm and HeNe 633 nm) for the simultaneous excitation of Alexa Fluor™ 488 or SYTOX® Green, TRITC, EthD-1 or Mitotracker®Red, and TO-PRO_3 or AlexaFluor™633 using 488/568/650 nm excitation/barrier filter combinations. To avoid cross-talk of the acquired images in the photomultiplier channels, specimens were scanned using a sequential scanning-mode. Images were recorded digitally and processed using Adobe®Photoshop 5.5 software (Adobe Systems Inc., Mountain View, CA, USA).

RESULTS

A total of 371 in vitro matured horse oocytes with a first polar body and an intact oolemma, were subjected to ICSI and cultured for various time intervals. By the time of CLSM analysis after culture, however, 24 of the oocytes (6%) displayed completely aberrant and uninterpretable chromatin and cytoskeletal patterns and were therefore considered to be degenerate and were excluded from further analysis. In total, thus, 347 oocytes (94%) were analyzed after ICSI by CLSM imaging (Fig. 1), and a further 60 oocytes were analyzed after sham injection.

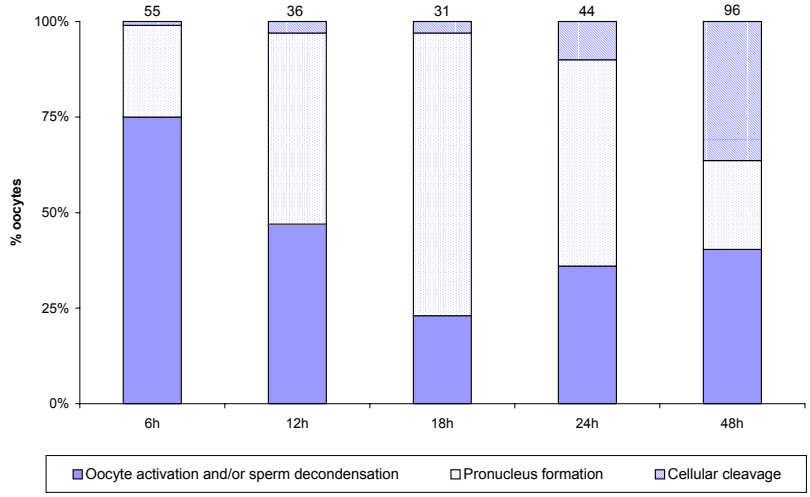

Fig. 1. Graphic representation of the dynamics of cytoskeleton and chromatin reorganization in horse oocytes fertilized by ICSI and incubated for a further 6 to 48 h. The series of cellular changes that occurred during fertilization have been categorized as: a) oocyte activation and/or sperm decondensation; this encompasses the events beginning with the progression of oocytes from arrest in metaphase II of meiosis to formation of a female pronucleus, and decondensation of sperm chromatin to eventual formation of the male pronucleus, b) pronucleus formation; including migration and apposition under the direction of the sperm aster, c) cellular cleavage; includes all fertilized oocytes that progressed to the two-cell stage or beyond. The number of oocytes analyzed at each time point is shown above each column. Oocytes that failed to complete fertilization were excluded from the figures.

Cytoskeleton and chromatin organization in horse oocytes after ICSI

In oocytes examined shortly after injection and while still arrested at metaphase II, microtubules were detected in the meiotic spindle and the first polar body only, as reported previously [29]. At this stage, the spindle appeared as a barrel-shaped conglomeration of microtubules with two anastral poles and with the chromosomes aligned along the meiotic plate (Fig. 2A). The polar body appeared as an amorphous mass of microtubules intertwined with chromatin (Fig. 2A, 2B). At 6 h after injection, densely stained microtubules could be observed radiating from the base of the decondensing sperm head (Fig. 2B, 2C). As the sperm chromatin continued to decondense to form the male pronucleus, the microtubules elongated further to form the radial sperm aster which had a distinct nucleation site at the sperm centrosome (Fig. 2I). The majority of oocytes displayed signs of activation 6 h after sperm injection (75%: 41/55; Fig. 1).

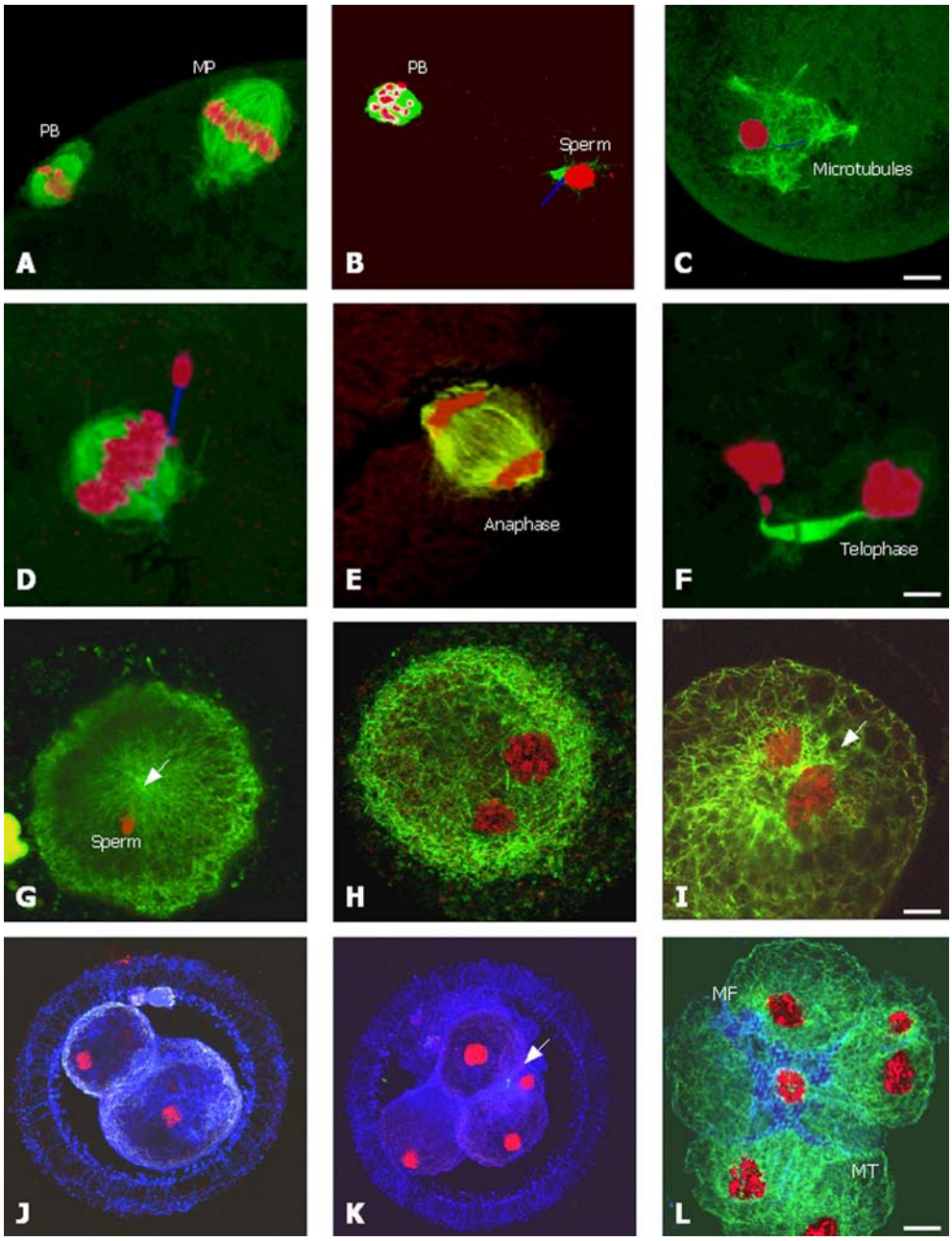
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Fig. 2. Laser-scanning confocal images of horse oocytes during fertilization and early embryonic development, after ICSI. In each case, microtubules are represented in green, microfilaments in blue and chromatin in red. Shortly after ICSI, microtubules were seen in the meiotic spindle of the metaphase-II oocyte or in the first polar body (A: metaphase plate: MP; polar body: PB) and alongside the incorporated sperm (B). The sperm head began to decondense while still attached to the tail (B). The sperm aster formed as a microtubular array nucleating from the base of the decondensing sperm head which would later develop into the male pronucleus (C). In M-II arrested oocytes, microtubules were confined to the polar body and to the spindle, which held the maternal chromosomes along the meiotic plate. Sperm injection initiated oocyte activation which was characterized by a resumption of meiosis during which the maternal chromosomes enlarged (D) and begin to migrate along the spindle towards the poles (E). At the telophase stage, the astral microtubules were found between the decondensing sets of female chromosomes (E). During the formation of the sperm aster, a distinct microtubule nucleation site (arrow) was detected adjacent to the sperm head and, during pronuclear migration, this array of microtubules expanded to fill the whole cytoplasm until it formed a microtubule matrix without a distinct nucleation site and surrounding both the male and female pronuclei (G, H). During pronuclear apposition prior to syngamy, a dense microtubule array (arrow) without a distinct bipolar centrosomal appearance, was detected between the male and female pronuclei (I). In two and four-cell embryos, the microfilaments were concentrated in the cortex of the daughter cells and at the cleavage furrows (arrow) (J, K). Microtubules formed a network surrounding the interphase nucleus of the daughter cells and extending throughout the cytoplasm (L; MF: microfilaments; MT: microtubules). Figures A, D, E, F: bar = 6 μm; B, C, L: bar = 10 μm; G-K: bar = 20 μm.

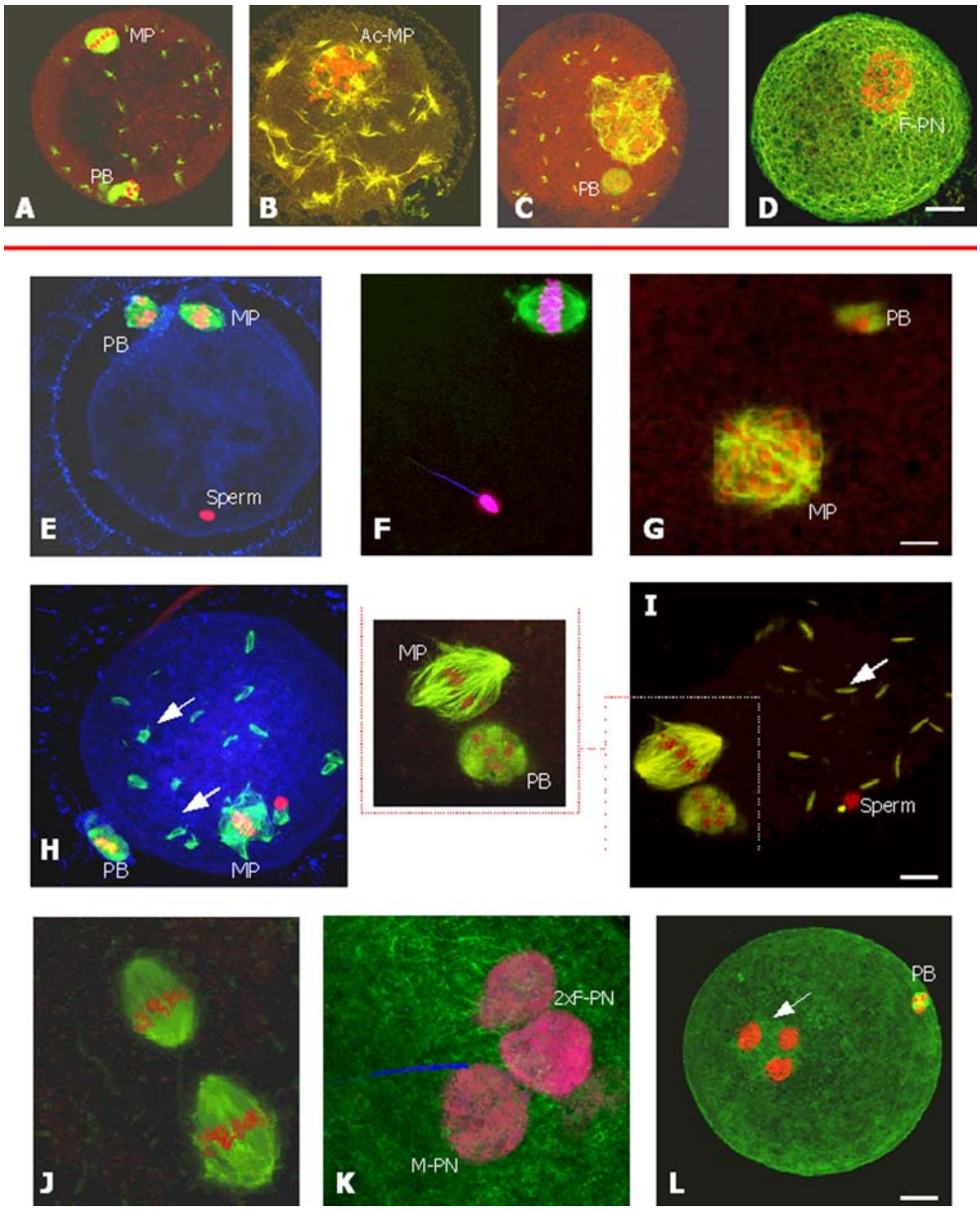
During activation, the maternal chromosomes, that were initially compact and aligned along the meiotic plate (Fig. 2A), began to enlarge (Fig. 2D) and, thereafter, the oocyte proceded through anaphase (Fig. 2E) and entered telophase (Fig. 2F) of the second meiotic division. This resulted in the formation of the second polar body; at this point, the microtubules were still visible in the meiotic midbody between the newly formed polar body and the developing female pronucleus (Fig. 2F). After 12 h of incubation, 50% (18/36; Fig. 1) of the injected oocytes had reached the pronuclear stage, while the remainder of the cells were still in earlier stages of activation and sperm decondensation. At 18 h postinjection, most of the presumptive zygotes (74%: 23/31; Fig. 1) had reached the pronuclear stage. During the development of pronuclei, the sperm aster continued to enlarge until it filled the entire cytoplasm (Fig. 2G). The sperm aster was not orientated preferentially toward the female nucleus but instead assumed a perinuclear distribution around both parental pronuclei and, at this stage, no distinct nucleation sites were visible (Fig. 2H). By 18h, the male and female pronuclei had enlarged and, presumably assisted by the sperm aster, had migrated to become apposed in an eccentric position within the cytoplasm, with an extremely dense array of microtubules between them (Fig. 2I). At 24 h postinjection, pronuclear apposition was still the dominant feature of most zygotes (55%: 24/44; Fig. 1), although they

had proceeded further towards syngamy, with the microtubules now concentrated at the poles of the adjacent pronuclei. In addition, a small number of zygotes had entered the first mitotic metaphase (5%; 2/44) during which the microtubule array developed into a bipolar structure that formed the mitotic spindle and held the now condensed chromatin along the mitotic plate. Although some two-cell embryos were detected as early as 6 h postinjection, it was not until 48 h that a significant proportion of the injected oocytes (36%: 35/96; Fig. 1) had undergone cellular cleavage. The resulting two to four-cell embryos had most of their microfilaments concentrated in the cell cortex and, sometimes, a distinct microfilamentar cleavage furrow was visible at the intercellular junction (Fig. 2J and K). The microtubules were organized in a network that spread throughout the cytoplasm of the daughter cells but was particularly prominent around the decondensed chromatin of the interphase nuclei (Fig. 2L).

Microtubule and DNA patterns in sham-injected oocytes

Of the 60 MII-stage oocytes examined by confocal microscopy 24 h after sham-ICSI, 13 (22%) showed no apparent signs of activation or further development; their microtubules remained concentrated in the second meiotic spindle and first polar body, and their chromosomes remained aligned at the metaphase plate (Fig. 2A). In 43% of sham-injected oocytes (26/60), multiple microtubule arrays were detected, distributed randomly throughout the cytoplasm (Fig. 3A).

Fig.3. Laser-scanning confocal images of horse oocytes during parthenogenesis induced by sham-injection (A-D) and sperm injected oocytes that failed to progress through fertilization (E-L). (A) In early horse parthenotes, microtubules were present in the MII spindle, the polar body (PB) and in multiple foci distributed throughout the cytoplasm (A; metaphase plate: MP). These microtubule foci extended and coalesced to form a dense meshwork of microtubules extending from the remnants of the meiotic spindle, while the chromosomes began to decondense (B,C; Activated metaphase plate: Ac-MP). In this parthenote, a distinct female pronucleus (F-PN) was seen surrounded by a dense network of disarrayed microtubules (D). The most common recorded cause of fertilization failure after ICSI was defective oocyte activation (E, F). The oocytes failed to resume meiosis and retained an intact metaphase plate, although the maternal chromosomes often became dispersed along the spindle away from the meiotic plate (G). In addition, the sperm chromatin frequently remained condensed in oocytes that failed to activate (F). In some cases of failed fertilization, multiple microtubule asters (arrow) were detected in the cytoplasm of injected oocytes, presumably originating from fragmentation of a defective meiotic spindle (H, I). The enlargement from figure (I) shows microtubular threads detaching from the spindle. Further defective oocytes, displayed two meiotic spindles due to a premature condensation of the sperm chromatin after ICSI (J), or three pronuclei (Digyny) due to failure to extrude the second PB, which was retained as a second female pronucleus (K, L: arrow indicate the microtubule domain concentrating between the pronuclei). Figures A-E, H, I and L: bar = 20 μm; F, G and J: bar = 10 μm; K and inset from I: bar = 6 μm.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In addition, while most meiotic spindles remained in the intact MII form, in some oocytes the microtubular spindle was wider than normal and the meiotic plate seemed to have begun reorganization, a change usually associated with the early stages of oocyte activation. Seventeen per cent of sham-injected oocytes (10/60: Fig. 3B, C) displayed more advanced features of activation, including microtubule aster assembly and formation of a microtubule network extending throughout the cytoplasm and accompanied by a resumption of meiosis and progression to telophase II. Female pronucleus formation was observed in 13% of the Sham-injected oocytes (8/60), as a mass of decondensed chromatin surrounded by a network of disarrayed microtubules (Fig. 3D: F-PN). In two cases (3%), the sham-injected oocytes developed gynogenetically with the second polar body remaining within the oocyte to form a second female pronucleus.

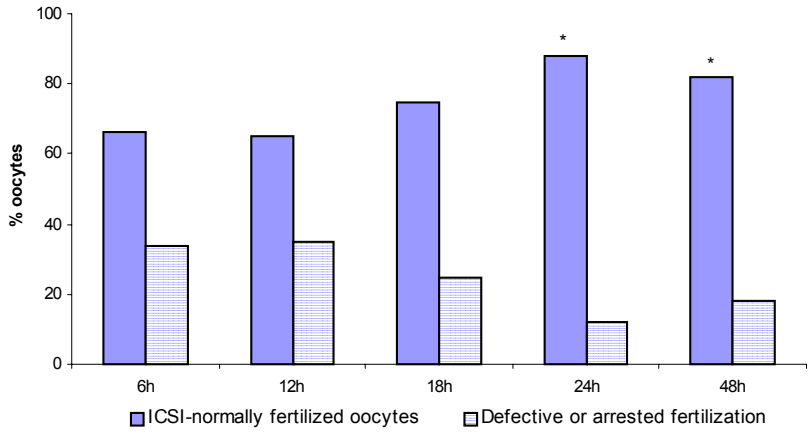

Fig. 4. The proportion of sperm-injected, in vitro matured horse oocytes progressing normally or showing signs of defective fertilization 6, 12, 18, 24 and 48 h after ICSI. The different stages of fertilization were identified by CLSM analysis of cytoskeletal and chromatin configurations, and based on the sequences described in Fig.1. Oocytes that displayed obviously abnormal cytoskeletal or chromatin patterns were considered to have suffered fertilization failure (defective or arrested oocytes). By 24 and 48 h after injection a higher proportion of ICSI derived oocytes showed signs of progression through normal fertilization that at 6, 12 or 18h (* $P<0.05$, Chi-square test). Nevertheless, a significant proportion of those fertilized oocytes had not progressed beyond the early events of oocyte activation and/ or sperm decondensation despite the time after sperm injection. However, since no abnormalities could be identified in their cytoskeletal or chromatin patterns, those oocytes were categorized as fertilized rather than arrested.

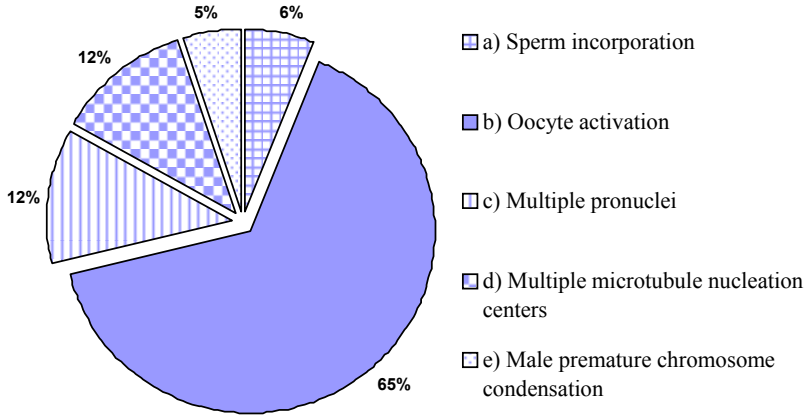

Fig. 5. The contribution of different anomalies to fertilization failure after ICSI of IVM horse oocytes. These failures were categorized by comparison to the patterns observed in normally developing ICSI-fertilized horse zygotes, and to abnormalities reported following ICSI of human oocytes. Basically, the causes of defective fertilization were: a) failure of sperm incorporation, b) failure to complete meiotic maturation and/or defective oocyte activation, c) abnormal microtubule nucleation giving rise to a truncated or otherwise defective sperm aster, d) inappropriate activation of the maternal spindle causing premature condensation of the male chromosomes, and e) failure to extrude the second polar body leading to retention of both sets of maternal chromosomes within the cytoplasm and a second female pronucleus (abnormal tripronuclear stage: Digyny).

Fertilization failures and developmental arrest in horse zygotes after ICSI

On the 347 oocytes injected, 262 (76%) displayed cytoskeletal and chromatin patterns consistent with normal fertilization; the remaining 85 oocytes (24%: see Fig.4 for the effect of incubation time) showed signs of failed fertilization or developmental arrest. Since no reports are available on fertilization failure after ICSI in the horse, the findings were described according to the sequence of events reported above (summarized in Fig. 6) and comparing those with the abnormalities of fertilization reported following ICSI in man [36-38]. The majority of injected oocytes that failed to complete fertilization did not even reach the pronuclear stage.

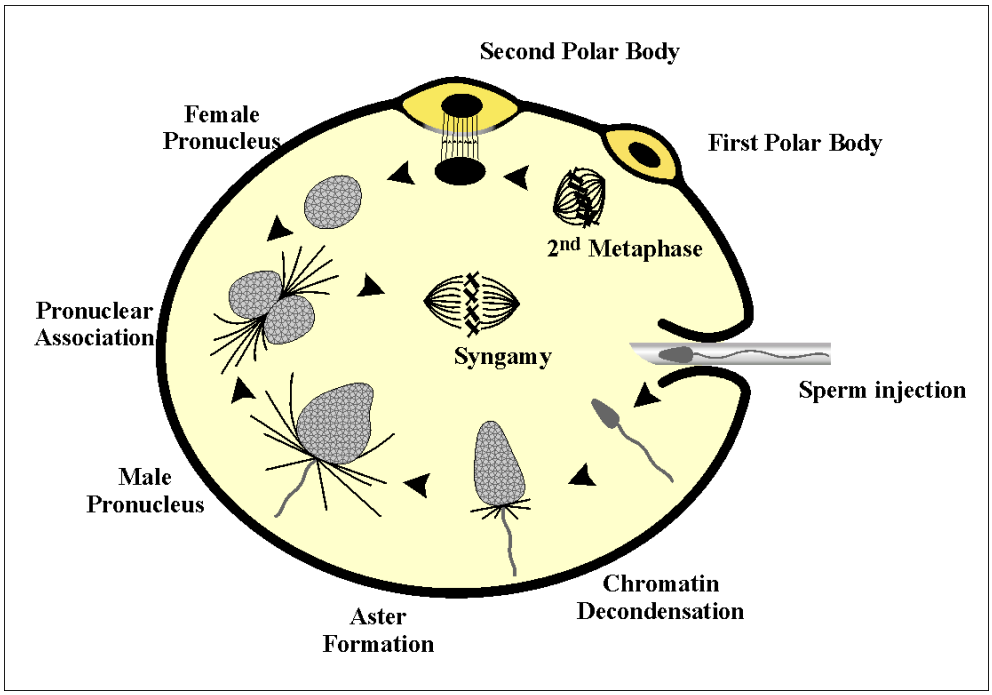

Fig. 6. A schematic representation of the microtubule and chromatin reorganizations that occur in horse oocytes fertilized by ICSI. A mature oocyte is injected while arrested in metaphase of the second meiotic division, when it contains a second meiotic spindle and a first polar body. After sperm injection, a microtubule aster forms at the base of the decondensing sperm head and the arrested oocyte is activated, progresses through meiosis and forms the second polar body. Annexation of the second polar body from the zygote is aided by microtubules aligned at the midbody of the second meiotic spindle. As the male and female pronuclei continue to decondense, the sperm aster enlarges to assist their migration and apposition. The microtubule aster that surrounds the adjacent pronuclei becomes concentrated at their interface. Following syngamy, the parental chromosomes line up at the equator of the zygote's first mitotic spindle.

Indeed, most defects (88%: 75/85; see Fig. 5) were associated with aberrant sperm integration and failure of the oocyte to complete meiosis after sperm incorporation. In total, 55 of the 85 fertilization failures (65%; Fig. 5) were characterized by the inability to progress properly through the second meiotic division. Affected oocytes displayed either an intact meiotic spindle (Fig. 3E and F) or a defective spindle with disorganized microtubules and the chromosomes displaced from the meiotic plate (Fig. 3G). In several of these oocytes, the sperm chromatin remained

condensed or only partially decondensed (Fig. 3F), presumably due to failure of the sperm plasma, acrosomal or nuclear membranes to breakdown. Another occasional defect was the presence of disarrayed microtubules in the cytoplasm of the injected oocyte (Fig. 3H and I). Such multiple microtubule nucleation centres were detected in 12% (10/85; Fig.5) of arrested oocytes, and seemed to arise from fragmentation of a defective meiotic spindle, particularly in those oocytes that remained arrested in metaphase-II (Fig. 3I and its inset). On rare occasions, two spindles were detected in the injected oocytes (6%: 5/85; Fig. 3J). These oocytes had failed to resume meiosis and it is assumed that the paternal chromosomes had instead condensed prematurely to form a paternal meiotic spindle. Those oocytes that did reach the pronuclear stage but failed to enter the mitotic cycle were all found to possess three pronuclei and a weakly stained first polar body, with only one of the pronuclei associated to a sperm tail (abnormal tripronuclear zygote; Digyny: 12%: 10/85; Fig. 3K and L). Finally, in 5 of the 85 defective zygotes (6%), no sperm was detected and the only DNA visible was that present in the meiotic spindle and in the first polar body (as in Fig. 2A).

DISCUSSION

This study examined the cytoskeletal reorganization and chromatin configuration in horse oocytes during fertilization by intracytoplasmic sperm injection, and demonstrated clearly that migration and fusion of the male and female genomes, and cell cleavage during early embryonic development, are accompanied by complex rearrangements of the cytoskeleton (summarized in Fig. 6). Analysis of the stage at which ICSI failed revealed that the majority of fertilization failures were due to failure of the injected oocyte to activate (65%). Furthermore, failure of oocyte activation was often accompanied by incomplete sperm decondensation, suggesting inadequate communication between the gametes during the fertilization process. Moreover, this study also demonstrated that during fertilization of horse oocytes, microtubule organization is initiated by the sperm midpiece which, via a distinct nucleation site, orchestrates formation of the sperm aster. Thus, the zygotic centrosome in horses appears to be primarily paternally inherited, just as it is in many other mammalian species (e.g. sheep [26], cow [24], pig [25], rhesus monkey [23], rabbit [39] and man [22]). However, microtubule assembly was also observed in 76% of sham-injected horse oocytes, mostly in the form of multiple asters dispersed randomly throughout the cytoplasm. Occasionally, these asters assembled into a more organized microtubule network which allowed parthenogenetic development (16%). Since cell division has also been reported

after parthenogenetic activation of horse oocytes [40, 41], it is concluded that the oocyte itself contains sufficient material to form a functional centrosome, just as parthenogenetic mouse zygotes are able to construct a maternal centrosome capable of duplicating and forming a functional mitotic spindle [21]. By contrast, studies on fertilization and polyspermy in cattle [24], pig [25], human [22] and rhesus monkey [23, 42] zygotes have demonstrated that the sperm aster is the most prominent, and usually the only, microtubule-containing structure in the zygote. This does not, however, rule out the possibility that the zygotic centrosome consists of both paternal and maternal components. Indeed, Simerly et al. [43] showed that in human oocytes the zygotic centrosome must be composed largely of maternally derived γ-tubulin (a centrosomal protein essential for nucleation of microtubules) because the modest amount of γ-tubulin present in the spermatozoa was insufficient for microtubule assembly. The recruitment of maternal γ-tubulin appeared to be an important factor in the transformation of the sperm centrosome into a functional zygotic centrosome. Studies on cattle [24] and rabbit [44] zygotes and parthenotes have also supported a biparental contribution to the zygotic centrosome, and our observations on microtubule nucleation in horse oocytes after ICSI or sham injection strongly support a biparental origin of the zygotic centrosome in this species.

The variability in chronology of the cytoskeletal and nuclear rearrangements observed in horse oocytes after ICSI probably reflected both the heterogeneity of the oocyte population after collection from abattoir-derived ovaries and maturation in vitro [45], and the fact that the oocytes were not subjected to any specific activation treatment after ICSI. Despite, or maybe aided by this asynchrony, the culture of injected oocytes during different time periods (i.e. 6, 12, 18, 24 and 48h) after ICSI allowed visualization of many different stages of fertilization and helped to unravel the sequence of cytoskeletal and nuclear remodeling that occurs during this process in horse zygotes. Of course, in order to establish the efficiency of ICSI in the horse, it is necessary to compare the events that accompany fertilization by ICSI with those occurring during fertilization in vivo. In the current study, injected oocytes began to show signs of activation, such as reorganization of the meiotic spindle and progression through meiosis II, within 6 h of sperm injection. Unfortunately, little is known about the events occurring as early as 6h after sperm penetration in vivo, and nor it is clear how long after ovulation sperm penetration occurs. Enders et al. [17] and Bézard et al. [1] were unable to detect any evidence of fertilization, e.g. sperm incorporation or oocyte activation and progression through metaphase II, in oocytes collected from the oviducts of mares earlier than 10 h after mating. However, the time required for sperm to be transported to the

site of fertilization and to capacitate so that they are ready to bind to and penetrate an oocyte is unknown, but could easily be longer than 6 h [46]. Nevertheless, once sperm transport is complete, it is likely that fertilization proceeds rapidly since Torner et al. [19] noted sperm head decondensation as early as 2 to 4 h after the onset of coincubation of spermatozoa with IVM oocytes in an IVF system.

Large differences between oocytes were observed in the timing of male chromatin decondensation and formation of the sperm aster, which might suggest a loss of synchrony between the male and female gametes during fertilization by ICSI. The presence of the sperm acrosome and perinuclear theca on injected sperm, structures that are removed at the oolema during normal fertilization [37, 47], would presumably tend to delay sperm decondensation. With regard to the time required for horse zygotes to reach the pronuclear stage, in the current study only 50% of presumptive zygotes had reached this stage at 12 h postinjection but the majority had done so at 18 h (see Fig.1). On the other hand, Grøndhal et al. [18] showed that pronucleus formation in vivo is completed as early as 12 h after ovulation. The apparent delay in the formation of the pronuclei after ICSI may relate to the need for greater sperm remodeling, although Torner el al. [19] reported that pronucleus formation did not peak until 16-24 h after conventional IVF. In the current study, only 10% of the presumptive zygotes had undergone cleavage 24 h postinjection but at 48 h the proportion of 2-cell or later stage embryos had risen to 36% (Fig. 1). By contrast, the first cellular cleavage in vivo has been reported to occur about 20-24h postovulation [1, 17] with most embryos reaching the 4 to 6-cell stage by 48 h [1]. On the other hand, Choi et al. [14] reported that only 10% and 4% of oocytes injected with fresh and frozen-thawed spermatozoa using a Piezo drill had undergone cellular cleavage by 20 h postinjection while Torner et al. [19] did not detect cleavage until 32 h after the onset of conventional IVF. In conclusion, there appear to be a similar delay in the early events of fertilization and embryo development after fertilization of IVM oocytes by conventional IVF or by ICSI, and it is therefore possible that the irregularities reflect not only a delay in sperm decondensation and male pronucleus formation but also defects arising during the process of in vitro oocyte maturation. Indeed, IVM horse oocytes are known to be developmentally compromised [48].

In the current study, a high proportion (25%) of oocytes or zygotes showed signs of fertilization failure after ICSI, and the principal reason for failure appeared to be failed oocyte activation (65%; Fig. 5). The mechanism by which oocytes activate after ICSI is itself unclear, but by analogy with other mammalian species, it must be assumed that metaphase II arrest in horse oocytes is maintained by high concentrations of metaphase-promoting factor (MPF: [49]). Inactivation of MPF is one of the critical events of oocyte activation because it “unblocks” the cell cycle

and allows the oocyte to complete the second meiotic division. Physiologically, MPF inactivation and oocyte activation are induced by entry of the sperm, which triggers a release of calcium from oocyte intracellular stores and sets off a series of signaling events that use calcium as a second messenger [50]. During ICSI, the injected sperm must elicit these calcium oscillations (reviewed by Tesarik [51]). However, it has been recently reported that the occurrence of sperm-induced calcium oscillations in both in vivo and in vitro matured horse oocytes subjected to ICSI were inconsistent [52]. Any perturbation or change in the pattern of calcium oscillation can cause incomplete MPF inactivation and thereby abnormalities of oocyte activation that are reflected in abnormal or incomplete fertilization and, in particular, a failure of the oocyte to progress through meiosis and of the sperm chromatin to properly decondense [51]. Sperm chromatin decondensation could also be negatively affected by a deficiency of those cell cycle proteins specifically required for decondensation, such as glutathione and nucleoplasmin [53,54]. The fact that failure of sperm chromatin decondensation was observed particularly in oocytes that failed to activate, suggests a failure of the sperm to adequately transmit its activating signal to the oocyte [51], or a failure of the oocyte to respond to this signal.

Other causes of fertilization failure as described in the present study, include the presence of multiple microtubule foci in the cytoplasm of injected oocytes. This defect in microtubule assembly may have been associated to incorrect reconstruction of the zygote's centrosome, as seen in arrested human IVF oocytes [55]. In this respect, defects in microtubule motor proteins, such as dynein, have been reported to result in detachment of the aster from the male pronucleus [21]. Based on the current observations, we suggest that the multiple microtubule foci originated by fragmentation of a defective meiotic spindle in oocytes that remained in meiotic arrest despite sperm incorporation. However, it cannot be ruled out that some oocytes were activated and that the sperm assisted in microtubule organization despite remaining condensed. To date, there is no information on the contribution of aging of the horse oocyte to the microtubule patterns. In a previous study [29] we showed that in our experimental conditions 25% of the oocytes can be expected to reach MII by 24h and an additional 17% will be in MII by 36h after IVM. This would suggest that an initial 25% of the oocytes are aged 12h by the time that they were selected for ICSI. However, we did not observed any obvious spindle or chromosome desegregation abnormalities in the MII oocytes by 24 and 36 h of IVM that would preclude further defects in their microtubule patterns. In addition, the cytoskeletal network was also presumably disrupted in those injected oocytes that displayed three pronuclei (12%). Digyny results from failure to form a second polar body and consequent retention of both sets of maternal chromosomes

as pronuclei [56]. In the present study, 5% of arrested oocytes formed, in addition to the maternal meiotic spindle, an anastral paternal spindle associated with the condensed paternal chromosomes. Such premature condensation of the male chromosomes has been reported in human zygotes [57] and appears to result from failed activation of the oocyte after sperm injection and the continued presence of active chromatin condensing factors (e.g. MPF) in the ooplasm. This, in turn, prevents the transformation of the sperm nucleus into a male pronucleus and causes the sperm chromatin to condense (reviewed by Zenzes and Casper [58]).

In summary, in this study confocal laser scanning microscopy was used to reveal the way in which the cytoskeletal and nuclear events that occur during fertilization of horse oocytes by ICSI are choreographed and to demonstrate the significance of highly integrated cytoskeletal changes in the migration and fusion of the parental genomes. The comparison of the microtubular structures in zygotes and parthenotes suggests that the sperm contributes the centrosomal template during fertilization but that the oocyte contributes structural entities to the functional zygotic centrosome and to the cytoplasmic microtubule network. Failure of fertilization after ICSI was due primarily to failure of gamete activation during the very early fertilization events and the high rates of failure observed in this study presumably relates, at least in part, to inadequacy of the in vitro matured oocytes used. Nevertheless, until conventional IVF becomes reliable, ICSI may be the best way to produce zygotes for offspring production, fundamental research into the cellular and molecular events of fertilization and as a tool for investigating infertility and for understanding the cellular basis of early pregnancy failure in horses.

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Chapter 6

Effects of in vitro production on horse embryos morphology, cytoskeletal characteristics and blastocyst capsule formation

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ABSTRACT

Attempts to produce horse embryos *in vitro* (IVP) have resulted in low blastocyst rates. In addition, a characteristic and vital feature of blastulation in equids, namely capsule formation, does not occur *in vitro*. The aim of this study was to evaluate the impact of IVP on horse embryo development and blastocyst capsule formation. IVP embryos were produced by intracytoplasmic sperm injection of *in vitro* matured oocytes, followed by either culture in synthetic oviductal fluid (SOF) or temporary transfer to the oviduct of a progesterone-treated ewe. As controls, *in vivo* embryos were flushed from the uterus of mares on days 6-9 after ovulation. Embryo morphological quality and the degree of cytoplasmic fragmentation were investigated by light microscopy, and then by multiphoton scanning confocal microscopy. During the latter, microfilament distribution was visualized by means of AlexaFluor-Phalloidin staining, while the rate of apoptosis was examined using the TUNEL assay for DNA fragmentation combined with DAPI staining to evaluate nuclear morphology and total cell number. To examine the influence of culture on blastocyst capsule formation, embryos were stained with a monoclonal antibody (OC-1) specific for capsular glycoproteins.
The blastocyst rate was significantly higher after transfer of 2 to 4 cell embryos to a sheep's oviduct (16%) than after culture in SOF medium (6.3%). Compared to similarly aged *in vivo* embryos, Day 7 IVP embryos were small with low cell numbers, and compact with a small, or no, blastocoele and an indistinct inner cell mass. In addition, IVP embryos had high percentages of apoptotic cells (10% versus 0.3% for *in vivo* embryos) and irregular microfilament distribution, which highlighted the heterogeneity of cell size and shape. Finally, although IVP embryos secreted capsular glycoproteins, the latter failed to coalesce and form a confluent capsule enveloping the embryo, but instead adhered to and permeated into the zona pellucida or remained in patches on the trophectoderm surface. This confirmed that the initial layer of capsule is made up of OC-1 reactive glycoproteins, and suggests that the missing element for capsule formation in vitro is a suitable microenvironment for crosslinking and coalesence of mucin-like glycoproteins.

INTRODUCTION

A variety of assisted reproductive techniques, including conventional *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), oocyte transfer (OT), and nuclear transfer, have been performed or attempted with equine gametes (Palmer *et al*., 1991; Carnevale and Ginther, 1995; Squires *et al*., 1996; Galli *et al.*, 2002; Li *et al*., 2002). However, the relatively low efficiency of the *in vitro* culture

stages has limited their commercial application in this species. In particular, the current techniques for *in vitro* oocyte maturation, conventional IVF and culture of zygotes to the blastocyst stage are inadequate. For example, while only two foals have been produced via conventional IVF (Palmer *et al*., 1991; Bézard *et al*., 1992), the adoption of intracytoplasmic sperm injection (ICSI: Squires *et al*., 1996; Meitjes *et al*., 1996) has, by circumventing the need for sperm to penetrate the zona pellucida (ZP), resulted in the birth of several foals (Squires *et al*., 1996; Cochran *et al*., 1998; McKinnon *et al*., 2000; Li *et al*., 2001; Galli *et al.*, 2001a,b). Still, the majority of ICSI pregnancies have resulted from transferring immediate post-cleavage zygotes to the oviduct of recipient mares (Squires *et al*., 1996; Cochran *et al*., 1998; McKinnon *et al*., 2000) while only a few have been produced via *in vitro* culture to the blastocyst stage and transfer to the uterus (Li *et al*., 2001; Galli *et al*., 2001a). In support of the apparent negative effect of culture, McKinnon *et al*. (2000) reported that even incubation for 26-28h between ICSI and oviductal transfer resulted in a lower pregnancy rate than transfer of presumptive zygotes 4-8 h after ICSI. Thus, if a viable complete *in vitro* horse embryo production (IVP) system is desired, and on grounds of cost and recipient welfare it is, one critical step is to devise a culture system that supports embryo development to a stage suitable for uterine, rather than oviductal, transfer. To date, two approaches to improving horse embryo culture have been explored. The first involved coculture of presumptive zygotes with somatic cells or their products (oviduct epithelial cells, Battut *et al*., 1991; cumulus cells, Li *et al*., 2001; equine trophoblast monolayer conditioned medium, Choi *et al*., 2001) and the second involved culturing zygotes in defined cell-free culture media such as synthetic oviductal fluid (SOF; Azuma *et al*., 1995; Galli and Lazzari, 2001), Dulbecco's modified Eagles medium (DMEM; Li *et al*., 2001) or Human Tubal Fluid (HTF; Dell'Aquilla *et al*., 2001). In all of the above systems, however, only a low percentage of zygotes developed to the blastocyst stage (average 15%). Indeed, the fact that temporary transfer of ICSI-derived horse zygotes to the oviduct of progesterone treated ewes remains the most successful system for producing horse blastocysts *ex vivo* (rates approach 50%; Galli *et al*., 2002) emphasizes the inadequacy of current *in vitro* culture systems.

In vitro-produced mammalian embryos tend to exhibit characteristic differences to their *in vivo* counterparts, including lower cell numbers, an altered inner cell mass: trophectoderm ratio, irregularly sized blastomeres and an increased incidence of cytoplasmic fragmentation, all of which are related to reduced developmental competence (pig – Kikuchi *et al*., 1999; cow - Kruip and den Daas, 1997). Programmed cell death (apoptosis) is a feature observed in both *in vivo* and *in vitro* produced mammalian blastocysts (e.g. mouse - Brison and Schultz, 1997; man -

Hardy, 1999; cattle - Byrne *et al*., 1999) and has been proposed as a means of eliminating cells that are damaged, nonfunctional, abnormal or misplaced (Hardy, 1999; Meier *et al*., 2000). It has been further suggested that apoptosis is a major contributor to embryonic arrest in sub-optimal culture conditions, such as an excess embryo: medium ratio (Brison and Schultz, 1997), heat shock (Paula-Lopes and Hansen, 2002), excess oxygen free radical concentrations (Van Soom *et al*., 2002) or following exposure of embryos to a high concentration of spermatozoa during IVF (Jurisicova *et al*., 1995). Since IVP embryos exhibit relatively high levels of apoptosis (with varying degrees of cytoplasmic fragmentation) and a high incidence of developmental arrest during culture, parameters such as cell number and apoptosis rate may be valuable indicators of the health and developmental capacity of pre-implantation embryos.

Studies in the hamster (Barnett *et al*., 1997), rat (Matsumoto *et al*., 1998) and pig (Wang *et al*., 1999) have indicated that the actin filament organization of IVP embryos differs from that of *in vivo* embryos. Of course, the actin cytoskeleton plays an important role in the migration of cytoplasmic organelles and the nucleus, and in cell cleavage during mitosis (Alberts *et al*., 1994). Given that actin filament dynamics can be affected by environmental conditions such as temperature, pH, and ion concentrations, it would not be surprising if culture conditions affected embryo cleavage or compromised further development. Previous studies have indicated the likely roles of actin filaments during horse oocyte maturation, fertilization and early embryo development (Tremoleda *et al.,* 2001, 2002), and disruption of the microfilament cytoskeleton has been proposed as a reason for the poor viability of frozen-thawed embryos (Huhtinen et al., 2001). However, it is not known if the actin cytoskeleton of IVP horse blastocysts differs significantly from that of *in vivo* embryos.

An unusual and vital feature of early embryonic development in the horse is the formation of an acellular blastocyst capsule beneath the zona pellucida (ZP) on days 6-7 after ovulation, soon after the blastocyst enters the uterus (Flood *et al.*, 1982). After the loss of the ZP, the capsule remains to envelop the conceptus throughout the second and third weeks of pregnancy (Betteridge, 1989). Although the precise functions of the capsule are not known (see Betteridge, 1989 for a review), it is thought to provide vital mechanical protection during the period when the conceptus is mobile and propelled throughout the uterine lumen by myometrial contractions (Ginther, 1985), where the resulting intrauterine migration is essential for the conceptus to distribute its maternal recognition of pregnancy signal to, and inhibit $PGF_{2\alpha}$ secretion from, sufficient endometrium to ensure luteostasis (McDowell *et al.*, 1985). Production of the glycoprotein capsule appears to be

primarily, or exclusively, a function of trophoblast cells (at least after day 11 of gestation: Oriol *et al*, 1993a; Albihn *et al*., 2003). However, the presence of large quantities of the endometrial lipocalin, P19 (Stewart *et al*., 1995), associated to the capsule argues that there may also be a maternal uterine contribution to capsule formation, as does the finding that equine embryos that blastulate *in vitro* are not able to produce a visible capsule (McKinnon *et al*., 1989; Hinrichs *et al*., 1990). On the other hand, the suggestion that the ZP is an essential requirement for capsule formation (Skidmore *et al*., 1989) has been disproven by the demonstration that zona-free bisected blastocysts develop an apparently normal capsule after transfer to the uterus of recipient mares (McKinnon *et al*., 1989). In any case, it has yet to be established whether the initial capsule is composed of the same trophoblast-produced molecules that predominate at later stages, and are recognized by the antibody OC-1 (Oriol *et al.,* 1993a). Neither has the influence of *in vitro* culture on capsule glycoprotein production and coalesence been examined.

The aim of this study was to document the impact of *in vitro* production on the morphological and ultrastructural characteristics of horse embryos. IVP embryos were produced by ICSI followed by one of two different culture systems; the first was based on synthetic oviduct fluid (SOF: Galli *et al*., 2001b) and the second involved temporary transfer to the oviduct of a progesterone-treated ewe. As controls, i*n vivo* produced embryos were flushed from the uterus of mares on days 6-9 after ovulation. Embryo morphology and degree of cytoplasmic fragmentation were analyzed by light microscopy, while multiphoton scanning confocal microscopy was used to evaluate total cell numbers, nuclear morphology (after DAPI counterstaining) and rates of cell apoptosis, where the latter was taken as the incidence of nuclear fragmentation or of DNA fragmentation as indicated by the deoxynucleotidyl dUTP terminal transferase (TUNEL) assay. To examine the influence of culture on cytoskeletal morphology and on capsule formation, day 6-9 *in vivo* and day 7 IVP late morulae, early blastocysts and IVP embryos cultured for a further 3 days, with or without horse adult fibroblast cells, were analysed with respect to microfilament distribution and capsular glycoprotein expression.

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (Milan, Italy) and plastic-ware from Nunc (Roskilde, Denmark).

In vitro production of Day 7 embryos

Collection and Culture of Cumulus Oocyte Complexes
Ovaries from slaughtered mares were transported to the laboratory in PBS at 25°C. Within 4 h of slaughter, cumulus oocyte complexes (COCs) were recovered by scraping the inside of follicles between 0.5 and 3.0 cm in diameter with a Jacobson curette. The scrapings were washed into Petri dishes using TCM199 supplemented with 25 mM HEPES, 1 mg/ml BSA and 10 μg/ml heparin. COCs were classified as either compact (Comp) or expanded (Exp) depending on cumulus and granulosa cell morphology, as described by Hinrichs *et al.* (1997). Thus, compact COCs were those surrounded by a dense cellular mass with a smooth cumulus hillock and homogenous colouration, whereas expanded COCs were those with cells protruding from the surface of the cumulus hillock and matrix visible between the cumulus cells. COCs were matured by culture for 22-24 h in TCM199 supplemented with 10% fetal calf serum (FCS), 1 μl/ml ITS (insulin, transferin, sodium selenite), 1mM sodium pyruvate, 100 ng/ml Long-IGF1, 50 ng/ml Long-EGF, and 0.1 IU/ml each of FSH and LH (Pergovet, Serono, Italy), in 4-well plates at 38.5°C in an atmosphere of 5% CO_2 in air (Galli *et al*., 2001b, Lazzari *et al*., 2002).

Preparation of oocytes and sperm for ICSI
After culture, oocytes were separated of their cumulus cells by incubation in 25 μg/ml hyaluronidase in Hepes-buffered SOF medium (H-SOF; Galli *et al*., 2001b) and then 2.5 mg/ml trypsin in PBS for 2 min, before aspirating them through a fine pipette. Oocytes with an intact cell membrane were returned to maturation medium for 2-4 h, after which those with an extruded first polar body were selected for sperm injection.
Intracytoplasmic sperm injection (ICSI) was performed with frozen/thawed ejaculated sperm from a stallion of proven fertility. One hour before injection, the semen was thawed and the spermatozoa were rinsed free of cryoprotectant by centrifuging them at 750g through a discontinuous Percoll density gradient (45%: 90%) for 40 min at room temperature. The viable spermatozoa recovered from the bottom of the tube were washed in Ca^{2+} free TALP (Parrish *et al*., 1988) and re-pelleted by centrifugation at 400g for 10 min. This second sperm pellet was suspended at a concentration of 4 million sperm/ml in Hepes-buffered SOF medium supplemented with 6mg/ml fatty-acid-free (FAF) BSA, modified Eagle's medium (MEM) amino acids, 1 μg/ml heparin, 20 μM penicillamine, 1 μM epinephrine, and 10 μM hypothaurine (SOF IVF medium: Lazzari *et al*., 2002).

Just before ICSI, the sperm suspension was diluted 1:1 (v/v) with a 12% solution of PVP in SOF-IVF medium.

ICSI
Sperm injection was performed as described by Kimura and Yanagimachi (1995) using a Piezo micropipette-driving unit (Prima Tech, Japan) fixed on a micromanipulator (Narishige, Japan) and mounted on an inverted microscope equipped with a 37°C heated stage. A pipette with inner and outer diameters of 50 and 150 µm, respectively, was used to hold oocytes, and a pipette with a tip inner diameter of approximately 5 µm was used for sperm injection. A motile sperm was aspirated into the injection pipette and immobilized by applying two or three piezo-pulses to the tail-midpiece junction. The oocyte was held on the holding pipette by suction, with the polar body orientated to the 6 or 12 o'clock position, and the injection pipette was advanced through the ZP at the 3 o'clock position using the piezo-drilling motion. The core of ZP so excised was expelled into the holding medium and, finally, the injection pipette was advanced through the oolema using one piezo-pulse and the sperm was released into the ooplasm.

Culture of injected oocytes in vitro
After sperm injection (Day 0), oocytes were cultured in groups of 20 in 20µl droplets of SOF medium supplemented with MEM amino acids and 16 mg/ml FAF BSA (SOF-BSA-AA; Lazzari *et al*., 2002), under mineral oil at 38.5°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. On Day 2 of incubation, the cleavage rate was determined and morphologically normal two and four-cell embryos were selected for further culture. The selected embryos were incubated in one of two culture systems, namely: 1) *In vitro* culture (IVC) in 20 µl droplets of SOF-BSA-AA which was partially replaced (by adding 20 µl fresh medium and then removing 20 µl of the mix) with SOF-BSA-AA on Day 3 and with TCM199-BSA on Day 6; 2) Sheep-oviduct culture after surgical transfer of embryos embedded in agar chips to the ligated oviduct of a ewe implanted with an intravaginal progesterone-releasing device (EAZI-BREED CIDR; InterAg, Hamilton, New Zealand) on the day of transfer, as previously described by Willadsen (1979). For the IVC-group, both expanded and compact COCs were used to produce embryos, whereas for the Sheep-oviduct group only compact COCs were used. After 5 further days in culture, embryos in the IVC-group were assessed, and those lacking cellular compaction and displaying irregular embryonic cell sizes were removed from culture, fixed and stained to examine their developmental status. Sheep oviduct embryos were harvested 5 days after transfer into the oviduct, and the rate and quality of blastocysts from both systems was scored according to the criteria outlined in the Manual of the International Embryo Transfer Society (1998).

In vitro production of Day 10 embryos

A proportion of the morphologically normal Day 7 blastocysts produced via the sheep oviduct system were maintained for a further 3 days in one of two different culture systems: 1) A semi-defined culture system consisting of 20 µl droplets of a 1:1 (v:v) solution of DMEM (Gibco BRL, Paisley, Scotland, UK) and TCM199, supplemented with 5% FCS and 5% Serum Replacement (Knockout ™ SR; Gibco BRL) and incubated under mineral oil. The medium was partially replaced on Day 8 of culture. 2) In a cell co-culture system consisting of adult horse skin fibroblast cell monolayers (SFC) and 300 µl DMEM: TCM199 supplemented with 5% FCS and 5% SR, in 4-well plates. The SFCs were prepared from a small piece of subdermal tissue harvested from the chest of a 5-yr-old Haflinger mare, under local anesthesia. The tissue was sliced finely and digested for 30 min at 38°C in 0.5% trypsin-EDTA in PBS (v:v). The digested fragments were then washed twice in PBS by centrifugation at 700g for 10 min, and the resulting pellet of cells was resuspended in DMEM supplemented with 10% FCS (v:v) and cultured at 38.5°C in 5% CO_2, 5% O_2, and 90% N_2. Cells used for co-culture had been through 1-5 passages.

Collection of in vivo produced horse embryos

Sixteen early horse conceptuses were recovered from 5-10 year old Dutch Warmblood mares by non-surgical uterine lavage with modified Dulbecco's phosphate-buffered saline (DPBS) supplemented with 1% FCS, as described by Imel *et al*. (1981). The conceptuses were recovered on days 6, 7 or 9 after ovulation detected by daily ultrasonographic examination of the ovaries and were thus 6.5, 7.5 and 9.5 ± 0.5 days old at the time of collection.

Embryo Evaluation

In vitro and *in vivo* horse embryos of similar developmental stages were compared as follows: In Experiment 1, Day 7 *in vivo* embryos and day 7 IVP embryos from both the IVC and sheep-oviduct groups were analyzed for total cell number, apoptotic rate and microfilament distribution. In Experiment 2, Day 10 IVP embryos from both the semi-defined and SFC-monolayer culture systems were evaluated for total cell number, microfilament organization and blastocyst capsule formation. Four Day 7 embryos derived from SOF (3) or sheep-oviduct culture (1) and a range of day 6-9 *in vivo* embryos were also analysed in this way. In all cases,

embryos were measured and assessed morphologically immediately after collection or harvest and before further fixation and labelling.

Morphological assessment
Morulae and blastocysts produced *in vitro* (Day 7 and 10) or *in vivo* (Day 6, 7 and 9) were measured using a stereomicroscope fitted with an eyepiece micrometer.

Table 1: System used to grade the quality of equine embryos produced *in vitro**

Grade 1	**Excellent or good**: Symmetrical and spherical embryo mass with cells of uniform size, colour and density. Embryo mass has clear edges without indentation. No evidence of cellular fragmentation or loose cytoplasmic granules in the perivitelline space.
Grade 2	**Fair**: Moderate irregularities in the overall shape of the embryo, for example a minor degree of cytoplasmic fragmentation evident as slightly convolution of the edges of the embryo and small cytoplasmic granules in the perivitelline space.
Grade 3	**Poor**: Major irregularities in the shape of the embryonic mass or in the size, colour or density of the constituent cells. High degree of cytoplasmic fragmentation with extrusion or degeneration of cells of variable size reflected by highly lobulated, irregular edges of the embryonic mass and a large perivitelline space.
Grade 4	**Degenerate or dead**: High degree of cytoplasmic fragmentation. Embryonic cells of irregular size and colour. Absence of cellular compaction and failure to form a clear embryonic mass. This group includes embryos with irregularly sized cells of a low number (<4) inconsistent with the expected stage of development.

* Adapted from McKinnon AO and Squires EL (1988), Hardy (1999) and the Manual of the International Embryo Transfer Society (1998).

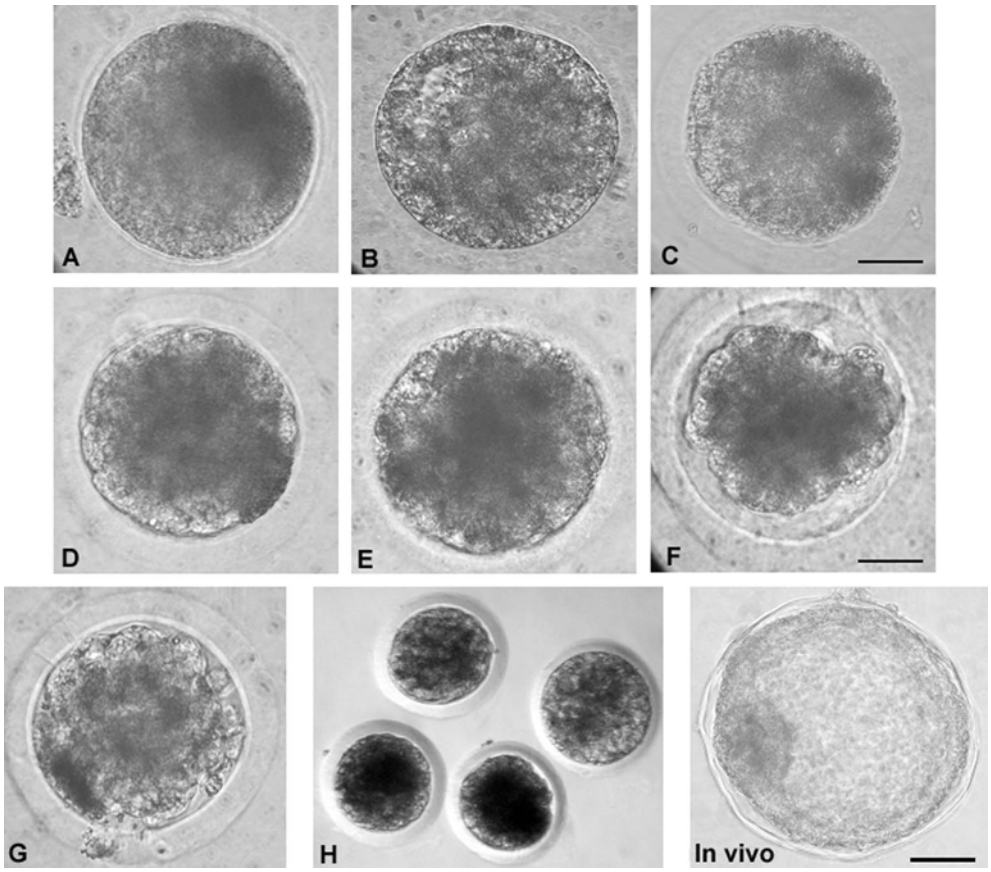

Fig 1: Photomicrographs to illustrate the quality scoring of day 7 IVP horse embryos (Grades 1-4: good to degenerate). Grading was based on criteria layed down in the manual of the International Embryo Transfer Society and included degree of compactness, size and appearance of the perivitelline space, colour, presence of extruded cells, and the degree of cell granulation and cytoplasmic fragmentation.

A and B: GRADE 1
B and C: GRADE 2
D: GRADE 2-3
E: GRADE 3
F: GRADE 4
H: A group of grade 1-2 embryos cultured in SOF medium.
I: A grade 2 *in vivo* early blastocyst for comparative purposes.

Scale: In A-G bar = 45 μm, in H bar = 90 μm and in *In vivo* bar = 75 μm

For Day 10 IVP embryos, separate measurements were made of the part of the embryo that remained enclosed within the ZP and the part that had herniated through the hole made during ICSI. In addition, each embryo was graded morphologically on a scale of 1-4, where 1 was good quality and 4 was indicative of degeneration. In this respect, *in vivo* embryos were graded using the system described by McKinnon and Squires (1988), while an adapted scale based on criteria published in the Manual of the International Embryo Transfer Society was developed for assessing IVP embryos (Table 1; Fig.1). This latter scale included parameters such as degree of compactness, size and appearance of the perivitelline space, colour, presence of extruded cells and the degree of cell granulation and cytoplasmic fragmentation (Hardy, 1999). After morphological assessment, embryos were fixed in 4% paraformaldehyde for 24 h and then stored at 4°C in PBS prior to staining.

Cell Number, Apoptosis index and Microfilament Distribution (Experiment 1)
Apoptosis was detected using the TUNEL-labeling technique for DNA fragmentation. First, fixed embryos were incubated twice for 15 min in PBS containing 150 mM glycine and 1 mg/ml of polyvinylacohol (PVA), to reduce free aldehydes and block non-specific reactions. Next, they were permeabilized by immersion for 15 min at 4 °C in 0.1% (v:v) Triton X-100 in PBS. The permeabilized embryos were then washed twice in PBS containing 1 mg/ml of PVA (PBS-PVA; pH 7.4) before being incubated in 20 µl drops of fluorescein-conjugated dUTP and TdT (TUNEL reagents; Boehringer Manheim, Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 37 °C in a dark, moist chamber. Following TUNEL labeling, embryos were washed three times in 0.5% (v:v) Triton X-100 in PBS containing 1 mg/ml PVA (PBS-TX100-PVA). To enable microfilament detection, embryos were then incubated for 1 h with 15 IU/ml AlexaFluor™ 568 Phalloidin (Molecular Probes Europe BV, Leiden, The Netherlands; A-12380) in PBS-TX100-PVA. Finally, to enable DNA visualization, the embryos were washed twice in PBS-PVA and incubated with 0.1 µg/ml of 4,6-diamino-2-phenyl-indole (DAPI) in PBS for 10 min.

Cell Number, Microfilament Distribution and Capsule immunolocalization (Experiment 2)
Fixed embryos were washed and permeabilized in 0.1% Triton X-100, as described above. Capsular glycoproteins were labeled using a monoclonal antibody raised against day 13.5-15.5 equine capsule in mouse: OC-1 (Oriol *et al*., 1993a). For the labeling, embryos were first incubated for 45 min in a blocking solution (0.1M glycine, 1% v:v goat serum, 0.01% Triton X-100 and 0.5% w:v BSA) and then exposed to a 1:100 dilution of mAb OC-1 in PBS-TX100-PVA, for 1 h at 37 °C.

Next, the embryos were incubated with a 1:300 dilution of a goat anti-mouse IgG coupled to Alexa Fluor™ 488 (Molecular Probes; A-11029)in PBS-TX100-PVA, and then washed twice in PBS-PVA before incubation with AlexaFluor™ 568 Phalloidin (15 IU/ml in PBS-TX100-PVA) to label the microfilaments. Finally, the embryos were counterstained with DAPI (0.1µg/ml in PBS) to label the nuclei. As controls, a few embryos were incubated with labeled goat anti-mouse secondary antibody without pre-exposure to OC-1. In addition, Day 6, 7 and 9 *in vivo*-produced embryos on which the capsule was clearly identifiable by light microscopy were stained with mAb OC-1 as positive controls, while *in vitro* and *in vivo* matured horse oocytes were labeled to control for cross-reactivity of OC-1 staining with the ZP.

Fluorescence and Multiphoton Laser Scanning Microscopy (MPLSM)
Stained embryos were mounted on glass slides with an antifade (Vectashield, Vector Lab. Burlingame, CA, USA). To avoid excessive pressure being exerted on the embryos, coverslips were supported at each corner by thick droplets of a Vaseline-wax mixture. Embryos were examined first using a Multiphoton Excitation Microscopy (MPEM) system combined with a Confocal Laser Scanning Microscope (CLSM; Bio-Rad Radiance 2100 MP) mounted on a Nikon TE300 inverted microscope. Imaging was performed using a 488-Argon-ion laser and a 543-HeliumNeon laser to simultaneously excite fluorescein and Alexa Fluor™ 568 (Experiment 1) or Alexa Fluor™ 568 and Alexa Fluor™ 488 (Experiment 2), respectively. DAPI staining was imaged by using a 100 fs pulsed 780 nm excitation laser source (a mode-locked Titanium:Saphire laser: Tsunami, Spectra Physics, Mountain View, CA, USA). To avoid cross talk of the images in the photomultiplier channels, specimens were analysed using a sequential scanning mode. Images were recorded digitally and processed using Adobe®Photoshop 5.5 software (Adobe Systems Inc., Mountain View, CA, USA). After MPLSM, mounted embryos were flattened, by pressing on the coverslip, to enable easier counting of total nucleus numbers using a conventional immunofluorescence microscope equipped with an eyepiece counting grid.

The method for calculating apoptosis rates was adapted from Spanos *et a*l. (2000). Stained nuclei were categorized as follows: 1) Compact DAPI – interphase nuclei with a distinct round outline, uniform DAPI-staining and no TUNEL labelling (Fig. 2A). 2) Mitotic DAPI - nuclei in prophase, metaphase or anaphase with no TUNEL labelling. 3) Compact TUNEL - nuclei with strong DAPI and uniform TUNEL labelling. 4) Fragmented nuclei with TUNEL labelling. 5) Fragmented nuclei without TUNEL labelling. For each embryo, all nuclei were counted and

categorized in one of these 5 groups. The cells deemed to be apoptotic were all those with TUNEL-labelled nuclei plus those with fragmented nuclei but no TUNEL labelling. The following indices were then calculated:
a) apoptotic index = apoptotic nuclei / total nuclei x 100.
b) fragmented nucleus index = fragmented nuclei / total nuclei x 100
c) mitotic index = nuclei in mitosis / total nuclei x 100.

Statistical Analysis
All culture experiments included 5-7 replicates and statistical analyses were performed using GraphPad Prism® (San Diego, CA, USA). Cleavage and *in vitro* embryo development rates were compared using Fishers' exact contingency test. The effect of culture conditions on total cell numbers and on the apoptotic, fragmentation and mitotic indices was examined using either unpaired Student's tests or a one–way ANOVA followed by pair-wise multiple comparisons (Bonferroni *t*-test), after testing for normality (Kolmogorov-Smirnov test) and equivalence of variances (Levene Median test with Barletts test correction). Differences were considered statistically significant if $P<0.05$.

RESULTS

In vitro embryo development

A total of 666 oocytes displaying a first polar body after 24-26 h IVM were subjected to ICSI. Of these, 349 and 317 were derived from COCs categorized as respectively compact or expanded at the onset of IVM. There was, however, no effect of cumulus morphology at the onset of IVM on the cleavage rate after ICSI (61.3% versus 63.4%, Comp and Exp-COCs, respectively: Table 2). On the other hand, there was a significant effect of culture system on embryo development rate since zygotes from compact COCs incubated in the sheep-oviduct system yielding a significantly higher blastocyst rate (16% of injected and 23.7% of cleaved oocytes) than SOF-IVC with either compact (6.3% and 12.6%) or expanded (9.4% and 15%) COCs (Table 2).

Morphology and cellular characteristics of Day 7 Embryos (Experiment 1)

Of the 69 Day 7 embryos produced by ICSI and either culture in SOF (n = 41) or temporary transfer to the sheep oviduct (n = 28), 47 were analyzed with respect to cell number, apoptosis and microfilament organization. These were compared with 10 *in vivo* Day 7 embryos.

Table 2: Cleavage and development of horse embryos produced by ICSI of *in vitro* matured oocytes which had a compact or expanded cumulus at the onset of IVM, and subsequent culture for 7 days in SOF medium or the oviduct of a progesterone treated ewe.

Culture	No. oocytes	No. cleaved	Cleavage rate	No Day 7 embryos	Embryo/ oocytes (%)	Embryo/ cleaved (%)
SOF-IVC (Comp-COCs)	349	214	61.3 %	11	6.3 ± 1.9[a]	12.6 ± 4[c]
Sheep oviduct (Comp-COCs)				28	16 ± 3.3[b*]	23.72 ± 5.9[d*]
SOF-IVC (Exp-COCs)	317	201	63.4 %	30	9.4 ± 1.3[a]	15 ± 1.5[c,d]

Within a column, values with a different superscript differed significantly ([a,b] $P< 0.01$; [c,d] $P< 0.05$). * The embryo rate was adjusted to account for the number of cleaved embryos actually transferred into sheep oviducts (n=118).

Embryo diameter and total cell number

Day 7 *in vivo* embryos were significantly bigger than IVP embryos (mean ± sem embryo diameters: 374 ± 64.1, 139.9 ± 1.6 and 138.1 ± 2.5 mm for *in vivo*, SOF and sheep oviduct embryos, respectively: Table 3). Not surprisingly, *in vivo* embryos also contained significantly more cells (1736 ± 568: range 176-5720) than SOF (116 ± 15: range 20-363) or sheep oviduct (86 ± 9: range 31-141) produced embryos. With regard to the IVP embryos, COC morphology at the onset of culture did not affect eventual blastocyst diameter but did significantly influence day 7 cell number which was significantly higher for exp-COCs (133 ± 16) than for comp-COCs subsequently cultured via either the SOF or sheep oviduct systems (83 ± 31 and 86 ± 9, respectively: Table 3).

Apoptosis and nuclear morphology

Apoptosis occurred at a very low rate in *in* vivo embryos, with only 4 of the 10 embryos containing apoptotic cells and only 0.5% of all nuclei being classified as apoptotic. By contrast, all IVP embryos contained apoptotic nuclei and apoptosis rates were much higher. For this reason, the apoptotic, mitotic and fragmentation

indices were compared only between the different groups of IVP embryos. In this respect, the proportion of apoptotic cells was significantly higher in embryos produced from Comp-COCs and SOF (21.5 ± 6.4%: Fig. 2) than Exp-COCs and SOF (9.6 ± 1.3%) or Comp-COCs and sheep oviduct transfer (6.5 ± 1.5%).

Table 3: Diameter and total cell number of Day 7 horse embryos produced *in vivo* or *ex vivo* following ICSI of oocytes derived from compact and expanded COCs and cultured in SOF (IVC), or from compact COCs and temporary transfer to the oviduct of a sheep.

Embryo production Method	No.	Diameter (μm)	Number of cells (mean ± SEM)
SOF-IVC (Comp-COCs)	11	140.3 ± 3.2^{a}	82.91 ± 30.7^{c}
SOF-IVC (Exp-COCs)	21	139.7 ± 1.7^{a}	132.9 ± 15.9^{d}
Sheep oviduct (Comp-COCs)	15	138.1 ± 2.5^{a}	85.8 ± 9.3^{c}
In vivo	10	374 ± 64.1^{b}	1736 ± 567.9^{e}

Within a column, values that differ significantly have different superscripts (^{a,b} $P< 0.001$; ^{c,d,e} $P<0.05$).

A similar pattern was seen for the incidence of fragmented nuclei (13.3 ± 3.5% versus 4.9 ± 0.9% and 4.5 ± 1% for Comp-COCs-SOF, Exp-COCS-SOF and Comp-COCS-sheep oviduct, respectively), but there was no apparent effect of COC morphology or culture system on the mitotic rate. More detailed analysis of the different classes of apoptosis (condensed-TUNEL positive, fragmented-TUNEL positive or fragmented-TUNEL negative) demonstrated that the bulk of the between-IVP group difference was in the proportion of fragmented-TUNEL positive cells; indeed, the proportion of TUNEL negative apoptotic cells did not differ between groups (Fig. 3). When apoptosis rates were compared between IVP embryos of different quality, it was found that embryos considered transferable (grades I and II: 76% of all IVP embryos) had lower apoptosis rates (9% of cells)

than embryos not considered fit for transfer (grades III and IV: 22% apoptotic cells).

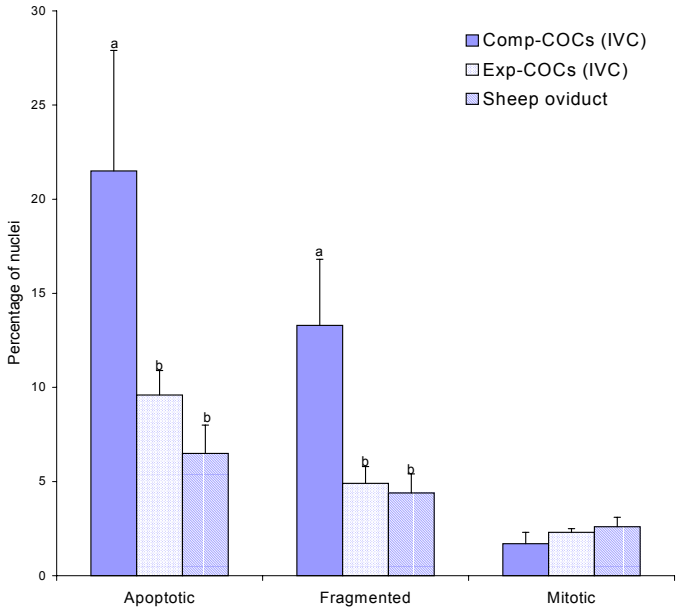

Fig.2. The effect of cumulus morphology at the onset of IVM (compact or expanded) and subsequent culture system (SOF medium or sheep oviduct) on the mean (+ SEM) percentages of cells showing evidence of apoptosis, nuclear fragmentation or cell division (mitosis) in Day 7 IVP horse embryos. Embryos derived from Comp-COCs and cultured in SOF had a significantly higher proportion of apoptotic and fragmented nuclei. [a,b] Within a group, different superscripts denote values that differ significantly ($P< 0.05$).

Microfilament organization

Representative MPLSM photomicrographs of Day 7 *in vivo* and *in vitro* embryos are shown in Fig. 4. IVP embryos were not only smaller and had fewer cells than their *in vivo* counterparts, they were more compact such that most were categorized as morulae (Fig. 4A, B) or early blastocysts (Fig. 4C, D), whereas *in vivo* day 7 embryos were mostly expanded blastocysts (Fig 4H, I). The distinction between inner cell mass (ICM) and trophoectoderm (TE) was thus more difficult to discern in day 7 IVP embryos, which did not have the clear nucleus-dense domain seen in *in vivo* embryos. Apoptotic cells were scattered and not concentrated in particular areas, but were seen in higher numbers in poor quality IVP embryos (Fig 4E, F).

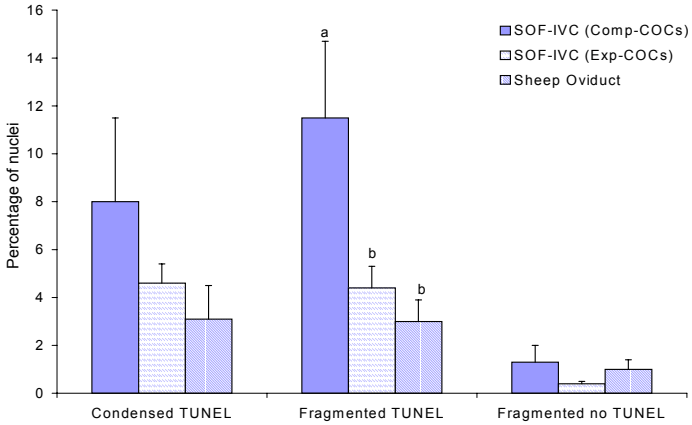

Fig. 3. The effect of cumulus morphology at the onset of IVM (compact or expanded) and culture system (SOF or sheep oviduct) on the rate of different categories of apoptosis in Day 7 horse embryos (mean ± SEM). Day 7 embryos derived from Comp-COCs and cultured in SOF had a significantly higher proportion of fragmented TUNEL-positive nuclei. (^{a,b} $P< 0.05$).

Microfilaments (MF) were primarily located along the inside of the cell plasma membranes, adjacent to the cell borders. In *in vivo* blastocysts, MF labeling of the contiguous cell borders was intense and homogeneous throughout the whole embryo. By contrast, in IVP embryos MF labeling was generally less intense and tended to be more marked around the periphery of the embryo (Fig. 4A, D) and weaker within, with some areas almost lacking labeling (Fig. 4G). On some occasions, the actin labeling in IVP embryos appeared to have conglomerated at the junctions of several cells (Fig 4E). The blastocoele cavity of IVP blastocysts tended to be small and irregular with its borders poorly stained for MF (Fig. 4C), especially when compared to the large, well delineated cavity of *in vivo* blastocysts (Fig. 4H).

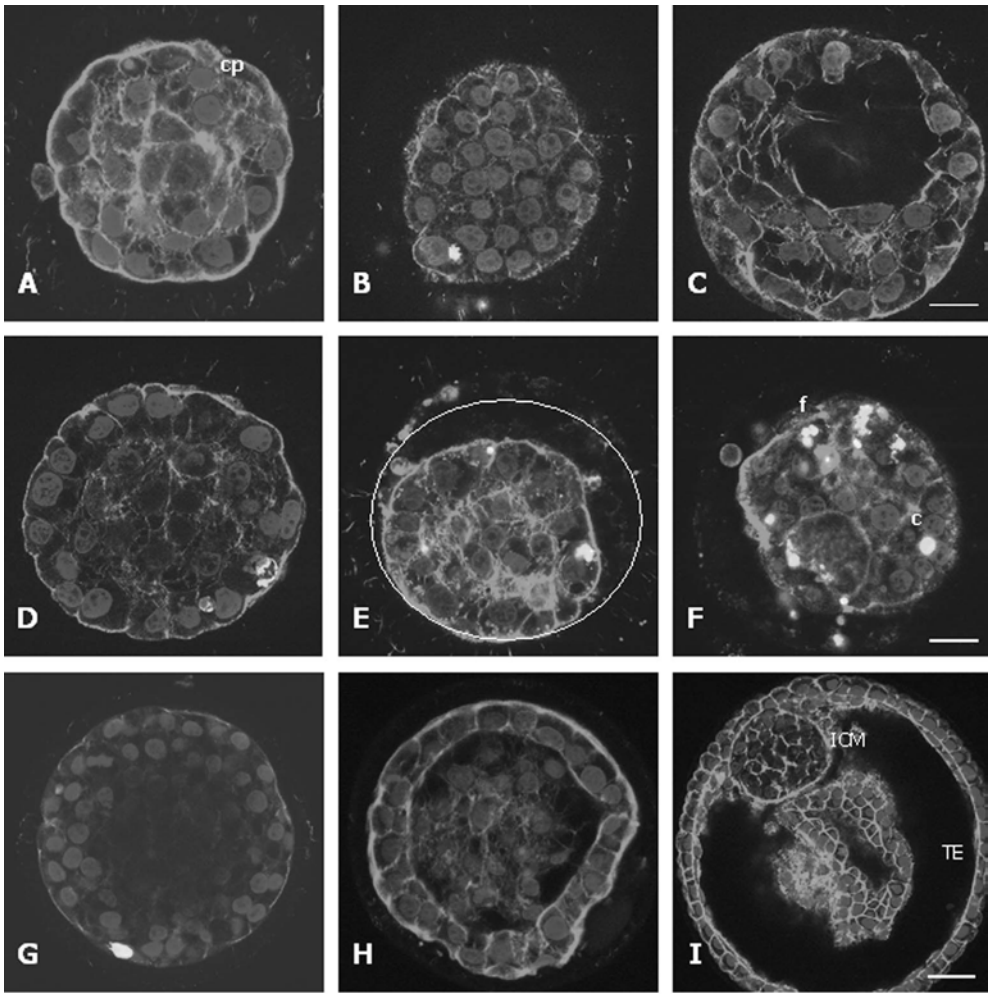

Fig 4: Confocal laser scanning photomicrographs to demonstrate the features of, and differences between, morphology and microfilament distribution in *in vivo* and IVP horse embryos. In all cases, nuclei are coloured blue, microfilaments red and TUNEL labelled nuclei green (these nuclei contain DNA breaks and are considered to be apoptotic).
IVP embryos had relatively few cells and were relatively compact, such that most were categorized as morulae (Fig. 4A, B) or early blastocysts (Fig. 4C, D). Day 7 *in vivo* embryos were mostly expanded blastocysts (Fig 4H, I) with a clear distinction between trophectoderm (TE) and inner cell mass (ICM). Apoptotic cells tended to be scattered but more numerous in poor quality IVP embryos (Fig 4E, F: f denotes a fragmented nucleus). Microfilaments (MF) lined the inside of the cell plasma membranes and were intense and sharp in *in vivo* embryos. In IVP embryos, MF labeling was

generally less intense but tended to be stronger around the periphery of the embryo (Fig. 4A, D) and weaker to almost absent within (Fig. 4G). On some occasions, the actin filaments were clumped at the junction of several cells (Fig 4E). The blastocoele cavity of IVP blastocysts tended to be small and much less clearly delineated by a MF border (Fig. 4C) than that of *in vivo* blastocysts (Fig. 4H).
Scale: In A-G bar = 30 µm, in H bar = 40 µm and in I bar = 120 µm.
Cp = compact DAPI-labeled nucleus; f and c = fragmented and compact TUNEL-labeled nuclei, respectively

Capsule glycoprotein expression (Experiment 2)

In total, twenty day 10 IVP embryos were stained with mAb OC-1 (Table 4) to examine capsule formation. These embryos had been produced by 5 day culture in sheep oviducts followed by 3 days either in a cell-free system (n=10) or with a monolayer of adult horse fibroblast cells (n=10). All day 10 IVP embryos were classified as partially hatched, since part of the embryo had herniated via the hole made during ICSI. In these embryos, a distinct layer of OC-1 positive 'capsule' was visible lining the inside of the ZP (Fig. 5A, B) and extending into the transzonal channels of the latter (Fig. 5E). When the ZP was separated from its contained embryo by micromanipulation, the capsular material remained stuck to the ZP and not to the trophectoderm (Fig 5F) demonstrating that it was more intimately associated to the former. In the area of embryo herniated from the ZP, capsular glycoprotein was present on the apical surface of the trophectoderm cells (Fig. 5A, B) as scattered small patches that were not assembled into a confluent layer (Fig. 5 I, H). Day 7 IVP embryos (3 from SOF and 1 from sheep-oviduct cultures) showed only weak OC-1 labeling in scattered patches on the apical surface of trophectoderm cells, again without assembly into a confluent capsule and, in these cases, with little infiltration into the substance of the ZP (Fig 5. C, D). By contrast, in two day 6 *in vivo* embryos examined (categorized as late morulae) a clear thick confluent capsule was sandwiched between the relatively thick ZP and the trophectoderm surface and there was no infiltration into the transzonal channels of the ZP (Fig. 5J). Older *in vivo* hatched and expanded blastocysts, had a complete capsule apposed tightly to the trophectodermal surface and these capsules displayed the classical bilaminar appearance (Fig. 5K) described previously by Oriol *et al*. (1993).
Because OC-1 showed such strong affinity for the ZP of IVP embryos, a number of *in* vitro matured COCs (n=4) were also stained with OC-1, to ensure that there was no cross-reaction with ZP after *in vitro* culture. In these cases, there was no OC-1 staining of any part of the ZP (Fig. 5L), demonstrating clearly that staining of the ZP in IVP embryos was a function of embryonic secretion of OC-1 reactive glycoproteins.

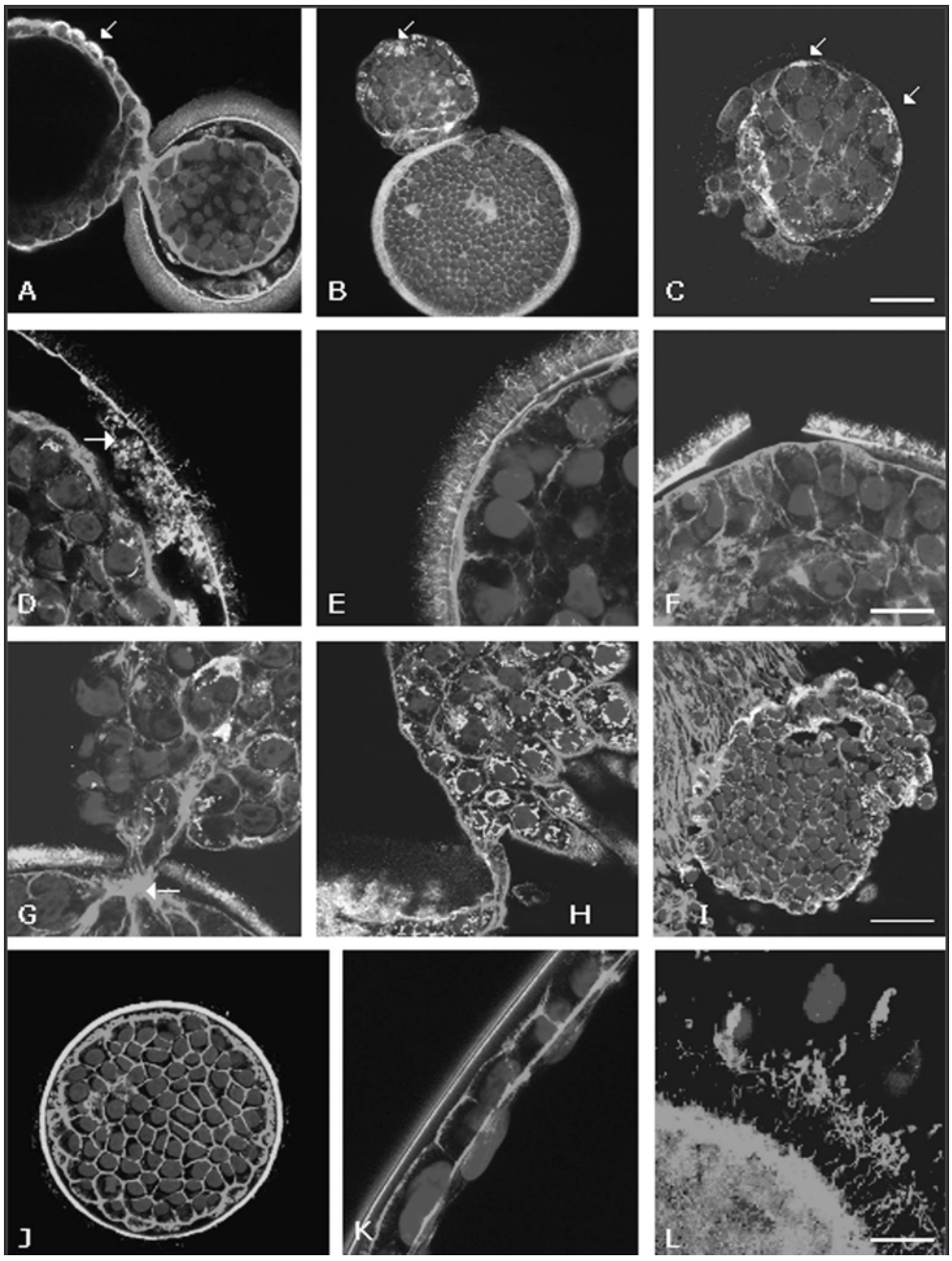
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Fig 5: Confocal laser scanning micrographs to show the distibution of OC-1 reactive molecules (capsular glycoproteins) in IVM oocytes, and IVP and *in vivo* embryos. In all cases, nuclei are labeled blue, microfilaments red and capsular glycoproteins green.

A. Capsule material lining the inside of and permeating through the ZP of a day 10 IVP embryo. OC-1 staining is also seen (arrow) on the outer surface of the trophectoderm cells that have herniated out of the ZP via the hole made during ICSI.
B. Day 10 IVP embryo with strong OC-1 staining on the herniated ZP-free trophectoderm cells. Cell density in the ZP enclosed portion of the embryo is much higher than in the 'unrestricted' herniated area.
C. Capsular material on the trophectodermal surface of a day 7 IVP embryo derived from sheep-oviduct culture.
D. Accumulation of capsule material beneath the ZP of a day 7 IVP embryo derived from *in vitro* culture..
E. Capsular material permeating through the transzonal channels of a day 10 IVP embryo.
F. Separation of the ZP from the trophectoderm by micromanipulation demonstrated that the 'pseudocapsule' was adhered to the ZP rather than the trophectoderm.
G. Day 10 IVP embryo with a dense actin 'neck' between the zona-contained and herniated parts of the embryo.
H. Herniated part of a Day 10 IVP embryo with considerable quantities of capsular material on the surface but without coalescence into a confluent layer.
I. A day 10 IVP embryo that, during culture with a fibroblast monolayer, attached to the surface of the well. It is presumed that failure of OC-1 reactive glycoproteins to coalesce prevented them from exerting the anti-adhesive properties that have been ascribed to the capsule.
J. Day 6.5 *in vivo* early blastocyst with a distinct well-defined capsule and a very clear microfilament cyoskeleton delineating individual cells.
K. High magnification image of the capsule and trophectoderm of a day 7 *in vivo* blastocyst to show the bilaminar appearance of the capsule after staining with mAb OC-1.
L. Cumulus oocyte complex stained after IVM to demonstrate that mAb OC-1 did not cross react with zona pellucida.

Scale: In A-C, I and K bar = 50 μm and in D-G, J and L bar = 10 μm

TABLE 4. Immunolocalization of capsular material on early *in vivo* and IVP horse embryos.

Origin	Stage	No.	Size (µm)	No. of nuclei	Site of mAb OC-1 staining
In Vivo	Day 6	2	200; 290	364; 979	Thin capsule sandwiched between ZP and trophectoderm
	Day 7	1	730	3939	Distinct capsule enveloping the conceptus
	Day 8	1	940	10.400	
	Day 9	1	1640	44.500	
In Vitro	Day 10 (DMEM:M199)	10	*165 ± 9.0 / 136.5 ± 5.0[a]	531.± 45.8[b]	Accumulation on the inside of the ZP and infiltration into the transzonal channels. Scattered labelling of trophectoderm cells of the "herniated" areas
	Day 10 (cell-coculture)	10	*148 ± 2.9 / 122 ± 29.1[a]	438.6 ± 61.1[b]	
	Day 7 (SOF-IVC)	3	137 ± 1.2	112 ± 61.7	Small patches of labelling on the surface of trophectoderm cells
	Day 7 (sheep oviduct)	1	140	53	
COCs	IVM Comp-COCs	2	130 ± 4.5	Metaphase II stage	No labelling
	IVM Exp-COCs	2			

mAb OC-1: monoclonal antibody raised against the capsule of day 13-15 horse conceptuses (Oriol *et al.*, 1993)
* Diameters of the embryonic portions within/herniated from the ZP.
[a,b] Within a column, values which did not differ significantly have the same superscripts (P>0.001).

DISCUSSION

This study demonstrated that in vitro produced horse embryos differ markedly from their in vivo produced counterparts by having fewer cells, lower morphological quality scores and a higher incidence of apoptotic cells (Table 3). These observations are similar to those for IVP bovine embryos which are consistently of lower quality than embryos produced in vivo, both in terms of morphological quality (Fair *et al*., 2001) and with regard to the expression of developmentally important genes (Rizos *et al*., 2002). In cattle, these differences have been shown to affect embryo survival following cryopreservation adversely and to compromise embryonic development (Rizos *et al*., 2001). On the other hand, because only a few foals have so far been produced from IVP embryos (Galli *et al*., 2001a, 2002; Li *et al*., 2001), it is too early to determine if and to what degree in vitro production adversely affects embryo development in this species. It is, however, clear that despite the apparently poor quality and low cell numbers of IVP equine embryos, they are able to establish pregnancies following transfer to recipient mares (Galli *et al*, 2001a; Li *et al*., 2001). With regard to embryo cell number, Allen and Pashen (1984) similarly demonstrated that embryos produced by the mechanical separation of blastomeres from 2-to-8 cell horse embryos and insertion into evacuated pig zona pellucidae were able to establish pregnancies despite low cell numbers. Together, these observations suggest that low cell number can be rapidly compensated for after transfer of embryos into the uteri of recipient mares.

As in previous studies (Galli and Lazzari, 2001; Galli *et al*., 2002), embryo development was more efficient when zygotes were transferred temporarily to the oviduct of progesterone treated sheep (23% blastocysts) than when they were cultured solely *in vitro* (14%). However, the number of cells in day 7 IVP embryos derived from compact COCs and either cultured in SOF medium or transferred to a sheep's oviduct did not differ. By contrast, cell numbers were higher in day 7 IVP embryos originating from expanded COCs and cultured in SOF medium. The effects of this relatively rapid increase in cell number *in vitro,* of embryos derived from expanded as compared to compact COCs, on later embryo development is however difficult to predict. At first glance, a higher cell number would appear to be positive sign of embryo viability. On the other hand, in other species, relatively rapid development *in vitro* has proven detrimental to the resulting offspring (van Wagtendonk-de Leeuw *et al*., 2000). That the rate of apoptosis (cells with DNA strand breaks and/or nuclear fragmentation) was significantly higher in SOF than sheep oviduct embryos (22 versus 7%), and much higher in both than *in vivo* produced embryos suggests a detrimental effect of culture on embryo development. Similarly increased rates of apoptosis have been observed in cattle (Byrne *et al*.,

1999) and pig (Kidson *et al.*, 2002) embryos produced *in vitro* and it has been further suggested that sub-optimal culture conditions may cause the proportion of affected cells to increase to a level where further embryonic development is critically compromised, particularly in embryos with low cell numbers (Brison and Schultz, 1997; Jurisicova *et al*., 1998). On the other hand, apoptosis has also been proposed to be a physiological process during mammalian preimplantation embryogenesis, presumably acting as a quality control mechanism to eliminate aberrant cells (Jurisicova *et al*., 1995; Meier *et al*., 2000). In either case, the marked difference in the proportion of apoptotic cells between *in vivo* and IVP horse embryos, suggests that culture adversely affected embryo development.

IVP embryos were produced at similar rates from oocytes that had a compact or an expanded cumulus at the onset of IVM, although the overall percentage of injected oocytes developing into blastocyts was low (mean=14%). Previous studies have suggested a greater ability of oocytes with an expanded cumulus at recovery to form a male pronucleus after ICSI (Dell'Aquilla *et al*., 1997, 2001), although no differences in nuclear maturation rates were observed. One possible explanation for this finding was that COCs with an expanded cumulus had better cytoplasmic maturation and were thereby better able to support embryo development. Although the current study did not show any significant difference in cleavage rates between Exp- and Comp-COC oocytes, embryos resulting from SOF culture after ICSI of Exp-COC oocytes had more cells and a lower incidence of apoptosis than those from Comp-COCs. Interestingly, the major difference in the proportion of apoptotic cells was a higher level of cells with fragmented nuclei in Comp-COC derived embryos. Apoptosis is considered to progress from condensation of chromatin and cytoplasm to DNA fragmentation accompanied by indentation and convolution of the nuclear membrane. TUNEL labels the DNA breaks *in situ* and thus identifies cells fairly early in the apoptotic process (Gavrieli *et al*., 1992) whereas fragmentation of the convoluted nucleus with blebbing and fragmentation of the whole cell into membrane bounds apoptotic bodies occurs much later in the apoptotic process (for review see Wyllie *et al*., 1980). The raised incidence of nuclear fragmentation in Comp-COC derived embryos is therefore indicative of more advanced apoptotic changes which could relate to a higher sensitivity to culture conditions and/or presence of more serious internal defects in these embryos (Jurisicova *et al*., 1995). However, the quality (based on apoptotic index) of embryos derived from Comp-COCs was significantly improved when zygotes were transferred to and cultured in sheep oviducts, thereby identifying sensitivity to the culture conditions as the most likely critical factor. Overall, this data suggests that current IVM protocols are still sub-optimal with respect to the acquisition of

developmental competence by equine oocytes, especially those less advanced oocytes with a compact cumulus at the time of recovery.

Staining of the microfilament cytoskeleton helped to identify differences in cellular organization between *in vivo* and *in vitro* embryos. *In vivo* day 7 embryos were expanded blastocysts in which the microfilaments delineated the cell borders and highlighted the division between a distinct ICM and the TE layer (Fig 4F). By contrast, IVP embryos were compact without, or with only a small, blastocoele cavity and patchy microfilament distribution. Actin microfilaments play an important role in cell cleavage during embryo development (Maro *et al*., 1986) and are essential for the distribution of mRNA and organelles such as mitochondria and the Golgi apparatus between daughter cells (Bassell *et al*., 1994; Volderrama *et al*., 1998). The polymerization and depolymerization of actin filaments are, however, dynamic processes susceptible to disruption by environmental conditions (temperature, pH, culture medium; Barnett *et al*., 1997). Moreover, microfilament disruption can adversely affect the structural integrity of cells, with serious consequences for their metabolic activity. Abnormal actin filament distribution has been suggested as a reason for abnormal embryo cleavage in IVP pig embryos (Wang *et al*., 1999) because embryo division can be similarly blocked by cytochalasin D, an inhibitor of microfilament polymerization. In general, cells store a large pool of nonfilamentous actin (G-actin; Wang et al., 2000) to maintain their ability to quickly reorganize filamentous actin in response to environmental changes or need. Whether the pool of G-actin is low or impaired in IVP horse embryos, thereby decreasing their potential to overcome suboptimal culture conditions, has yet to be investigated.

The combination of monoclonal antibody OC-1 and confocal microscopy enabled three-dimensional imaging of capsule glycoprotein distribution in early embryos. In turn, this enabled us to demonstrate that the capsule assumes its classical bilaminar appearance around *in vivo* embryos from very soon after its initial formation (Fig. 5K); previously OC-1 expression and appearance of the capsule after OC-1 staining had not been reported for embryos recovered earlier than day 11 of gestation (Oriol *el al*., 1993a). Production of capsular glycoproteins by the trophoblast cells of IVP embryos was also demonstrated by OC-1 labeling of the apical surface of these cells, thereby confirming the hypotheses that the initial layer of capsule is formed from OC-1 reactive glycoproteins and that early trophoblast cells secrete capsular glycoproteins independent of a maternal (endometrial) input (Albihn *et al*., 2003). Intriguinly, *in vitro* the capsular glycoproteins failed to assemble into a normal and complete capsule enveloping the embryo, suggesting either that some aspect of the uterine environment is necessary for glycoprotein

coalesence or that some aspect of the *in* vitro environment otherwise hindered capsule formation. In the former respect, because the glycoproteins of the capsule are mucin-like, Oriol *et al*. (1993a) postulated that they, like other mucins, may coalesce to form a gel-like mucus layer by hydration and cross-linking (Verdugo, 1991) after they have accumulated in sufficient quantities on the surface of the trophectoderm. Oriol *et al*. (1993a) further suggested that failure of capsule formation *in vitro* might thus be due to dispersal of the glycoproteins into the culture medium, or unsuitability of the microenvironment for hydration and cross-linking. The current study suggests that the former is a minor problem since capsule glycoproteins were found on the surface of the trophoblast and lining and permeating through the ZP. Nevertheless, it cannot be discounted that a degree of dispersal prevented glycoprotein concentrations reaching a critical concentration needed to initiate coalescence. In this latter respect, it is also likely that the absolute production of capsular glycoproteins by IVP embryos was relatively low, due primarily to their low numbers of cells. There are, however, comparable reports of reduced secretion of other high molecular weight mucins in culture such that they fail to assemble into a mucus layer (Virmani *et al*., 1992). One interesting observation with regard to the proposed functions of the capsule, was that the herniated part (ZP-free) of one of the embryos cultured with an AFC monolayer adhered to the cell layer, despite the presence of capsular glycoproteins on the surface of the adhered cells (Fig 5I). The capsule has been proposed to confer anti-adhesion properties, by means of its high content of negatively charged sialic acid residues, on the mobile pre-day 17 conceptus (Oriol *et al*., 1993b; Chu *et al*., 1997). The adhesion of glycoprotein secreting trophectoderm cells to the monolayer suggests that the anti-adhesive property is acquired only after coalescence into a complete layer.

In the present study, considerable quantities of capsule material were detected as an accumulation between the trophoblast and the ZP of IVP embryos (Fig. 5D). This was reminiscent of the flocculent material detected between the trophoblast and ZP of *in vivo* embryos by Flood *et al.*(1982) and Wilson *et al.*(1986) which they postulated, but could not prove, to be capsule precursor material. An striking feature of IVP embryos was the level to which the ZP was lined and infiltrated with capsular glycoproteins, something not seen in *in vivo* embryos. The possibility that mAbOC-1 was cross-reacting with the ZP *per se* was ruled out by the failure to label the ZP of either *in vivo* embryos or IVM oocytes (Fig. 5L). Instead it appears that during culture *in vitro,* capsule material adheres to the inside of the ZP and permeates through the cumulus cell-created transzonal channels, presumably as a consequence of the failure of the glycoproteins to coalesce on the trophectodermal surface. By contrast, *in vivo* capsule formation does not require the presence of a

ZP since zona-free bisected blastocysts have been shown to form a capsule after transfer to the uterus of recipient mares (McKinnon *et al*., 1989). This again indicates that the greatest obstacle to capsule formation *in vitro* is probably that the microenvironment fails to replicate the conditions necessary for glycoprotein cross-linking. In day 10 IVP embryos, a 'pseudo'-capsule formed lining the inside of the ZP (Fig. 5E) that was not seen around ZP-free areas of embryo (Fig. 5H). This capsule was tightly adhered to the ZP, as demonstrated during removal of the latter by micromanipulation (Fig. 5F); the capsule peeled away with the ZP

In the current study, in vitro cultured embryos escaped from their ZP by herniating through a hole in the ZP (in this case the hole made during ICSI), as previously reported by Hinrichs *et al*. (1990) and Hochi *et al*. (1993). In other species, the exact mechanism of hatching from the ZP is also unclear, although it is thought that, in vivo, proteolytic enzymes released by the maternal endometrium are most likely to be responsible for ZP dissolution dissolution (O'Sullivan *et al.*, 2002). The current observations demonstrate that loss of the horse ZP in vitro is very different to the apparently rapid loss in vivo (intermediate stages are rarely found), in which the ZP is though to be attenuated and ruptured by a combination of a uterine zonalytic and blastocyst expansion (Betteridge *et al*., 1980; Flood *et al*., 1982). Although the uterus seems thus to critically influence the mode of zona loss from horse embryos, the existence of a uterine zonalytic has yet to be demonstrated in this species (NB there appears to be no uterine zonalytic in cattle; Betteridge *et al*, 1980). It is therefore tempting to speculate that the essential roles of the uterus during physiological zona loss and capsule formation may be linked. Finally, it has been proposed that the capsule is essential to the survival of horse embryos in vivo because embryos transferred after capsule removal either do not develop into pregnancies (Stout *et al*., 1997) or do so only after forming a new capsule (McKinnon *et al*., 1989). If IVP embryos do not form a normal capsule during culture then it is likely that transfer to the uterus of the recipient mare at a stage when capsule formation can still occur will be essential to the success of this procedure. Since the transfer of day 7 IVP late morulae or early blastocysts has resulted in pregnancy in mares, it must be assumed that subsequent capsule coalesence can and does occur.

In summary, the present study represents the first detailed description and comparison of the morphological, cytoskeletal and developmental characteristics of *in vitro* and *in vivo* produced horse embryos. Day 7 IVP embryos were smaller, had fewer cells and were more compact than *in vivo* embryos of similar age. In addition, Day 7 IVP embryos had a small or nonexistent blastocoele cavity, an indistinct inner cell mass and had still not properly (or normally) hatched after 10

days of culture. IVP embryos also displayed high percentages of apoptotic cells (10% compared to 0.3% for *in vivo* embryos), a disturbed pattern of microfilament distribution and irregularities in cell size and shape. Finally, while IVP embryos remained viable, continued to develop for at least 10 days *in vitro* and secreted capsular glycoproteins, the latter failed to coalesce to form a confluent capsule, a structure that is a prominent and apparently vital feature of *in vivo* blastulation.

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Chapter 7

Effect of the number of passages of fetal and adult fibroblasts on nuclear remodelling and first embryonic division in reconstructed horse oocytes after nuclear transfer

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SUMMARY

The effects of repeated passage *in vitro* of fetal fibroblast cells (FFC) and adult fibroblast cells (AFC) on nuclear remodelling and first embryonic division when used to reconstruct horse oocytes, and the reasons for the developmental block in progression to the two-cell stage, were investigated. A total of 463 metaphase II oocytes produced 427 fibroblast-cytoplasm couplets after nuclear transfer, which finally resulted in 319 reconstructed oocytes. With increasing numbers of passage, the rates of nuclear remodelling decreased in both types of donor cells; about half of the fused donor cell nuclei showed the S-G2-prometaphase stages of the first embryonic division 18-20 h after cell fusion treatment irrespective of the number of donor cells passages (FFC: 49%; AFC: 53%). The rates of first embryonic division in the reconstructed oocytes fell with increasing age of the donor cells (FFC: 32% - 26% - 23%; AFC: 27% - 23% - 24%) and these rates were significantly lower than those obtained from metaphase II oocytes activated parthenogenetically (79%, $P<0.05$). Microscopic analysis of the organization of the first embryonic division in the developmentally blocked oocytes reconstructed with either FFC and AFC showed that most of these (FFC: 78%; AFC: 92%) could not form the mitotic spindle and the metaphase plate of chromosomes. These findings indicate that either fetal or adult fibroblasts that have undergone relatively few passages *in vitro* are most suitable as donors. However, both these cells have lower potential to restart first embryonic development after nuclear transfer than do the equivalent cells in other species. Improvement in the rate of donor cell nuclear progression from S-G2-prometaphase to beyond the metaphase stage, and the normal organization of first embryonic division in reconstructed horse oocytes, might be the key to the production of cloned embryos in this species.

INTRODUCTION

Many factors influence the production of cloned animals when using the technique of nuclear transfer. One is the remodelling of the donor cell nucleus within the cytoplasm of the recipient to organize the first embryonic division. Usually, greater extents of donor cell nuclear remodelling and embryonic development can be achieved when transferring embryonic rather than somatic cell nuclei into the cytoplasm of metaphase II oocytes, although this general rule does not hold true for all species (Campbell *et al*., 1996a; Kato *et al*., 2000; Westhusin *et al*., 2001). The number of donor cell passages is another significant factor in cloning by nuclear transfer. Most reports of successful cloning in domestic animals have used cells of limited passage (3-9) as sources of donor nuclei (Wilmut *et al*., 1997; Kato *et al*.,

1998; Wells *et al*., 1999; Hill *et al*., 2000; Reggio *et al*., 2001). Roh *et al*. (2000) reported that nuclei from both early passage (8-16) and late passage (17-32) donor cells were capable of supporting *in vitro* development after nuclear transfer in cattle, although the rate of blastocyst formation was lower when using the late passage cells. Studies of nuclear transfer in the horse are presently in their infancy (Hinrichs *et al*., 2000; Li *et al*., 2000a, 2001a; Reggio *et al*., 2000) and it is necessary to investigate the differences that both the type and the rate of *in vitro* passage of the donor cells make on the whole process.

The present study used both fetal and adult fibroblasts as donor cells for nuclear transfer. The effect of the number of cell passage on nuclear remodelling, and the potential of the reconstructed oocytes to progress to the first embryonic division were investigated, together with attempts to trace the developmental block in progression to the two-cell stage.

MATERIALS AND METHODS

Culture media

TCM 199 (Gibco BRL, Grand Island, NY) was used as the basic medium for oocyte maturation and culture of the reconstructed oocytes after nuclear transfer. A monolayer of horse oviduct epithelial cells (OEC) was also used in co-culture with the oocytes during maturation and development. TCM199 was supplemented with 20% (v/v) heat-inactivated FBS (Gibco BRL), 10μg FSH ml^{-1} (Sigma Chemicals, St Louis, MO), 5μg LH ml^{-1} (Sigma) and lμg oestradiol ml^{-1} (Sigma) for oocyte maturation.
Culture drops (500μl) containing 20-30 cumulus-oocyte complexes (COCs) were made under mineral oil (Sigma) on the monolayer of OEC in a four-well Petri culture plate (Nunc, Roskilde). Groups of 5-10 reconstructed oocytes were cultured in 500μl drops of TCM medium on the monolayer of OEC in a four-well Petri culture plate, for development.

Oocyte collection and maturation culture

Horse ovaries were obtained from a commercial abattoir and transported to the laboratory within 20h in PBS containing 1μg gentamicin ml^{-1} (Gibco BRL) and maintained at a temperature of 10-20°C. COCs were recovered from the ovaries by scraping the walls of follicles of 0.5 -3.0 cm diameter. Groups of 20-30 COCs were matured *in vitro* by co-culturing them with OEC for 28-30 h at 38°C in an

atmosphere of 5% CO_2 in air (Li *et al*., 2000b, 2001b).

Preparation of donor cells

Fetal fibroblast cells (FFC) were derived from a 32-day-old Thoroughbred x Pony fetus and adult fibroblast cells (AFC) were obtained from subdermal biopsies recovered from a 4-yr-old Pony mare. Doubling of the population of cultured cells progressed to full confluence in four-well Petri culture plates at which time the cells were sub-passaged. After 2-3 sub-passages, cultured cells of both the FFC and AFC lines were frozen in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) containing 10% FBS and 10% dimethyl sulphoxide. The FFC and AFC were thawed and then cultured in DMEM with 10% FBS for totals of, respectively, 3-5, 6-10 and 11-15 passages. Analysis of the cell cycle by flow cytometry showed that the nuclei of 93.3% of FFC and 93.6% of AFC were at the G0-G1 phase of the cycle after starvation culture for 3-5 days in DMEM containing 0.5% (v/v) FBS.

Nuclear transfer

The nuclear transfer procedure (Li *et al*., 2002) was performed using a Transferman micromanipulator (Eppendorf, Hamburg) attached to an inverted microscope (Olympus IMT-2, Tokyo, Japan). AlI the manipulations were performed in a basic medium of Earle's balanced salt solution (EBSS, Gibco BRL) and 20% (v/v) FBS on a heated stage (CO 102; Linkam, Tadworth) that provided a working temperature of 30°C.

After 28-30 h maturation *in vitro*, metaphase II (MII) oocytes were selected after removal of their cumulus cells by treatment in 200 i.u. hyaluronidase ml^{-1} (Sigma) in EBSS-20% (v/v) FBS for 5 min followed by gentIe pipetting. The MII oocytes were placed for 10 min in EBSS-20% (v/v) FBS containing 5μg cytochalasin Bml^{-1} (CCB; Sigma) and 5μg Hoechst33342 ml^{-1} (Sigma) before they were enucleated. Oocytes were enucIeated in EBSS-20% (v/v) FBS with 5 μg ml^{-1} CCB and only those oocytes in which removal of both the polar body and the metaphase II nucleus was confirmed by observation under UV light for 3-5s were included in the study. For the donor cells, FFC or AFC were held in EBSS supplemented with 20% (v/v) FBS for1 or 2 h before a cell of 15-20 μm in diameter was selected and injected into the perivitelline space of the recipient enucleated oocyte, in combination with a small volume (two or three times the volume of the oocyte) of inactivated Sendai virus (VR-907, 1-3 x 103 U ml^{-1} ; LGC, Teddington).

Cell fusion and activation of the reconstructed oocytes

The fibroblast-cytoplasm couplets were aligned manually in 0.28 mmol mannitol fusion buffer l^{-1} in a 1.0mm fusion chamber and subjected to two DC pulses, each of 220-250 kv cm^{-1} for 30 μs and delivered by an ECM830 Electro Square Porator (BTX, San Diego, USA). Couplets that had fused successfully were then activated chemically by immersing them in PBS medium containing 5μmmol ionomycin l^{-1} for 5min, followed by culture for 4h in TCM199 medium containing 10μg ml^{-1} cycloheximide (Sigma). Metaphase II oocytes in the control group were induced to develop parthenogenetically using the same conditions applied to activate the reconstructed oocytes.

Nuclear analysis and development of the reconstructed oocytes to the two-cell stage

Groups of 5-10 reconstructed oocytes were co-cultured on the monolayer of OEC in 500μl drops of development medium at 38°C in an atmosphere of 5% CO_2 in air. Nuclear remodelling was considered to have occurred when the nucleus had enlarged to two to five times the size of that in the original donor ce11, or when the condensed chromatin had became organized in the chromosomes to prometaphase, metaphase and then to beyond metaphase stage of the first embryonic division. Nuclear remodelling was in 121 fused oocytes at 18-20 h after cell-fusion treatment by staining with 1 % (w/v) aceto-orcein (Li *et al*.2002). Development of the reconstructed oocytes to the two-cell stage was assessed after a further 10-12 h in culture, thus, 28-30 h after cell fusion and treatment with cycloheximide.

Analysis of chromatin, microtubules and microfilaments in the reconstructed oocytes at the first embryonic division block

Reconstructed oocytes from both FFC and AFC that had blocked at the first embryonic division 28-30 h after cell fusion were selected for assessment of cytoskeletal and chromatin structures (Tremoleda *et al*., 2001). Briefly, the oocytes were permeabilized by immersing them for 1h at 38°C in M medium (Simerly and Schatten, 1993), and then fixed for 30 min in 2.5% paraformaldehyde in PBS. The microtubules were then labelled, first by incubating the fixed oocytes for 90 min at 37°C in a 1:250 dilution of a monoclonal anti-α-tubulin antibody (Sigma), and then by incubating them for 1h in a blocking solution (Albertini *et al*., 1984). Next, the oocytes were exposed to a goat anti-mouse antobody for 1h at 37°C, conjugated to

tetramethylrhodamine isothiocyanate (TRITC) and diluted 1:250 in PBS containing 0.5% Triton X-100 and 0.5% BSA. The oocytes were then incubated for 1h in Alexa Fluor 488 phalloidin (15 i.u. ml^{-1}: Molecular Probes, Inc., Eugene, OR) to demonstrate the microfilaments. Finally, the oocytes were washed twice in PBS containing 0.1% BSA before they were incuabated in TO-PRO_3 (lμmol l^{-1} in PBS: Molecular Probes) for 15 min to enable visualization of the DNA. The stained oocytes were then mounted under a coverslip with antifade (Vectashield, Vector Lab. Burlingam, CA) and viewed under a confocal laser scanning microscope (Leica TCS MP, Heidelberg) that was mounted on an inverted microscope (Leica DM IRBE) equipped with krypto-Argon-HeNe ion lasers to excite fluoresence for microtubules (TRITC), microfilaments (Alexa Fluor 488) and DNA (TO-PRO_3), simultaneously. After treatment, the chromatin was highlighted in blue, the microtubules in red and the microfilaments in green.

Statistical analysis

Each experimental group comprised 3-5 replicates and the results were evaluated by chi-squared analysis. Differences between groups were considered statistically significant when $P<0.05$.

RESULTS

Nuclear remodelling of FFC and AFC

A total of 463 MII oocytes produced 427 fibroblast-cytoplasm couplets after nuclear transfer, which resulted in 319 reconstructed oocytes. The rates of nuclear remodelling 18-20 h after cell fusion when using FFC and AFC as donor cells for oocyte reconstruction are shown (Table 1). Representatives stages of the process are illustrated (Fig. 1). The rates of cell fusion between the recipient cytoplasm and either FFC and AFC were 73-82% and 68-73%, respectively. There were no significant differences when using cells that had been passaged 3-5, 6-10 or 11-15 times. Nor were there any differences between FFC and AFC.

Table 1. Influece of the number of donor cell passages on nuclear remodelling 18-20 h after cycloheximide culture when using fetal and adult fibroblasts to reconstruct enucleated horse oocytes

Donor cell		Total / Fused (%) of oocytes	Cell cycle of donor nuclear chromatin (% fused oocytes)		
Type	Passages		$G_0 - G_1$	S-G_2 /prometaphase	≥Metaphase
FFC	3-5	20 / 16 (80)	3 (19[a])	9 (56)	4 (25[a])
	6-10	28 / 23 (82)	6 (26[ab])	12 (52)	5 (21[a])
	11-15	30 / 22 (73)	8 (36[b])	9 (41)	5 (23[a])
	Total	78 / 61 (78)	17 (28)	30 (49)	14 (23)
AFC	3-5	32 / 22 (69)	4 (18[a])	12 (55)	6 (27[ab])
	6-10	26 / 19 (73)	5 (26[a])	8 (42)	6 (32[b])
	11-15	28 / 19 (68)	4 (21[a])	12 (63)	3 (16[a])
	Total	86 / 60 (70)	13 (22)	32 (53)	15 (25)

$G_0 - G_1$ = size was similar to the original (15-20μm) and showed condensed chromatin.
S-G_2/prometaphase = Nuclear size expanded to 40-60μm and the chromatin homogenized within a nuclear envelope.
≥ Metaphase = metaphase and beyond. Nuclear envelope broke down and the chromosomes arranged on the metaphase plate, or separating towards the two poles of the spindle.
FFC = fetal fibroblast cells
AFC = adult fibroblast cells
a,b Values with different superscripts are significantly different ($P<0.05$)

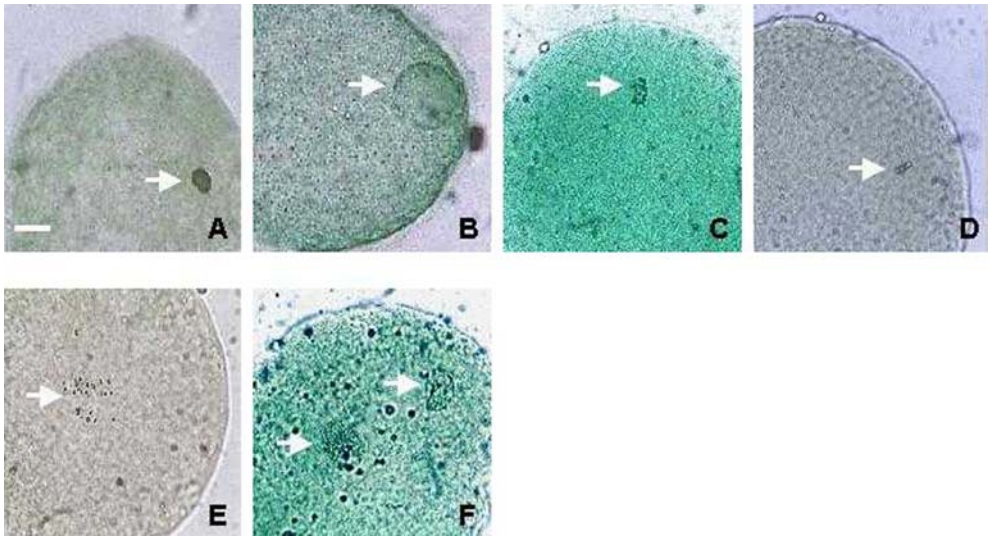

Fig. 1. Donor cell nuclear remodelling in reconstructed horse oocytes 18-20 h after cell fusion after nuclear transfer. (a) Original donor cell (G0-G1 stage); (b) swelling nucleus; (c) prometaphase stage; (d) metaphase stage; (e) anaphase stage (chromosomes separating); (f) two nuclei. Arrows indicate the position of the chromatin or nucleus. Scale bar represents 20 μm

When passaging the donor ceIIs, the number of nuclei that showed no change in G0-G1 configuration in the reconstructed oocytes after fusion with FFC increased significantly when using cells that had been passaged 11-15 times compared to those passaged 3-5 times (36% versus 19%, $P<0.05$). However, this was not the case for oocytes fused with AFC. With both types of cells, about half of the fused donor cell nuclei showed the S-G2-prometaphase stages of the first cell cycle (FFC: 41-56%; AFC: 42-63%), irrespective of the number of donor cell passages. On average, only 23% of the FFC and 25% of the AFC progressed to the metaphase stage (or beyond) of the first cell cycle after chemical activation treatment.

Cleavage of the reconstructed oocytes

After *in vitro* culture for 28-30h after cell fusion, the rates of first embryonic division in oocytes reconstructed with both FFC and AFC tended to decline with increasing number of passages of the donor cells (Table 2, FFC: 32% - 26% - 23%; AFC: 27% - 23% - 24%), although not significantly. Some abnormal two-cell

embryos showing a polynucleus, or a nucleus-free blastomere, or unequal cleavage with some fragments, were found after nuclear transfer using both FFC (8%) and AFC (8%). In the control group, 19 of 24 (79%) treated metaphase II oocytes developed parthenogenetically to the two-cell stage, a significantly higher proportion than that obtained with the reconstructed oocytes ($P<0.05$).

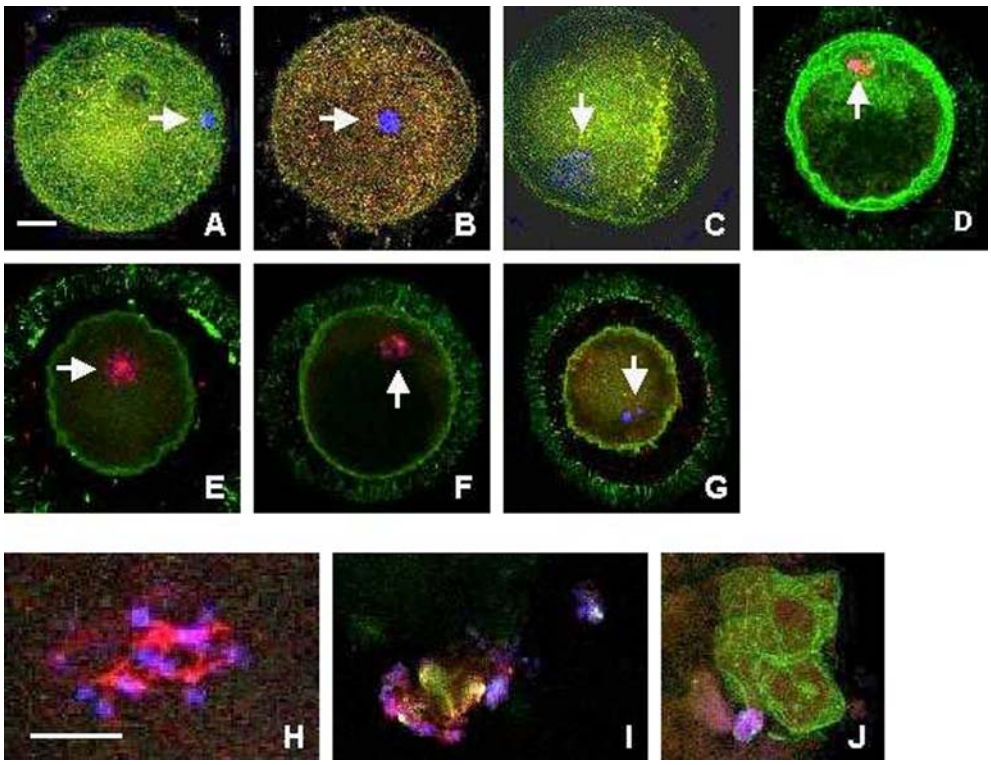

Fig. 2. Morphology of chromatin (blue), microtubules (red) and microfilaments (green) of developmentally blocked horse oocytes at first embryonic division after nuclear transfer. (a) Unchanged donor nucleus at the interphase stage; (b) swelling nucleus; (c) and (d) prometaphase stage; (e) metaphase stage; (f) and (g) anaphase stage (chromosomes separating); (h), (i) and (j): abnormalities of structure in the chromosomes and microtubular reorganization at the first embryonic division. Arrows indicate the position of the chromatin or microtubules. Scale bars represent 20 μm.

Table 2. Rates of first embryonic division 28-30 h after cell fusion in the nuclei of either fetal or adult fibroblast cells used to reconstruct enucleated horse oocytes

Donor cell		No.fused oocytes	No of 2-cell embryos (% fused oocytes) *		
Type	Passages		Total	Normal	Abnormal
Parthenogenetic control **		24	19 (79[a])	13 (54)	6 (25)
FFC	3-5	31	10(32[b])	7	3
	6-10	42	11 (26[b])	8	3
	11-15	30	7 (23[b])	5	2
	Total	103	28 (27)	20 (19)	8 (8)
AFC	3-5	36	10 (27[b])	7	3
	6-10	30	7 (23[b])	4	3
	11-15	29	7 (24[b])	5	22
	Total	95	24 (25)	16 (17)	8 (8)

FFC = fetal fibroblast cells.
AFC = adult fibroblast cells.
* 2-cell embyos were evaluated mophologically as normal or abnormal.
* * Metaphase II oocytes were induced to develop parthenogenetically using the same culture conditions employed when attempting to activate the reconstructed oocytes.
a,b Values with different superscripts are significantly different ($P<0.05$).

Analysis of the organization of the first embryonic division in the developmental block oocytes

The organization of chromatin, microtubules and microfilaments in the reconstructed oocytes blocked in the first embryonic division is illustrated (Fig. 2) and summarized (Table 3). Overall, the microtubules in 56% of the FFC and 50% of the AFC reconstructed oocytes were associated with the chromatin of the donor cell. However, most of these (including all those at interphase and prometaphase) could not form the whole mitotic spindle necessary to proceed to the next metaphase stage (FFC: 78%; AFC: 92%;). Thus, 39% in the FFC group and 42% in the AFC group showed the prometaphase of the first embryonic division in which chromatin had condensed as a start to the formation of chromosomes with associated concentrated microtubules. In 22% of the FFC-constructed oocytes and 8% of the AFC-constructed oocytes, the chromosomes were at metaphase or were beginning to separate, but could not complete the first embryonic division. Microfilaments had concentrated to form a rich domain surrounding the area of chromatin-microtubules complexes in the prometaphase-stage oocytes whereas they formed a network throughout the ooplasm in the oocytes blocked at other stages.

DISCUSSION

The present study compared the competence of two types of cells (FFC and AFC) to undergo nuclear remodelling and the first embryonic division after nuclear transfer. Results showed average rates of cell fusion and nuclear remodelling of about 70 - 78% when using both fetal and adult fibroblast, with no significant differences between the two cell types in their ability to act as a donor nuclei to reconstruct enucleated oocytes. This finding is similar to those in other domestic species used for nuclear transfer experiments, and it establishes that the nuclei of both fetal and adult horse fibroblasts have similar potential to induce remodelling in oocytes matured in *vitro*. Furthermore, after 28-30h of culture after cell fusion, the average rates of embryonic cleavage to the two-cell stage in the reconstructed oocytes were again not different between those made with fetal versus adult fibroblasts (27% and 25%).

However, when comparing these rates of first embryonic division in the reconstructed horse oocytes with those in other domestic species, large differences are evident, as 25-27% is only about half the rate achieved routinely in cattle (Kato

et al., 2000*),* sheep (Campbell *et al*., 1996a; Wilmut *et al*., 1997), goats (Baguisi *et al*., 1999; Keefer *et al*., *2001)* and pigs (Lai *et al*., 2001; Park *et al*., 2001). The remodelling of the donor cell nucleus and the first embryonic division in the reconstructed oocytes has been characterized in mice, rabbits and cattle, and this has indicated that the state of the cytoplasm in the recipient oocyte is a key-factor in the production of cloned embryos and normal offspring (Campbell *et al*., 1993 and 1996b; Cibell *et al*., 1998; Kono, 1998; Robl, 1999; Shin *et al*., 2002). Co-culture of horse COCs with oviduct epithelial cells has improved cytoplasmic maturation to support fertilization and embryonic development better after intracytoplasmic sperm injection (Li *et al*., 2001b). Therefore, this study used the same *in vitro* culture system to provide the metaphase II oocytes used as recipient cytoplasts. In addition, induction of parthenogenesis showed that 79% of metaphase II oocytes that had matured under the same conditions used in nuclear transfer progressed to two-cell stage embryos. This result indicates the presence of different requirements for horse cytoplasmic competence in fertilization, parthenogenesis and nuclear remodelling. The molecular mechanisms involved in reprogramming donor nuclei after somatic cell nuclear transfer have not been fully elucidated. Thus, the question remains as to how best to provide more physiologically mature metaphase II oocytes from abattoir ovaries for horse nuclear transfer studies.

The number of donor cell passages is another significant factor when attempting to clone by nuclear transfer. Kubota *et al*. (2000) found no differences in the development of nuclear transfer embryos when using adult fibroblasts that ranged in passage numbers from five to 15. In the present study, the rate of nuclear remodelling decreased significantly when using fetal fibroblasts of increasing passage number. During cell passage, both genetic and epigenetic alterations that might affect nuclear remodelling would be expected to accumulate in the cultured cells, and other possible disruptions to the regulation of imprinted genes could also be induced by repeated culture, thereby leading to perturbations in embryonic and fetal development (Walker *et al*., 1996; Wakayama *et al*., 1999). Furthermore, it would be sensible to try other cell types obtained from different somatic tissues for use as cell donors in this species.

Successful cell division requires the formation of the metaphase spindle after DNA replication during the normal mitotic cell cycle. By confocal microscopic analysis of non-cleaved oocytes in the present study, it was possible to demonstrate that the microtubules in 78% of the FFC-constructed oocytes, and in 92% of the AFC-

constructed oocytes, did not form a mitotic spindle-like structure and so were unable to progress to later stages of the first cell cycle. Furthermore, even the 22% of the FFC-constructed oocytes and 8% of the AFC-constructed oocytes in which the chromosomes were at the metaphase stage or were beginning to separate, could not complete the first embryonic division due either to structural defects of the spindle or the distribution of the chromosomes, or both. Tremoleda et al. (2001) reported that changes in the microtubules and the organization of the microfilaments during *in vitro* maturation of horse oocytes are similar to those seen in other species. Thus, it can be speculated that the underlying cause of the first embryonic division block in the reconstructed horse oocytes created by nuclear transfer may be a deficiency of the cytoplasmic transition factors in the recipient cytoplasm, such as those in the cell cyclin family and other cyclin-dependent factors, that are involved specially in the progression of the introduced donor nucleus from S-G2-prometaphase to metaphase. In addition, in contrast to the situation in mitotic cells, meiotic spindles in mammalian oocytes lack centrioles. Hence, the introduction of a foreign centrosome during somatic cell nuclear transfer, results in the presence of the centrioles which plays an indispensable role in reorganization of first embryonic division in the reconstructed oocytes (Navarra *et al*., 1994: Shin *et al*., 2002). Whether these factors are relevant to horse nuclear transfer remains to be clarified.

In summary, the present study investigated the rates of nuclear remodelling and two-cell embryonic development, and examined the reasons for the developmental block in progression to the two-cell stage in horse oocytes reconstructed using both fetal and adult fibroblasts that had undergone different number of passage in *vitro*. The findings indicate that fetal and adult fibroblasts that have undergone few passages (3-10) are equally suitable for use as nuclear donor cells. However, a marked improvement in the rate at which the reconstructed oocytes progress from the S-G2-prometaphase stage to beyond metaphase is needed before attempts to produce viable cloned embryos in this species are likely to be successful.

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Chapter 8

Summarizing discussion

Developments in assisted reproduction have revolutionized the treatment of sub-fertility in man and provided valuable tools for selective breeding in animals. In addition, they have created the means to preserve genetic material from endangered species for the subsequent production of offspring, should that prove necessary. In horses, techniques such as artificial insemination and embryo transfer are successful and, in some countries, used extensively to aid genetic progress. By contrast the commercial application of other assisted reproductive techniques, such as IVP, has been severely restricted by their low efficiency. During the last decade, however, there has been increasing scientific interest in the physiological requirements of early equine embryos, in the hope of developing culture systems that can support embryo development *in vitro* to a stage suitable for transfer to the uterus of recipient mares. Unfortunately, progress has been slow and the state-of-the-art lags a considerable distance behind that in species like cattle. The work presented in this thesis has focused on fundamental aspects of the events involved in the production of equine embryos under *in vitro* conditions. This final chapter briefly summarizes the major findings and considers their implications for future developments in equine embryo production, at both fundamental and applied levels.

In vitro maturation of horse oocytes; not only a nuclear event

When immature horse oocytes, arrested in the dictyate stage of the first meiotic division, are released from their follicular environment and cultured *in vitro*, they resume meiosis and progress to metaphase of the second meiotic division (Hinrichs *et al*., 1995). However, for the oocyte to become fertilizable and developmentally competent, these nuclear changes must be supported by appropriate cytoplasmic reorganization. And since IVM horse oocytes have proven less developmentally competent that their *in vivo* counterparts (Scott *et al*., 2001), recent studies have focused on finding out what goes wrong during culture (for review see Hinrichs, 1998). In cattle, it has been suggested that 'preculturing' oocytes under conditions that maintain meiotic arrest but allow cytoplasmic maturation to proceed, may improve their developmental competence (Sirard, 2001). Using this rationale, in Chapter 2 horse oocytes were cultured with different components of the follicle wall and it was shown that theca cells play an important role in maintaining meiotic arrest, most likely via production of a secreted inhibitory factor. Loose granulosa cells alone failed to maintain oocytes in meiotic arrest but, when attached to an oocyte, they enhanced the meiosis inhibiting effect of theca cells in a synergistic fashion. Overall, these culture conditions (i.e. oocytes connected to sheets of granulosa cells in the presence of theca cells or their products) resemble the *in vivo* situation in small and medium-sized antral follicles, and they could provide a useful basis for an *in vitro* prematuration system in which to improve the

developmental competence of IVM horse oocytes. The fact that FSH failed to overcome the suppressive effect exerted by theca cells even though FSH receptor mRNA is present in cumulus and granulosa cells, suggests that FSH does not play an important role in chromatin reorganization; we speculate that it may instead be more relevant to cytoplasmic maturation.

Completion of oocyte growth and prematuration prior to nuclear maturation appear, thus, to be essential for the oocyte's ability to develop into an embryo and, eventually, a healthy offspring (Hyttel *et al*., 1997; Sirard, 2001). In this respect, oocytes collected from medium and large follicles appear to be more developmentally competent than those collected from small follicles, presumably because they have progressed further through prematuration (Merchal *et al*., 2001). Hinrichs and Schmidt (2000) similarly reported that horse oocytes recovered from non-atretic follicles > 20 mm in diameter had high rates of successful nuclear maturation, although they did not examine the influence of follicle size on embryo or foal production. Similarly, oocytes with an expanded cumulus at recovery, and therefore presumably from more mature follicles, have been reported to have higher meiotic competence and activation rates in response to calcium ionophore, than oocytes with a compact cumulus (Hinrichs *et al*., 1995; Hinrichs and Williams, 1997, 2000). Nevertheless, research into horse IVM is usually performed with oocytes with a compact cumulus because they form a more homogeneous population. Clearly, there is a need to carefully characterize follicular and cumulus parameters and to clarify their relationship with the developmental competence of the contained oocyte.

Research into IVM of horse oocytes has focused on nuclear changes, i.e. GV breakdown and progression to MII, and little is known about the accompanying cytoplasmic events. A complex interdependency of nuclear and cytoplasmic maturation was, however, suggested by the cytoskeletal restructuring that accompanied and almost certainly enabled nuclear reorganization during IVM (Chapter 3). Indeed, microtubules and microfilaments, the major cytoskeletal components of a mammalian ovum, appear to play critical roles in chromosomal alignment and segregation during meiosis. Examining the cytoskeleton may, therefore, prove useful when assessing the effects of IVM conditions on the likelihood of subsequent embryonic development.

Clearly, the most reliable criterion for evaluating IVM oocytes is their ability to be fertilized and develop into a viable embryo. The development of ICSI (Li *et al*., 2001; Galli and Lazzari, 2001; Galli *et al*., 2002; Choi *et al*., 2003) has finally provided the means to assess fertilisability. But until the whole IVP system is up

and running, transfer of oocytes into the oviduct of inseminated mares (oocyte transfer; Carnevale *et al*., 2000) may be a more than useful way to compare the developmental competence of *in vitro* and *in vivo* matured oocytes.

Conventional *in vitro* fertilization; an unsolved challenge

The first study on conventional *in vitro* fertilization (IVF) of horse oocytes was reported more than 12 years ago (Bézard *et al*., 1989). Since then only two foals have been produced from oocytes matured *in vivo* (Palmer *et al*., 1991; Bézard, 1992) and none from oocytes matured *in vitro* (Hinrichs, 1998; Alm *et al*., 2001; Hinrichs *et al*., 2002). The commonly proposed reasons for the failure of sperm to penetrate the oocyte are culture-induced changes in the oocyte coverings and/or inadequate capacitation of stallion sperm *in vitro*. In chapter 4, sperm were shown to bind to but not penetrate the ZP of both *in vivo* and *in vitro* matured oocytes, indicating that failed zona penetration is most probably due to inadequate sperm activation. Interestingly, most of the ZP-bound sperm had a mottled, swollen acrosomal cap but did not undergo a normal acrosome-reaction (AR) even in the presence of progesterone, a reported inducer of the AR and enhancer of sperm-zona binding *in vitro* (Cheng *et al*., 1998a). The failure of progesterone to trigger oocyte penetration may also relate to inadequate capacitation of stallion sperm under *in vitro* conditions, and could involve failure to expose plasma membrane progesterone receptors, since this is a capacitation induced event (Cheng *et al*., 1998b) that is important to fertility (Rathi *et al*., 2000) Overall, conventional IVF with equine gametes remains an unresolved problem that warrants further investigation. The findings presented in this thesis suggest that this research should focus on the molecular and signaling events that regulate sperm capacitation, zona binding and the acrosome reaction both *in vivo* and *in vitro*.

***In vitro* embryo production; new approaches**

Intracytoplasmic sperm injection (ICSI) has resulted in the birth of several foals (Squires *et al*., 1996; Cochran *et al*., 1998; McKinnon *et al*., 2000; Li *et al*., 2001; Galli *et al*., 2002). However, the need for specialized equipment and expertise, allied to poor foaling rates, mean that it is still not suitable for a large-scale commercial venture. Nevertheless, it has proven an excellent tool for producing equine embryos *in vitro* on which to study early embryo development. In chapter 5, we studied the nuclear and cytoskeletal events that occur in horse oocytes during and after fertilization. Sperm incorporation and subsequent fusion of the parental genomes were shown to involve a complex series of cytoskeletal changes, and comparison of zygotes and parthenotes showed that while the sperm contributes the zygote's centrosomal template, the oocyte contributes essential structural elements to both the centrosome and the associated cytoplasmic microtubule network.

Furthermore, we found that failure of fertilization was primarily due to failed gamete activation, presumably as a result of inadequate maturation *in vitro*. Of course, until conventional IVF becomes successful and reliable, ICSI will remain the preferred manner of IVF, and a potential treatment for both male and female infertility or for salvaging the germ line of dead or castrated stallions.

The next hurdle to IVP is to improve culture conditions for ICSI-derived zygotes to ensure that the resulting embryos are able to develop to term. To date, the majority of ICSI pregnancies have resulted from immediate transfer of post-cleavage zygotes to the oviduct of recipient mares ((Squires *et al*., 1996; Cochran *et al*., 1998; McKinnon *et al*., 2000), and only a few from transfer of cultured blastocysts to the uterus (Li *et al*., 2001; Galli *et al*., 2002). On the grounds of cost, practicality and animal welfare, a culture system that supports embryo development to a stage when they can be transferred non-surgically to the uterus would of course be preferable. However, culturing equine embryos *in vitro* has proven difficult and few develop into blastocysts (average 15%; Li *et al*., 2001; Galli *et al*., 2002; Choi *et al*., 2003), certainly many fewer than after temporary transfer to the oviduct of progesterone-treated ewes (blastocyst rate around 50%: Galli *et al*, 2002). In addition, the impact of *in vitro* culture of horse embryos on fetal and post-natal development has yet to be examined. Chapter 6, provides the first description of the morphological, cytoskeletal and developmental characteristics of IVP horse embryos produced by ICSI followed by culture in defined medium or temporary transfer to a sheep's oviduct. Day 7 IVP embryos, were smaller, had fewer cells and were more compact than *in vivo* embryos of a similar age. In addition, they had a small blastocoele, an indistinct inner cell mass and had not properly hatched. In addition, IVP embryos had high rates of apoptosis, a disrupted pattern of microfilament distribution and irregularities in cell size and shape. In addition, the influence of culture on capsule formation, a vital and unique feature of early horse embryo development (Betteridge, 1989), was examined by labeling day 10 IVP embryos with the monoclonal antibody OC-1 (Oriol *et al*., 1993), to detect capsular glycoprotein expression. This proved that IVP embryos secrete capsular material but that, in culture, the latter fail to coalesce into a complete layer enveloping the embryo. Nevertheless, since the transfer of day 7 IVP embryos has resulted in normal pregnancies, it must be assumed that capsule coalescence can still occur if the embryos are transferred to a mare's uterus. These findings may prove useful in understanding how the embryo and the uterine environment together contribute to the formation and function of the equine capsule. And despite all the apparent abnormalities of IVP embryos, the foals thus far produced demonstrate that at least some can develop normally after transfer (Li *et al*., 2001; Galli *et al*., 2002).

Studies on a larger number of pregnancies and foals resulting from IVP embryos will be needed to confirm that they are not compromised by a period in culture.

There are few reports of somatic cell nuclear transfer to enucleated horse oocytes, and development rates of the resulting constructs are low compared to those in cattle (Choi *et al*., 2002; Li *et al*., 2002). Chapter 7 describes the effect of the number of passages and the nature of the donor cells on nuclear reprogramming and the first embryonic division in reconstructed horse oocytes; both are key factors in the development of cloned embryos. A large proportion of the reconstructed oocytes remained arrested at the first embryonic division and did not progress beyond the metaphase stage. Analysis of the arrested constructs identified several structural defects in their spindle apparatus, suggesting defective microtubule and/or microfilament organization. However, whether this related to inadequate cytoplasmic maturation of the IVM oocytes or to alterations in the mechanism of cell division due to enucleation needs further investigation. In the future, a cloned equine will almost certainly be produced, but it is impossible to predict what effects the DNA and epigenetic reprogramming will have on the health of this animal (Rideout *et al*., 2001).

Future considerations

Our ability to produce horse embryos *in vitro* has improved dramatically over the last few years, but much has still to be done. Future studies should focus on why conventional IVF still yields such poor results in the horse. The basic methods successful in other species have failed, and new approaches must therefore take account of unique features of gamete physiology in the equids. This important challenge should endorse this species as an interesting experimental model in which to investigate fundamental aspects of fertilization. Another important hurdle is to unravel what an oocyte requires to become fertilized and capable of supporting embryonic development. Although IVP embryos obtained from IVM oocytes are capable of developing into live offspring, the fact that their developmental competence is low highlights the need to investigate how an oocyte's nuclear and cytoplasmic entities reorganize to support early embryonic development and how this is affected by culture. To aid this process, current protocols for oocyte and follicle selection could be standardized across laboratories to ensure that comparable data is produced.

In conclusion, progress in reproductive technology has enabled the production of horse embryos *in vitro* and thereby provided an invaluable tool for investigating fundamental aspects of early embryo development. However, before these techniques are truly ready for commercial exploitation, their effects on the health

of the resulting offspring and, in particular, on the expression of developmentally important genes need to be investigated. There are, therefore, still fundamental gaps that need to be filled to ensure that the application of IVP to horse breeding is efficient and, more importantly, does not adversely affect the welfare of the animals involved.

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Samenvatting

De toepassing van moderne voortplantingstechnieken heeft een ommekeer gebracht in de behandeling van verminderde vruchtbaarheid bij de mens. Daarnaast is deze toepassing van grote waarde gebleken voor een versnelde genetische vooruitgang bij de fokkerij van dieren. Ook kan met behulp van deze technieken genetisch materiaal van bedreigde diersoorten worden geconserveerd ten einde dat materiaal, indien nodig, later te gebruiken voor het verkrijgen van nakomelingen. Technieken als kunstmatige inseminatie en embryotransplantatie zijn bij het paard met succes toegepast en worden in sommige landen intensief gebruikt ter bevordering van de genetische vooruitgang. Echter, de commerciële toepassing van andere voortplantingstechnieken bij deze diersoort, zoals *in vitro* fertilisatie (IVF) en *in vitro* productie van embryo's (IVP), is nog zeer beperkt, met name door de lage slagingskans. Gedurende de laatste tien jaar is de wetenschappelijke interesse voor IVF en IVP bij het paard sterk toegenomen. Daarbij werd beoogd de *in vitro* procedures en kweekomstandigheden zodanig te verbeteren dat met een redelijke kans op succes een *in vitro* geproduceerd embryo na transplantatie naar de baarmoeder van een draagmerrie zal resulteren in dracht en uiteindelijk een veulen. De vooruitgang die hierbij tot nu toe werd behaald is echter gering en de ontwikkelingen bij het paard liggen ver achter bij die bij andere diersoorten zoals bijvoorbeeld het rund. Het onderzoek beschreven in dit proefschrift is vooral gericht op de fundamentele aspecten van de processen die plaatsvinden tijdens de productie van paarden embryo's onder *in vitro* omstandigheden. Dit laatste hoofdstuk vat kort de belangrijkste bevindingen van dit onderzoek samen en bespreekt, zowel op fundamenteel als praktisch niveau, de toepassing ervan voor toekomstige ontwikkelingen van de *in vitro* productie van paardenembryo's.

***In vitro* maturatie van paardeneicellen: meer dan kernrijping alleen**

Wanneer onrijpe eicellen - die voor wat betreft de progressie van de meiose (reductiedeling) gestopt zijn in het dictyate stadium van de eerste meiotische deling - uit hun folliculaire omgeving worden gehaald en *in vitro* worden gekweekt zal de meiose worden hervat en zullen de eicellen zich verder ontwikkelen tot aan de metafase van de tweede meiotische deling. Echter, om een zich goed ontwikkelende eicel te verkrijgen die met succes bevrucht kan worden, zullen de veranderingen van de kern moeten worden ondersteund door een adequate ontwikkeling en reorganisatie van het cytoplasma. Aangezien *in vitro* gematureerde (IVM) paardeneicellen zich minder goed ontwikkelen dan *in vivo* gematureerde eicellen is de laatste jaren het onderzoek vooral gericht op de problemen die

optreden tijdens de kweek. Er wordt verondersteld dat bij runderen “voorkweken” van eicellen, waarbij de voortgang van de meiose tijdelijk wordt verhinderd terwijl de cytoplasmatische rijping wel doorgaat, de kwaliteit en de ontwikkelingspotentie van de eicel bevordert. Op grond van deze gedachtegang, zijn in hoofdstuk 2 paardeneicellen gekweekt in aanwezigheid van verschillende componenten van de follikelwand. Hierbij is aangetoond dat theca cellen een belangrijke rol spelen bij het handhaven van de tijdelijke stopzetting van de meiose, waarschijnlijk doordat deze cellen een remmende factor uitscheiden. Het toevoegen van losse granulosa cellen aan deze kweek blijkt de tijdelijke stopzetting van de meiose niet te kunnen handhaven. Echter, wanneer zij aan een eicel vastzitten verbeteren zij het remmende effect van de theca cellen op de meiose op synergistische wijze. Deze kweekomstandigheden (eicellen verbonden met granulosa cellen, in het bijzijn van theca cellen of hun producten) weerspiegelen de *in vivo* situatie binnen kleine en middelgrote antrale follikels. Dit kan een nuttige basis vormen voor een *in vitro* systeem ter verbetering van de ontwikkelingscapaciteit van IVM paardeneicellen. De bevinding dat follikel stimulerend hormoon (FSH) niet in staat was het remmende effect van de theca cellen te compenseren (ondanks de aanwezigheid van mRNA van de FSH receptor in cumulus en granulosa cellen), suggereert dat FSH geen belangrijke rol speelt bij de reorganisatie van het chromatine maar vermoedelijk meer betrokken is bij de cytoplasmatische rijping.
De voltooiing van de groei van de eicel en de rijping die voorafgaat aan de kernrijping blijken dus essentieel te zijn voor de ontwikkeling van eicel tot embryo en uiteindelijk een gezonde nakomeling. In dit opzicht lijken eicellen die uit middelgrote en grote follikels worden gewonnen, beter in staat tot verdere ontwikkeling dan eicellen die komen uit kleine follikels, waarschijnlijk omdat eerstgenoemde eicellen verder zijn in het rijpings proces. Recent onderzoek heeft aangetoond dat bij paardeneicellen verkregen uit non-atretische follikels met een diameter > 20 mm, het proces van kernrijping relatief succesvol verliep, alhoewel het effect van follikel grootte op de uiteindelijke productie van embryo’s en veulens niet werd vermeld. Ook blijken eicellen, die bij winning een geëxpandeerde cumulus hebben en dus waarschijnlijk uit verder gematureerde follikels afkomstig zijn, een betere potentie te hebben om de meiose te voltooien en door calcium ionophoor geactiveerd te worden, dan eicellen met een compacte cumulus. Desalniettemin wordt onderzoek naar *in vitro* maturatie van paardeneicellen meestal uitgevoerd met eicellen met een compacte cumulus omdat deze een meer homogene populatie vormen. Er is in elk geval behoefte aan een nauwkeurige karakterisering van follikel- en cumulus- parameters en hun relatie tot de ontwikkelingspotentie van de eicel.

Onderzoek naar IVM van paardeneicellen is gericht op veranderingen van de kern, oftewel het ontbinden van de GV en de verdere ontwikkeling naar het MII stadium. Er is weinig is bekend over de begeleidende processen op cytoplasmatisch niveau. De herstructurering van het cytoskelet, die gepaard gaat met een nucleaire reorganisatie en deze zeer waarschijnlijk ook mogelijk maakt (hoofdstuk 3),veronderstelt een complexe interactie tussen en onderlinge afhankelijkheid van nucleaire en cytoplasmatische rijping. Microtubuli en microfilamenten, de voornaamste bestanddelen van het cytoskelet van de eicel, blijken dan ook een essentiële rol te spelen bij chromosomale heroriëntatie en de daaropvolgende segregatie tijdens de meiose. Derhalve zou onderzoek naar het cytoskelet van groot belang kunnen blijken bij de bestudering van de effecten van IVM omstandigheden om de kans op een succesvolle embryonale ontwikkeling te vergroten.
Het is duidelijk dat het meest betrouwbare criterium voor de evaluatie van IVM eicellen hun vermogen tot bevruchting en uitgroei tot een levensvatbaar embryo is. Met de ontwikkeling van de intra-cytoplasmatische sperma injectie (ICSI) is een methode beschikbaar gekomen om dit vermogen te evalueren. Echter, tot het tijdstip waarop IVP als methode routinematig beschikbaar komt, zou voor wetenschappelijk onderzoek wellicht het plaatsen van eicellen in de eileider van geïnsemineerde merries (oocyte transplantatie) een betere manier zijn om de ontwikkelingscapaciteit van *in vitro* en *in vivo* gerijpte eicellen te vergelijken.

Conventionele *in vitro* fertilisatie: nog altijd een uitdaging
Het eerste onderzoek naar conventionele IVF van paardeneicellen werd 12 jaar geleden beschreven. Sindsdien zijn er slechts 2 veulens voortgekomen uit eicellen die *in vivo* zijn gerijpt en geen enkel veulen uit *in vitro* gerijpte eicellen. De meest genoemde oorzaken voor het mislukken van de penetratie van de eicel door de spermacel zijn veranderingen in de omhulling (zona pellucida, ZP) van de eicel, geïnduceerd door de kweekomstandigheden, en inadequate capacitatie van de spermacellen van de hengst *in vitro*. In hoofdstuk 4 wordt aangetoond dat spermacellen wel aan de ZP, van zowel *in vivo* als *in vitro* gematureerde eicellen binden maar niet penetreren, hetgeen impliceert dat de spermacellen onvoldoende geactiveerd zouden zijn om penetratie te bewerkstelligen. Interessant genoeg vertoonden de meeste spermacellen die aan de ZP gebonden waren een onregelmatige, gezwollen kopkap zonder een normale acrosoom reactie (AR) te ondergaan. De AR vindt zelfs niet plaats in de aanwezigheid van progesteron, terwijl beschreven is dat progesteron de binding tussen spermacellen en zona pellucida *in vitro* bevordert en de AR induceert. Het feit dat progesteron niet in staat is de eicel penetratie te faciliteren kan ook veroorzaakt worden door onvoldoende capacitatie van spermacellen onder *in vitro* condities maar ook doordat de progesteron receptoren van de plasma membraan van de zaadcel niet

worden geëxposeerd. Dit laatste proces wordt ook wel beschouwd als een onderdeel van capacitatie en is positief gecorreleerd aan de fertiliteit van de hengst. Tot nu toe blijft conventionele IVF met paardengameten een onopgelost probleem dat verder onderzoek vereist. Op grond van de bevindingen vermeld in dit proefschrift, zou dit onderzoek zich moeten richten op de moleculaire processen die de sperma capacitatie, de binding van de zaadcel aan de ZP en de AR, zowel *in vivo* als *in vitro*, regelen.

***In vitro* embryo productie: nieuwe wegen**

Intracytoplasmatische sperma(cel) injectie (ICSI) heeft tot nu toe slechts geresulteerd in de geboorte van een gering aantal veulens. Dit beperkte succes en de hiervoor benodigde hoog gespecialiseerde apparatuur en expertise hebben er toe geleid dat deze techniek nog niet op grote schaal commercieel wordt toegepast. Desalniettemin is het een uitstekende manier gebleken om paardenembryo's *in vitro* te produceren en de vroege ontwikkeling hiervan te onderzoeken. In hoofdstuk 5, worden de processen beschreven die plaatsvinden in de kern en het cytoskelet van paardeneicellen tijdens en na de bevruchting. De versmelting van de zaadcel met de eicel en daaropvolgende fusie van de ouderlijke genen blijken een serie complexe, opeenvolgende veranderingen van het cytoskelet in te houden. Bovendien wordt door vergelijking van zygoten met spontaan delende eicellen duidelijk dat de eicel essentiële elementen levert voor zowel het centrosoom als het daarmee verbonden cytoplasmatische netwerk van microtubuli, maar ook de spermacel bijdraagt aan de centrosomale structuur van de zygote,. Verder is het mislukken van de bevruchting in eerste instantie te wijten is aan een gebrekkige gameet aktivatie, waarschijnlijk door een onvoldoende maturatie *in vitro*. Tot het tijdstip waarop conventionele IVF betrouwbaar en succesvol blijkt, zal ICSI de voorkeur genieten als IVF methode en zo een mogelijke behandeling zijn voor zowel mannelijke als vrouwelijke onvruchtbaarheid bij het paard. Ook zou het in de toekomst mogelijk moeten zijn, via ICSI nakomelingen te verkrijgen van gestorven of gecastreerde hengsten, wanneer spermatogene stamcellen geconserveerd en gekweekt kunnen worden.

Een volgend nog op te lossen probleem bij IVP bij het paard is de verbetering van kweek omstandigheden voor via ICSI geproduceerde zygoten zodat de uiteindelijk verkregen embryo's kunnen uitgroeien tot een stadium waarin zij voor transplantatie geschikt zijn. Tot nu toe zijn de meeste ICSI-drachten voortgekomen uit directe transplantatie van zygotes (eventueel na deling), naar de eileider van ontvangst merries en slechts een enkele uit transplantatie van gekweekte blastocysten naar de baarmoeder. Uit het oogpunt van ethiek, bestaande regelgeving en de hoge kosten is een kweekmethode nodig, waarbij het embryo zich kan ontwikkelen tot een stadium waarin het op niet-chirurgische wijze in de

baarmoeder kan worden gebracht. Het kweken van embryo's *in vitro* is echter moeilijk gebleken en slechts een beperkt deel van de zygoten ontwikkelt zich tot blastocysten (gemiddeld 15%). Deze aantallen zijn in ieder geval veel lager dan wanneer de transplantatie tijdelijk plaatsvindt naar de eileider van met progesteron behandelde ooien (aantal blastocysten omstreeks 50%). Bovendien moet nog onderzocht worden of het *in vitro* kweken van paardenembryo's nadelige effecten heeft op de foetale en postnatale ontwikkeling. In hoofdstuk 6 worden voor het eerst de morfologische, ontwikkeling karakteristieken en veranderingen in het cytoskelet van IVP paarden embryo's beschreven, die geproduceerd zijn door middel van ICSI en vervolgens *in vitro* gekweekt zijn of tijdelijk zijn overgebracht naar een schapeneileider. Dag 7 IVP embryo's zijn kleiner, bevatten minder cellen en zijn compacter dan *in vivo* embryo's van dezelfde leeftijd. Ook hebben ze een klein blastocoel, een weinig duidelijke afgetekende "inner cell mass" en vindt "hatching" onvolledig plaats. Bovendien wordt apoptose dikwijls bij de IVP embryo's waargenomen, zijn cellen onregelmatig van vorm en grootte en is het patroon van de filament verdeling onderbroken. De invloed van de kweek op kapselvorming, een vitaal en uniek kenmerk van vroege embryonale ontwikkeling bij het paard is onderzocht door middel van labeling van dag 10 IVP embryo's met het monoklonale antilichaam OC-1 om zo de expressie van het kapsel glycoproteine zichtbaar te maken. IVP embryo's scheiden weliswaar kapselmateriaal uit maar tijdens het kweken smelt dit niet samen tot een structuur die het embryo omgeeft. Toch moet worden aangenomen dat het kapsel alsnog gevormd kan worden nadat het embryo is overgeplaatst in de baarmoeder van een draagmerrie, aangezien er normale drachten zijn voortgekomen uit transplantaties van D7 IVP embryo's. Deze bevindingen zouden zeer nuttig kunnen zijn bij het vergaren van kennis en begrip omtrent de interactie tussen embryo en baarmoeder en beider bijdrage aan de vorming en functie van het kapsel van het paardenembryo. Ondanks alle duidelijke afwijkingen van IVP embryo's bewijzen de tot nu toe geproduceerde veulens dat sommige embryo's zich in ieder geval normaal kunnen ontwikkelen na transplantatie. Verder onderzoek naar grotere aantallen drachten en veulens van IVP embryo's is nodig om te bevestigen dat er geen nadelige gevolgen optreden tijdens de *in vitro* procedure.

Er zijn slechts enkele beschrijvingen van de transplantatie van de kern afkomstig van een somatische cel naar een paardeneicel waarvan de kern eerder was weggenomen. De ontwikkelingspotentie van de op deze manier gemaakte "zygote" blijkt laag te zijn in vergelijking met de procedure bij het rund. Hoofdstuk 7 beschrijft het effect van het aantal passages en de aard van de donor cellen op de kern- programmering. Ook hun invloed op de eerste embryonale deling van aldus gereconstrueerde paardeneicellen wordt beschreven. Beide zijn essentiële factoren bij de ontwikkeling van gekloonde embryo's. Een groot deel van de

gereconstrueerde eicellen blijft in de ontwikkeling stilstaan bij de eerste embryonale deling en komt niet verder dan de metafase. Een analyse heeft een aantal structurele defecten van de kernspoel aan het licht gebracht, hetgeen duidt op een afwijkende organisatie van microtubuli en/of microfilamenten. Er is verder onderzoek noodzakelijk om vast te stellen of dit gerelateerd is aan een onvoldoende cytoplasmatische rijping van de IVM eicellen of aan veranderingen in het mechanisme van de celdeling veroorzaakt door de enucleatie. In de toekomst zal waarschijnlijk wel een paarden kloon worden geproduceerd. Het is echter niet te voorzien welke effecten de DNA- en de epigenetische herprogrammering op de gezondheid van dit dier zullen hebben.

De toekomst

De laatste jaren is men steeds vaardiger geworden in de *in vitro* productie van paardenembryo's, maar er is nog heel veel te verbeteren. Toekomstig onderzoek zou zich moeten richten op de redenen waarom met conventionele IVF bij het paard nog steeds zulke slechte resultaten worden bereikt. De methoden die normaal gesproken bij andere diersoorten met succes worden toegepast, blijken bij het paard onbruikbaar te zijn. Een andere, nieuwe aanpak die rekening houdt met de unieke eigenschappen van de fysiologie van de gameten van het paard is daarom vereist. Deze belangrijke uitdaging maakt het paard tot een interessant onderzoeksmodel om fundamentele aspecten van de bevruchting nader te bestuderen. Ook moet onderzocht worden welke nog niet bekende factoren noodzakelijk zijn om een eicel *in vitro* te bevruchten en vervolgens de eerste ontwikkeling *in vitro* door te laten maken, waarna uiteindelijk de verdere ontwikkeling succesvol *in vivo* voltooid wordt. IVP embryo's kunnen zeker worden verkregen na bevruchting van IVM eicellen en zich ontwikkelen tot levende nakomelingen. Echter, het feit dat hun ontwikkelings potentie laag is, betekent dat meer onderzoek gedaan moet worden naar de wijze van reorganisatie van de kern en het cytoplasma die noodzakelijk is voor de eerste embryonale groei onder kweekomstandigheden. Om dit proces te ondersteunen zouden protocollen voor eicel en follikel selectie tussen laboratoria onderling gestandaardiseerd moeten worden, om te bewerkstelligen dat geproduceerde data beter kunnen worden vergeleken.

Concluderend kan men zeggen dat de vooruitgang binnen de voortplantingstechnologie de productie van paardenembryo's *in vitro* mogelijk heeft gemaakt en hierbij een waardevol instrument ter beschikking is gekomen voor het onderzoek naar de fundamentele aspecten van vroege embryonale ontwikkeling. Voordat deze technieken echter volledig beschikbaar komen voor commercieel gebruik zal grondig onderzoek plaats moeten vinden naar de effecten op de gezondheid van de geproduceerde nakomelingen en in het bijzonder naar de expressie van de belangrijke genen die betrokken zijn bij de ontwikkeling. Er zijn

dus nog fundamentele hiaten in de kennis die opgevuld moeten worden alvorens IVP efficiënt in de paardenfokkerij kan worden toegepast zonder dat het welzijn van de betrokken dieren nadelig wordt beïnvloed.

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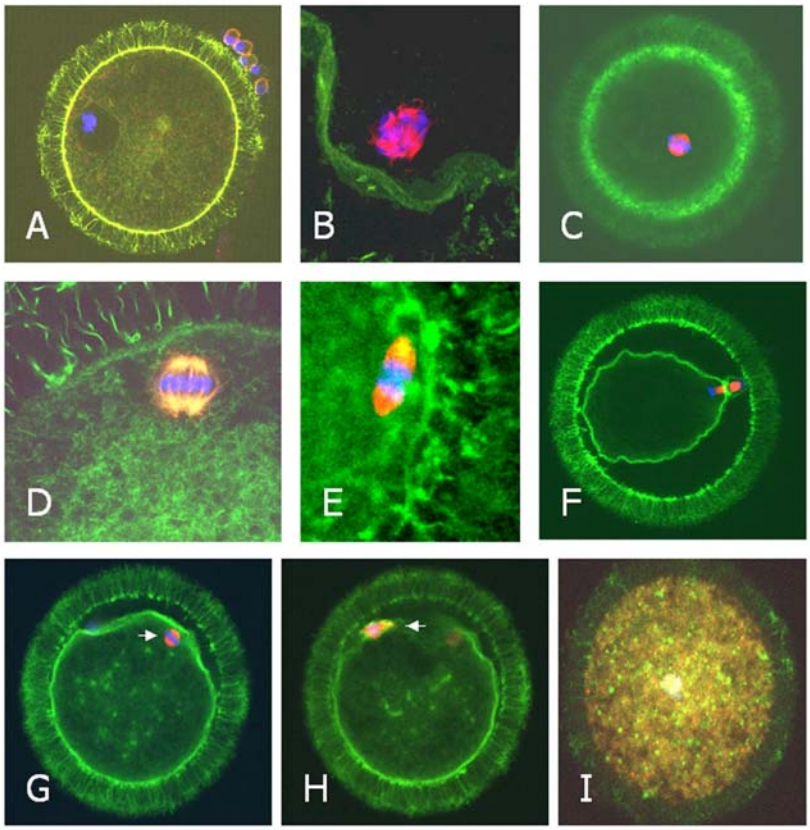
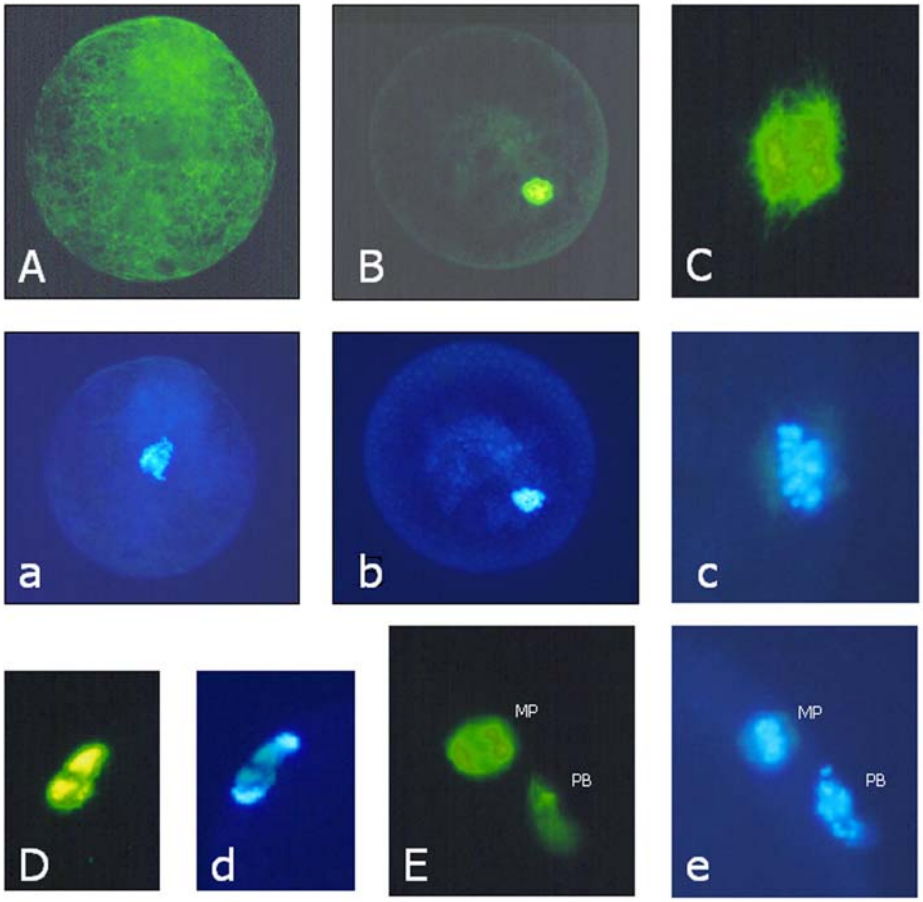
Curriculum Vitae

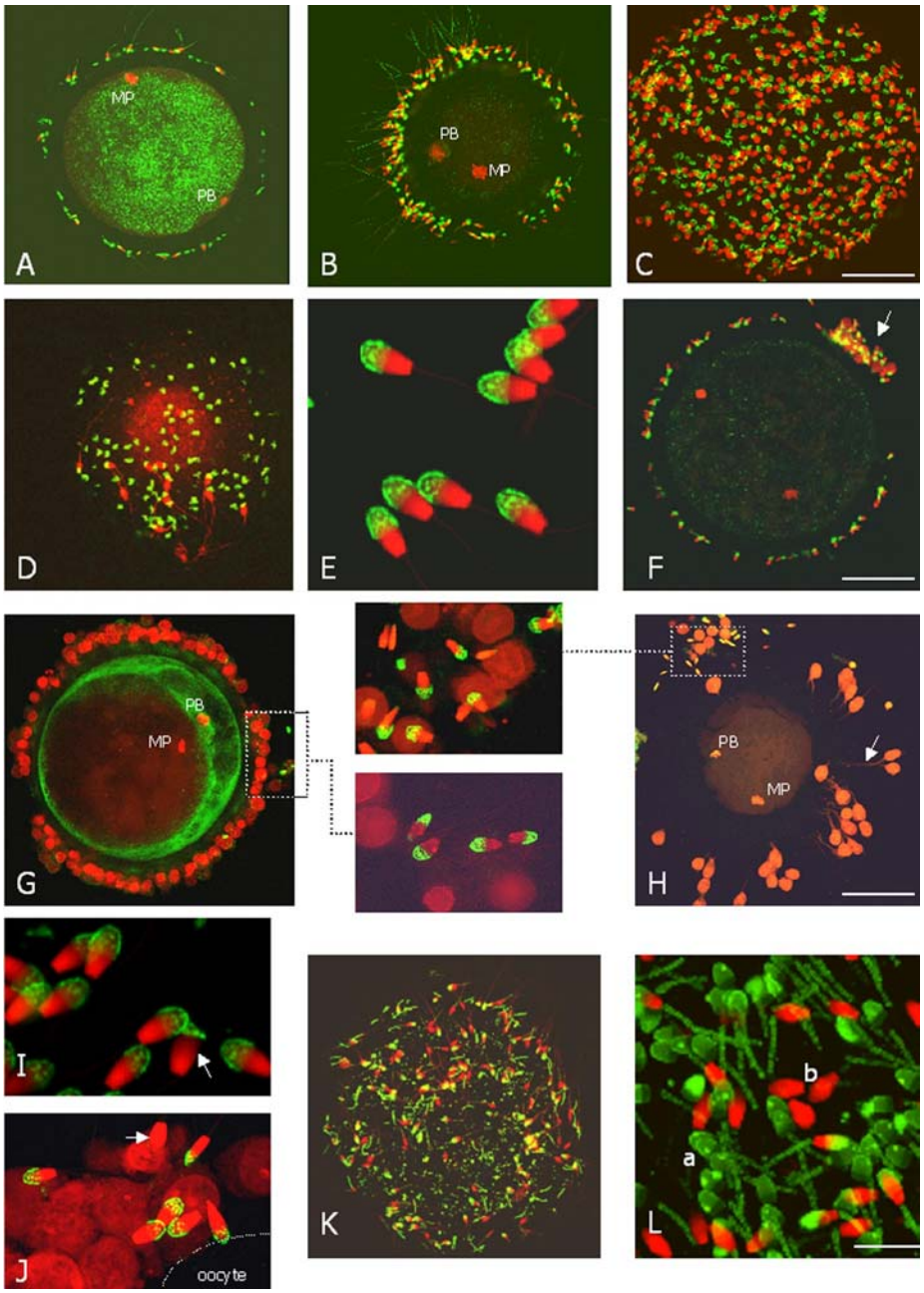
Jordi López Tremoleda was born on the 1st of December 1970 in Barcelona. Following his graduation from High School, he pursued studies in Veterinary Medicine at the "Universitat Autònoma de Barcelona". After graduation, and having completed his 15 months State Social Service, he traveled to England in 1995 to follow a student internship program in Equine Medicine, Surgery and Reproduction at the College of Veterinary Medicine at the University of Liverpool. Having developed an interest in the field of animal reproduction, he worked as a veterinary research assistant at the Department of Herd Health, Reproduction and Obstetrics at the Faculty of Veterinary Medicine, Utrecht University in 1996, and then traveled to Australia to work as a resident veterinarian at Alabar Australia Bloodstock in Victoria, during the southern hemisphere horse breeding season. Tremoleda then returned briefly to Utrecht University before moving on to the Equine Fertility Unit in Newmarket, England to work as a Veterinary Clinical and Research Assistant. After a year in Newmarket, he returned to Alabar Bloodstock for another breeding season and in January 1999 finally returned to Utrecht to start his PhD studies as a research assistant ("onderzoeker in opleiding") at the Department of Equine Sciences at the Faculty of Veterinary Medicine. Over the last 4 years, he has focused on the development of assisted reproductive techniques in horses.



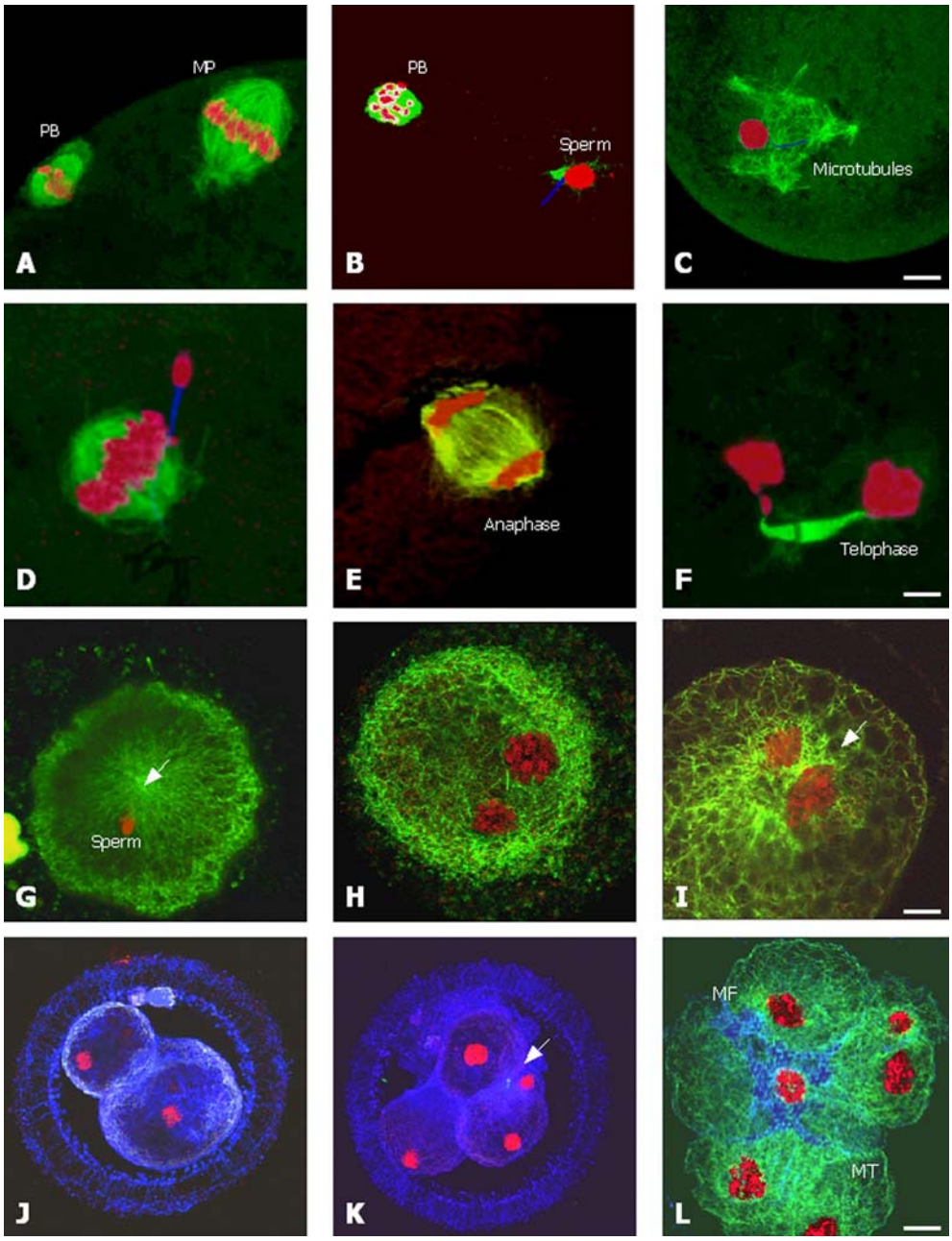
Chapter 3 - figure1



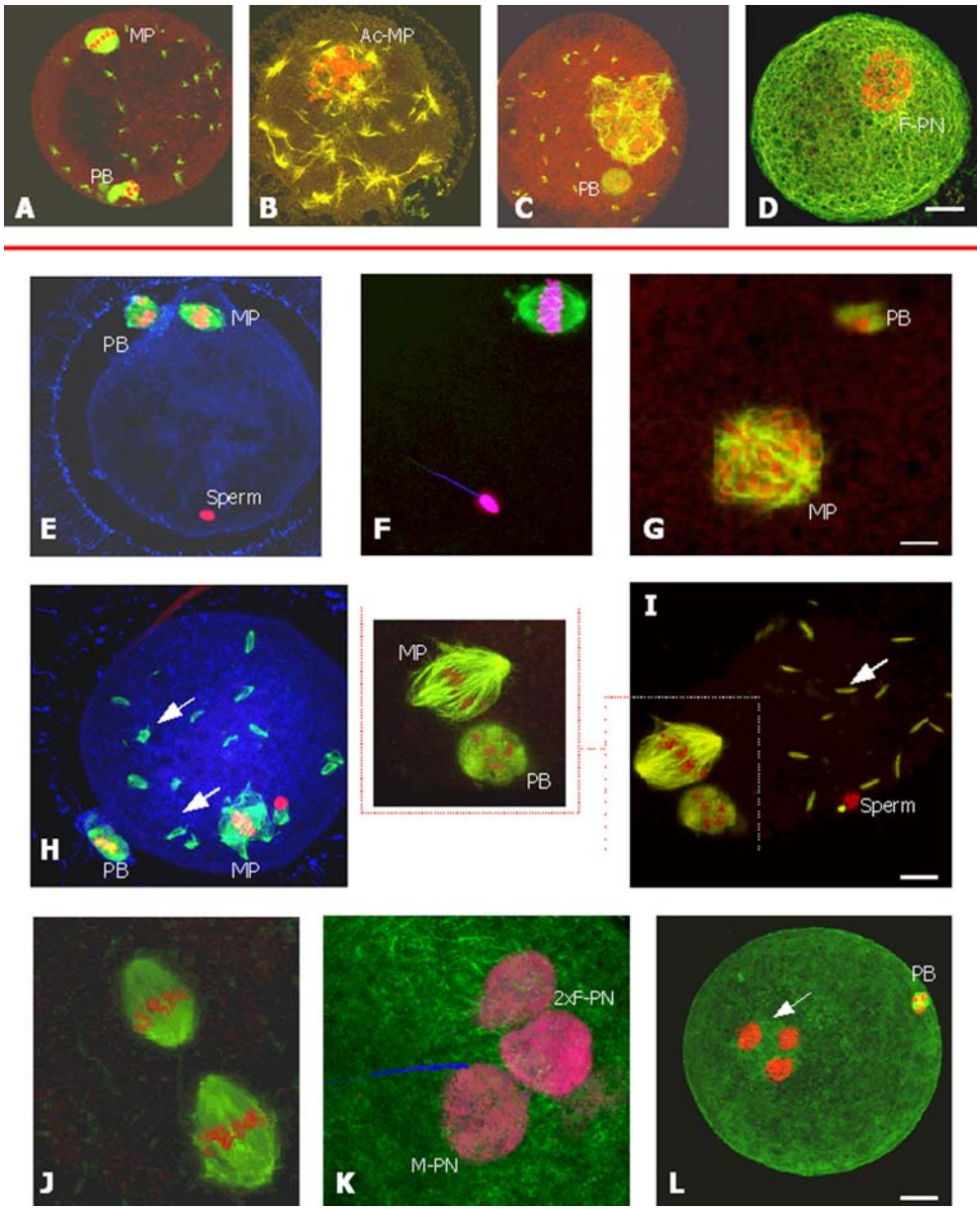
Chapter 3 - figure 2



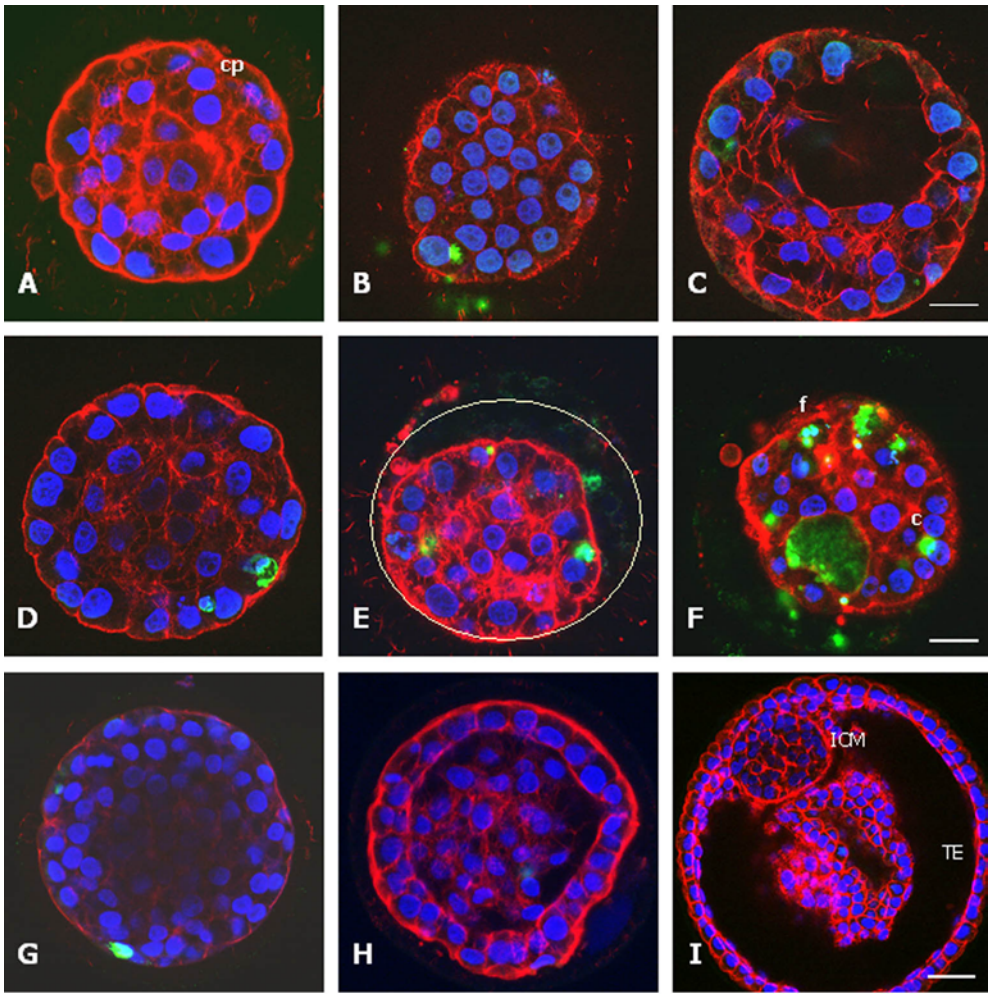
Chapter 4 – figure 1



Chapter 5 – figure 2

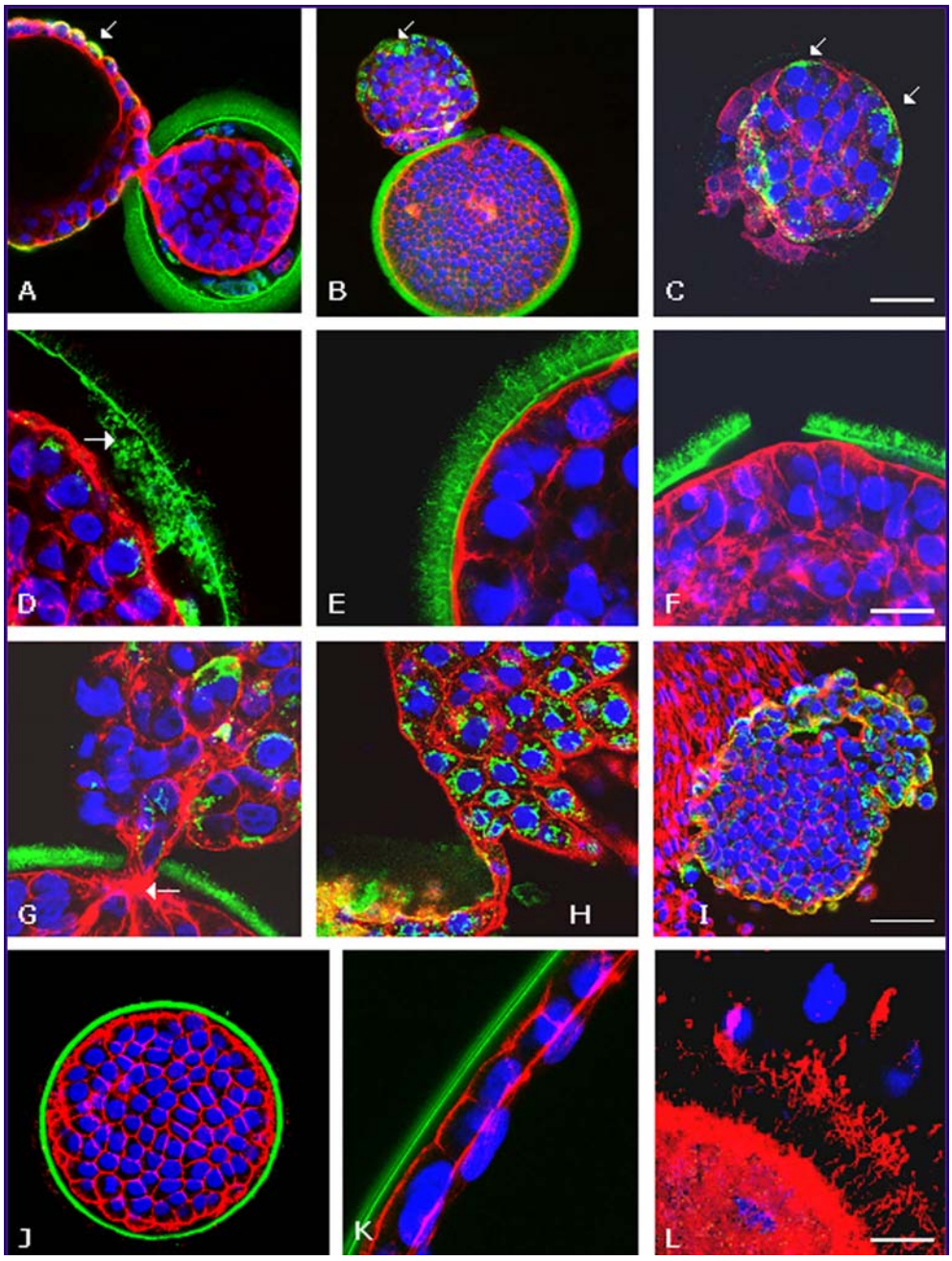


Chapter 5 – figure 3



Chapter 6 – figure 4

Chapter 6 – figure 5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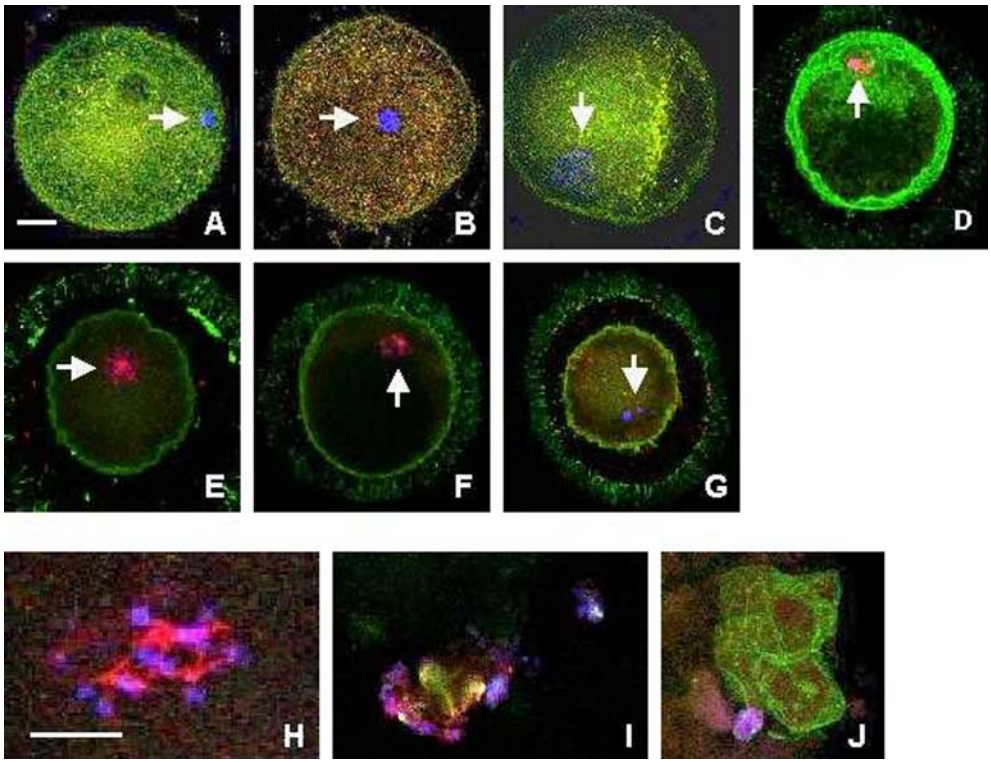

Chapter 7 – figure 2