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Developmental competence of equine oocytes after ICSI: Implications on technical, morphological and cellular aspects

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Dedicated To My Dear Mahboobeh, Brother, Sisters, &
My Parents in Love & Gratitude

Die Entwicklungskompetenz equiner Eizellen nach ICSI: technische, morphologische und zelluläre Aspekte inbegriffen

Die vorliegende Studie untersucht den Effekt der assistierten Eizellaktivierung mit Calcium Ionophor A23187 equiner Embryonen nach der intrazytoplasmatischen Spermainjektion (ICSI) auf die Entwicklungskompetenz. Es wurde eine Re-evaluierung des Einflusses der Kumulusmorphologie, der Glucose-6-Phosphat-Dehydrogenase (G6PD) Aktivität und der Beschaffenheit der Zona pellucida mit Hilfe der Polarisationslichtmikroskopie im Hinblick auf die präimplantative Entwicklungskompetenz durchgeführt. Eine Einteilung in zwei Gruppen wurde eine Stunde nach ICSI vorgenommen, wobei nur eine Gruppe mit Calcium-Ionophor A23187 behandelt wurde. Die mit Calcium-Ionophor A23187 behandelten Eizellen zeigten signifikant höhere Teilungs- und Blastozystenraten ($P < 0,05$) als jene, die nicht behandelt wurden. Die Kumulusmorphologie der ungereiften Eizellen (expandierter Kumulus (Ex) vs. kompaktierter Kumulus (Cp)) zeigte ebenfalls signifikante Unterschiede ($P<0,05$) in der Maturationsrate und der Blastozystenrate nach ICSI. Zur Messung der G6PD Aktivität der Eizellen wurden diese mit Brilliant Cresyl Blue (BCB) gefärbt. Der prozentuale Anteil der Eizellen mit einer geringeren G6PD-Aktivität und blauem Cytoplasma war in der Gruppe der Ex Eizellen signifikant höher ($P<0,01$) als in der Gruppe der Cp Eizellen. Des Weiteren wurden signifikant höhere ($P<0,05$) Maturations- und Entwicklungsraten der BCB+ Eizellen beobachtet verglichen mit Eizellen mit hoher G6PD Aktivität. Mit Hilfe der Polarisationslichtmikroskopie wurde die Zona pellucida der Eizellen mit unterschiedlicher Entwicklungskompetenz (Ex vs. Cp und BCB+ vs. BCB-) und in verschiedenen Reifungsstadien (Immature Eizellen und mature Eizellen mit und ohne Polkörper) vermessen. Unsere Ergebnisse zeigen, dass die Ex Eizellen verglichen mit den Cp Eizellen und die BCB+ Eizellen verglichen mit den BCB- Eizellen eine signifikant ($P<0,05$) dickere Zona pellucida und eine höhere Doppellichtbrechungsintensität aufweisen. Zusätzlich hatten die Eizellen, die nach der in vitro Maturation keinen Polkörper ausschleusten, eine signifikant ($P<0,05$) dickere Zona pellucida und eine signifikant ($P<0,05$) höhere Doppellichtbrechungsintensität als immature Eizellen und Eizellen, die einen Polkörper ausschleusten. Zusammenfassend zeigte sich dass sich die Aktivierung der Eizellen nach ICSI mit Calcium-Ionophor A23187 äußerst positiv auf die Entwicklung der equinen Embryonen auswirkt. Kumulusmorphologie wie auch G6PD Aktivität sind verlässliche Indikatoren für die Entwicklungskompetenz. Ebenfalls könnten die Dicke und die Struktur der Zona pellucida, gemessen an Hand der Doppellichtbrechungsintensität, als neue Methode zur Bestimmung des Entwicklungspotentials equiner Eizellen dienen.

Developmental competence of equine oocytes after ICSI: Implications on technical, morphological and cellular aspects

Using equine intracytoplasmic sperm injection (ICSI) procedure, the present study was performed, (i) to study the effect of assisted oocyte activation with calcium ionophore A23187 on developmental competence of oocytes, (ii) to re-evaluate the effect of cumulus morphology on meiotic and developmental competence of oocytes, (iii) to investigate the effect of glucose-6-phosphate dehydrogenase (G6PD) activity on meiotic and developmental competence of oocytes and (iv) to analyze zona pellucida (ZP) properties of equine oocytes of different quality and maturational status, quantitatively by polarization light microscopy. One hour after ICSI, injected oocytes were categorized into two groups: those treated with calcium ionophore A23187 and non-treated group. Cleavage and blastocyst rate were significantly (P<0.05) higher in treated group compared to non-treated group. When we categorized oocytes into expanded (Ex) and compact (Cp), depending on expansion of cumulus cells, meiotic competence after in vitro maturation (IVM) and developmental competence to blastocyst stage after ICSI was significantly (P<0.05) higher in Ex group compared to Cp group. Oocytes stained with brilliant cresyl blue (BCB) dye in order to measure the G6PD activity of the oocytes. Percentage of BCB+ oocytes (blue cytoplasm, low G6PD activity) of Ex group was significantly (P<0.01) higher than that of Cp group. The mean volume of BCB+ oocytes was significantly (P<0.01) higher than the mean volume of BCB– (colorless cytoplasm, high G6PD activity) oocytes. A significantly (P<0.05) higher maturation, cleavage and blastocyst rate were observed in BCB+ group compared to BCB– group. Using a polarization imaging system, ZP were analyzed in oocytes of different developmental competence (i.e., Ex *vs.* Cp and BCB+ *vs.* BCB–) and maturational status (i.e., immature, oocytes without and with polar body). Our results revealed that Ex oocytes compared to Cp oocytes and BCB+ oocytes compared to BCB– oocytes had a significantly (P<0.05) thicker ZP and exhibited greater birefringence. In addition, oocytes without polar body showed a significantly (P<0.05) thicker ZP and greater birefringence than both did immature oocytes and oocytes with polar body. In conclusion, activation of equine oocytes by calcium ionophore A23187 is beneficial following conventional ICSI procedure. Our results confirmed that G6PD activity of oocyte before IVM as well as cumulus morphology is a reliable predictor for subsequent development of equine oocytes. Likewise, the extent and pattern of ZP birefringence may constitute a new marker for oocyte quality assessment in equine in vitro embryo production system.

Contents

List of abbreviations

6-DMAP	:	6-dimethylaminopurin
AI	:	Artificial insemination
AOA	:	Assisted oocyte activation
ART	:	Assisted reproductive technology
ATP	:	Adenosine triphosphate
BCB+	:	Brilliant cresyl blue positive (Oocyte with blue cytoplasm)
BCB–	:	Brilliant cresyl blue positive (Oocyte with colorless cytoplasm)
BSA	:	Bovine serum albumin
°C	:	Degree celsius
Ca	:	Calcium
[Ca2+]i	:	Cytosolic free Ca2+ concentrations
CO2	:	Carbon dioxide
COC	:	Cumulus oocyte complex
CZB	:	Chatot, Ziomet, and Bavister medium
d	:	Day
DMEM-F12	:	Dulbecco's modified eagle's medium (DME) and Ham's F-12 Nutrient Mixture
DNA	:	Deoxynucleic acid
D-PBS	:	Dulbecco’s phosphate buffered saline
FCS	:	Fetal calf serum
FSH	:	Follicle stimulating hormone
g	:	Gram
G	:	Gauge
G1/G2	:	Gardner's 1/Gardner's 2 medium
GnRH	:	Gonadotropin releasing hormone
G6PD	:	Glucose-6-phosphate dehydrogenase
GSH	:	Glutathione
GV	:	Germinal vesicle
GVBD	:	Germinal vesicle break down
GV-TI	:	Germinal vesicle to telophase I
h	:	Hour

HEPES	:	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
ICSI	:	Intracytoplasmic sperm injection
IOIs	:	Interovulatory intervals
IU	:	International unit
IVC	:	*In vitro* culture
IVM	:	*In vitro* maturation
IVF	:	*In vitro* fertilization
IVEP	:	*In vitro* embryo production
L	:	Liter
LH	:	Luteinizing hormone
M	:	Molar
MII	:	Metaphase II
mg	:	Milligram
min	:	Minute
ml	:	Milliliter
MP	:	Metaphase plate
MPF	:	Maturation promoting factor
mRNA	:	messenger RNA
mtDNA	:	Mitochondrial DNA
Mw	:	Major wave
mw	:	Minor wave
MW	:	Molecular weight
n, No	:	Number
NADPH	:	Nicotinamide adenine dinucleotide phosphate
OV	:	Ovulation
PGCs	:	Primordial germ cells
PLCζ	:	Phospholipase C-zeta
POFs	:	Preovulatory follicles
PVA	:	Polyvinylalcohol
PVP	:	Polyvinylpyrrolidon
RNA	:	Ribonucleic acid
s	:	Second
SAS	:	Statistical analysis system

SD	:	Standard deviation
SOF	:	Synthetic oviductal fluid
TALP	:	Tyrode's albumin lactate pyruvate
TCM	:	Tissue culture medium
vs.	:	Versus
v/v	:	Volume by volume
w/v	:	Weight by volume
ZP	:	Zona pellucida
µm	:	Micrometer
µg	:	Microgram
µl	:	Microliter

List of tables

List of figures

1 Introduction

Assisted reproductive techniques in equine have been developed to obtain pregnancies from sub-fertile mares and stallions and to salvage gametes after death as well. In recent years, these procedures have been used for clinical cases with repeated success (Carnevale and Maclellan 2006). Although *in vitro* fertilization (IVF) has had only limited advance in equine, intracytoplasmic sperm injection (ICSI) has provided a valuable method to achieve fertilization *in vitro* for this species (Squires *et al.* 1996). Ideally, embryos resulting from ICSI could be allowed to develop *in vitro* until the late morula or blastocyst stage of the development, when they would be transferred into recipient uteri, cryopreserved, or transferred back into the donor's uterus (Carnevale and Maclellan 2006). Nevertheless, development of equine embryos *in vitro* is not yet optimal; reported blastocyst rates remained low, typically ranging from 4 to 16% (Galli *et al.* 2007).

To improve the blastocyst yield after ICSI, additional exogenous activation stimuli that induce intracellular calcium spikes (e.g., direct current, calcium ionophore A23187, ionomycin, or ethanol) have been applied in different species (Abdalla *et al.* 2009). It has been reported that in hamsters, mice and humans, during ICSI the mechanical disruption of the oocyte itself seems to be sufficient to trigger the cascade of events of oocyte activation (Sansinena *et al.* 2007). In contrast, other species such as the cow (Abdalla *et al.* 2009), sheep (Shirazi *et al.* 2009) and pig (Tian *et al.* 2006) require assisted activation of the oocyte following sperm injection. To date, it is controversial whether the activation of equine oocytes is beneficial following the ICSI procedure, especially when conventional ICSI is the method of sperm injection.

As an important factor in any assisted reproduction technology, oocyte quality profoundly affects the competence of equine oocytes to develop into preimplantation competent embryos. Oocytes derived from slaughterhouse ovaries comprise a heterogeneous population with regard to the quality and size of the follicle from which they are recovered (Carnevale and Maclellan, 2006). Due to the limited availability of equine oocytes, irrespective of the differences in oocyte characteristics; all non-degenerated oocytes are generally collected and cultured in maturation medium (Torner *et al.* 2007). These oocytes differ in cumulus morphology and can be classified as

having expanded (Ex) cumulus cells or compact (Cp) cumulus cells. Certain numbers of studies performed so far in equine have focused on effects of initial cumulus morphology on developmental competence of oocyte. However, the results of the different studies are inconclusive, particularly regarding the ability of these two oocyte categories to reach the blastocyst stage after ICSI (Galli *et al.* 2007; Matsukawa *et al.* 2007).

There is no single standard selection criterion for equine oocytes (Hinrichs *et al.* 2005). With the aim of describing an intrinsic predictor of oocyte quality, brilliant cresyl blue (BCB) dye has been used effectively to measure the glucose-6-phosphate dehydrogenase (G6PD) activity of oocyte (Tiffin *et al.* 1991), without deleterious effects on blastocyst formation rate. The G6PD enzyme is active in the growing oocytes (Mangia and Epstein, 1975), but has low activity in oocytes that have completed their growth phase. The BCB test is based on the capability of the G6PD to convert the BCB stain from blue to colorless. This non-invasive intrinsic approach has been successfully used to identify the more competent oocytes in various species (Alm *et al.* 2005; Bhojwani *et al.* 2007; Manjunatha *et al.* 2007; Pujol *et al.* 2004; Rodriguez-Gonzalez *et al.* 2003; Rodriguez-Gonzalez *et al.* 2002). Moreover, it has been shown that BCB screened oocytes differ in various oocyte quality markers at the cellular (El Shourbagy *et al.* 2006; Torner *et al.* 2008; Wu *et al.* 2007) and molecular (Ghanem *et al.* 2007; Torner *et al.* 2008) level. However, the predictive value of the BCB test for developmental competence of equine oocytes has not been studied so far and was, therefore, the focus of the present work.

The Zona pellucida and its properties as a reliable parameter for the assessment of oocyte quality has been a topic of recent debate in assisted reproduction. The introduction of a newly developed microscopy technique based on the detection of polarized light generated by birefringent cell structures was a great step forward since non-invasive analysis of zona pellucida in live oocyte/embryo became possible (Oldenbourg, 1996). The underlying concept is that polarization microscopy allows the distinction of three layers within the zona pellucida of oocytes and among them; the inner layer exhibits the highest amount of birefringence (Pelletier *et al.* 2004). For the past few years, several authors have focused on the relationship between these birefringent characteristics of the zona pellucida and oocyte/embryo competency to develop into blastocyst stage, healthy fetuses and live born babies (Madaschi *et al.*

2009; Montag *et al.* 2008; Rama Raju *et al.* 2007; Shen *et al.* 2005). Nevertheless, using this microscopic technique, the characteristic feature of equine zona pellucida and its correlation with quality-associated parameters of oocyte has still to be determined.

Therefore, the main objectives of this study are:

1. To study the effect of assisted oocyte activation (AOA) with calcium ionophore A23187 on developmental competence of equine oocytes after ICSI.

2. To re-evaluate the effect of cumulus morphology on meiotic and developmental competence of equine oocytes after ICSI.

3. To investigate the effect of G6PD activity on meiotic and developmental competence of equine oocytes after ICSI.

4. To analyze the zona pellucida of equine oocytes of different quality and maturational status quantitatively by polarization light microscopy.

2 Literature review

At the beginning of the 20th century, the embryologist E. B. Wilson formulated the increasingly emerging biological principle that development of the embryo is largely dependent on the process by which the oocyte is generated (*"embryogenesis begins during oogenesis")*, inspiring future generations of scientists.

Although at the moment we do have enough evidence that the spermatozoon markedly provides an essential contribution to the generation of a new individual (Barroso *et al.* 2009), the central and crucial role of the oocyte in the establishment of the fate of the embryo is nevertheless indisputable. This role is progressively acquired during oogenesis, through a range of cellular and molecular attributes that provide the oocyte with the ability to complete meiosis, ensure mono-spermic fertilization, decondense the sperm head, undergo preimplantation embryo development, and accomplish specific post-fertilization events as a consequence of the action of stored maternal mRNAs and proteins (Dedieu *et al.* 1996).

Accordingly, developmental failure is not exclusive due to oocyte competence and is attributable to multiple factors, including sperm dysfunctions (Barroso *et al.* 2000) and compromised uterine receptivity (Tranguch *et al.* 2006) as well. However, it is a fact that through the assisted reproduction techniques only a minority of equine oocytes are intrinsically able to generate *in vitro* embryos with full developmental competence (Carnevale and Maclellan 2006; Choi *et al.* 2008; Galli *et al.* 2007). Indeed, our immediate ability to improve oocyte quality is integrally tied with and dependent on the availability of reliable markers of oocyte quality (Combelles and Racowsky 2005). Therefore, it would be of paramount importance to reliably and non-invasively describe classification criteria for selection of oocytes with superior developmental competence. Attempts to determine morphological attributes associated with oocyte quality have achieved very limited success, since morphological criteria are subjective and insufficient to distinguish oocytes/embryos having different abilities to bring about a full-term development. Promisingly, recent studies have suggested that analyses conducted at the cellular and molecular level on diverse expressions of oocyte competencies could provide novel cues for the definition of more objective and convenient criteria of oocyte quality (Wang and Sun 2007). Most of these approaches

for oocyte selection cannot be applied directly, because their application does not preserve cell viability. Nevertheless, it has the potential to establish cellular standards for the generation of competent oocytes *in vivo* or *in vitro*. Little is known about the efficiency of such approaches in relation to assisted equine reproductive technologies, however. Thus, the criteria to be investigated here include new approaches for assessment of equine oocyte quality. This may enable us to identify a robust system for assessment of oocyte viability for *in vitro* embryo production (IVEP) system in equine.

2.1 Oogenesis

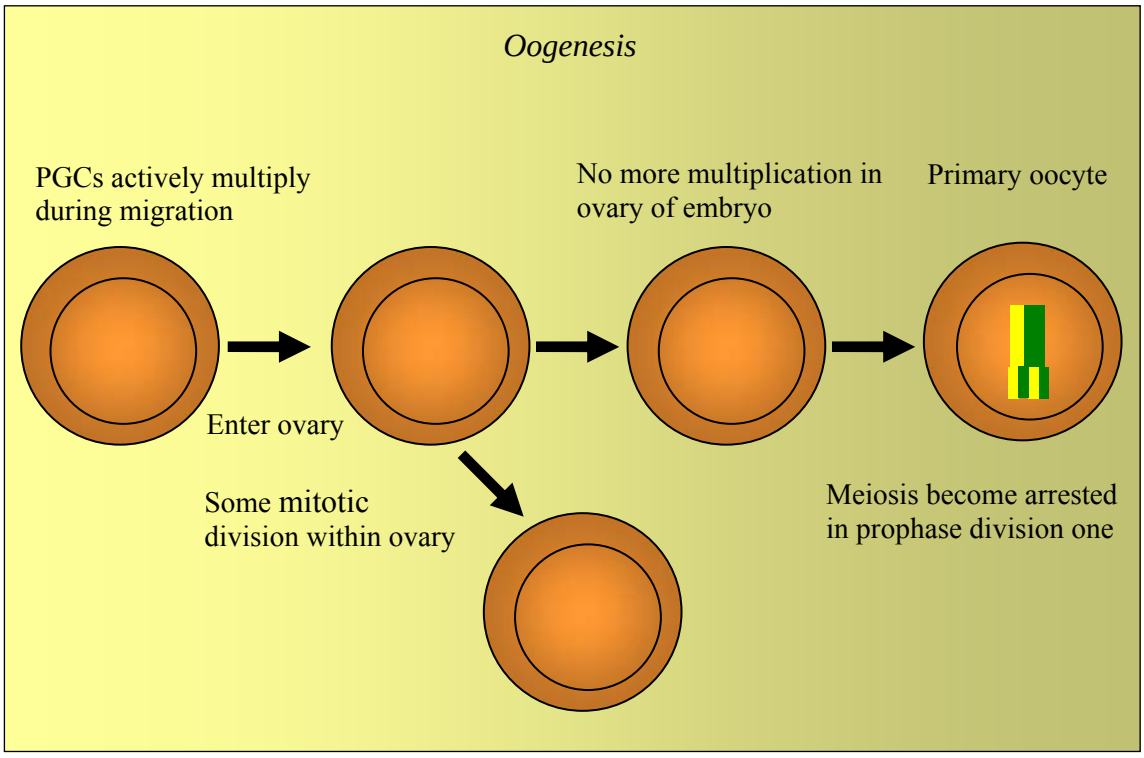

Figure 1: The sequential of stages in mammalian oogenesis, from multiplication of PGCs to the formation of primary oocytes.

Oogenesis is defined as the process of generation of oocytes, the female gametes and is a complex process that is initiated during fetal development, but not completed until after fertilization. The mechanism of oogenesis varies among species, but is similar in general pattern. Primordial germ cells (PGCs) migrate from the developing hindgut to the site (genital ridge) where the gonad will form and then divide by mitosis as they colonize the ovary (Figure 1). The germ cells, now called oogonia, undergo several

rounds of division and then enter meiosis, becoming oocytes. All oocytes of the fetal ovary arrest in diplotene stage of meiotic prophase I (Bendel-Stenzel *et al.* 1998; Borum 1961; Saito *et al.* 2004). Shortly after birth, oocyte nests dissociate and granulosa cells surround individual oocytes forming primordial follicles.

2.2 Folliculogenesis

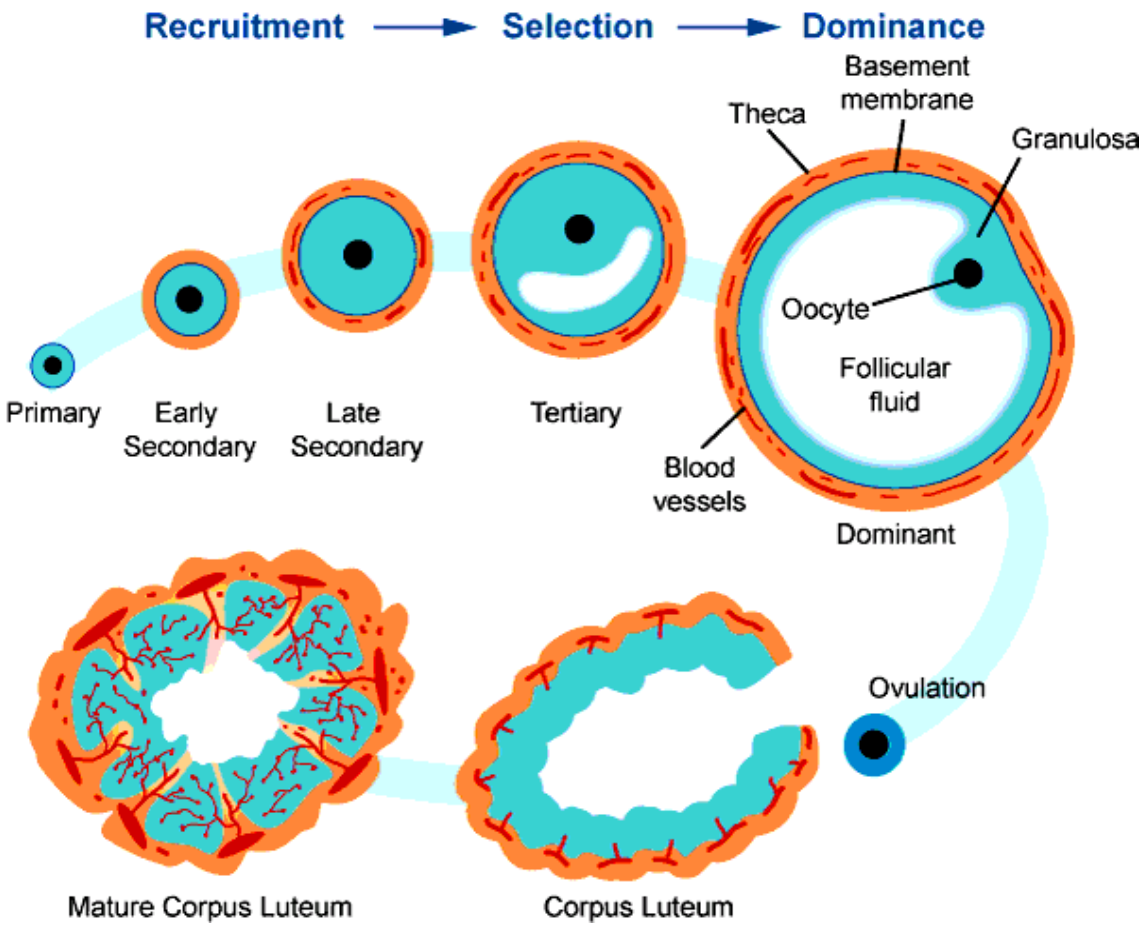

Figure 2 illustrates the categorization of stages during the process of follicular maturation and ovulation (from development of primary germ cells to production of a mature germinal vesicle stage oocyte in a dominant follicle) followed by formation of the corpus luteum (Fraser and Duncan 2009).

Ovarian folliculogenesis is a process of the follicular development starting from the smallest primordial follicles recruited into the growth pool through primary, preantral, and antral stages to the largest Graafian or preovulatory follicles (POFs) that ovulate (Figure 2) in response to the luteinizing hormone (LH) surge (Gougeon 1996; Knight

and Glister 2001). After ovulation, the remaining granulosa cells and theca cells within the POFs differentiate into luteal cells to form the corpus luteum (CL). However, in each cycle, only one or very few of the primordial follicles initially recruited are destined to become POFs; most of them undergo apoptotic death (atresia), mainly at the early antral stage.

Follicle-stimulating hormone (FSH) is the major survival factor that rescues the early antral follicles from atresia. The entire folliculogenesis process involves a complex network of paracrine, autocrine, and endocrine signals, including sex hormones, in a stage-dependent manner (Hu *et al.* 2004).

2.3. Dynamics of follicular growth and regression in mare

2.3.1 Reproductive cyclicity

The equine species exhibits distinct ovulatory and anovulatory seasons. The estrous cycle in mares is divided into 4 phases: (i) *Proestrus*, when an ovulatory follicle develops, (ii) *Estrus*, the period of 'sexual receptivity' during which time final follicular maturation and ovulation occurs, (iii) *Metestrus*, when the corpus luteum (CL) forms, and (iv) *Diestrus* when the CL is actively producing progesterone (Baerwald 2009). The follicular phase encompasses about 1/3 of the cycle (~7days). The lifespan of the dominant ovulatory follicle, defined as the time from emergence of the dominant follicle to ovulation, is ~14 days of the 21 day estrous cycle in horses (Ginther *et al.* 2004).

2.3.2 Initiation of follicle development

In equine, the primordial follicles, which have become arrested at meiotic prophase I during embryonic development, begin to leave the resting pool and enter into the pre-antral growth phase. Pre-antral follicles are believed to develop independent of gonadotropic support, but become more responsive to gonadotropins at the early-antral stages. With the onset of puberty, the cyclic growth of antral follicles occurs as a result of changes in hypothalamic and pituitary gonadotropin hormones (Craig *et al.* 2007).

2.3.3 Follicle recruitment

Follicle 'recruitment' describes the cyclic growth of a cohort of antral follicles. It is well documented that two waves of follicles develop during the equine estrous cycle (Ginther 1993). The wave pattern of follicular development refers to the periodic, synchronous, growth of a group of antral follicles. The emergence of each follicle wave is preceded by a rise in circulating FSH during the equine 'interovulatory intervals' (IOIs) (Ginther and Bergfelt 1992).

2.3.4 Selection of the dominant follicle

Follicle selection in mares is the process in which a single follicle from the recruited cohort undergoes preferential growth, while all other follicles in the cohort fail to develop and undergo atresia. The follicle that is selected is referred to as the 'dominant' or 'privileged' follicle. Atretic follicles have been termed 'subdominant' or 'subordinate' follicles.

A mare is monovular, which means that a single follicle from the pair of ovaries (as compared to multiple follicles) is selected for continued growth and ovulation. Follicles from the recruited cohort undergo a common growth phase prior to selection (Ginther *et al.* 2001a). The term 'deviation' describes the point at which divergence in the growth of the dominant and subordinate follicles occurs (Ginther *et al.* 1997). Deviation of the dominant follicle from the largest subordinate follicle occurs at a diameter of 23 mm in mares (Ginther *et al.* 2001a). Deviation in the ovulatory wave occurs, on average, 4 days after emergence of the largest follicle at 13 mm in mares (Ginther *et al.* 2004). The dominant follicle maintains a constant growth rate throughout the deviation process, while the subordinate follicles exhibit a reduction in growth rates (Ginther *et al.* 2001a). There is evidence in women, mares and heifers, that the dominant follicle exhibits an early size advantage over other follicles in the cohort, enabling it to establish dominance before the subordinate follicles reach a similar diameter (Ginther *et al.* 2001a; Ginther *et al.* 2004).

It has been reported that selection of a dominant follicle can occur 1-3 times during an IOI in mares. Major and minor waves of follicle growth have been characterized in mares. Major waves (Mw) are defined as those in which a dominant follicle is selected

for preferential growth, and minor waves (mw) are those in which dominance is not manifested (Baerwald 2009). A mare exhibits major and/or minor waves during the IOI and the final wave of the IOI is a major ovulatory wave. In other words, the preceding wave is usually an anovulatory minor or major wave; however, ovulation of the preceding wave has been reported (Bergfelt and Ginther 1993; Ginther 1990).

2.3.5 Physiological mechanisms underlying follicle dominance

Physiological selection of a dominant follicle is a complex phenomenon, which is regulated by endocrine, autocrine and paracrine factors. The rise in FSH responsible for stimulating follicle recruitment begins to decline in association with selection of the dominant follicle and atresia of subordinates (Ginther *et al.* 1997; van Santbrink *et al.* 1995). The duration and magnitude of the FSH rise above a critical threshold has been shown to determine the number of follicles selected from the recruited cohort. Similar to FSH, mares exhibit a small but significant transient increase in circulating LH around the time of deviation (Baerwald 2009).

The dominant follicle exerts both morphologic and functional dominance over other follicles of the wave. Concentrations of circulating estradiol increase with continued growth of the dominant follicle (van Santbrink *et al.* 1995). The follicular fluid of dominant follicles in mares contains greater estradiol and progesterone levels and lower androstenedione levels than subordinate follicles (Donadeu and Ginther 2002).

Dominant follicle estradiol production is believed to provide negative feedback on FSH and induce the formation of granulosa cell LH receptors, which initiates a shift from FSH to LH dependency in the dominant follicle (Gastal *et al.* 2000; Gastal *et al.* 1999; Xu *et al.* 1995; Yamoto *et al.* 1992). The dominant follicle then becomes unique in its ability to thrive despite decreasing FSH, while the subordinate follicles regress (Ginther *et al.* 2000). Follicles within the recruited cohort produce inhibin, which further acts to suppress FSH. The role of activin and follistatin in regulating follicle selection in domestic farm animals has been evaluated. However, results are inconclusive and further investigations are necessary (Baerwald 2009; Ginther *et al.* 2001b).

2.3.6 Preovulatory follicular growth

The dominant follicle grows at a rate of approximately 2.7 mm/day in mares following its selection until it ovulates at mid-cycle (Gastal *et al.* 1997). The dominant follicle in mare ovulates at a diameter of approximately 45 mm (Baerwald 2009). Growth of the ovulatory dominant follicle results in a rapid elevation of circulating estradiol (Gastal *et al.* 1999). Estradiol production peaks three days before the LH surge in mares. Estradiol provides a positive feedback at the hypothalamus and pituitary to stimulate the release of LH necessary for inducing ovulation. As LH levels rise in the late follicular phase, the preovulatory follicle shifts from an estrogen-secreting state into progesterone secreting state and transformation from follicular cells to luteal cells begins (Baerwald 2009).

2.4 Assisted reproduction in equine

Assisted reproduction technologies (ART) in equine include a number of procedures, all of which have the ultimate aim of assisting the 'infertile' mare to become pregnant and deliver a live offspring (Galli *et al.* 2007). The first application of ART in the horse dates back to the late nineteenth century with the establishment of the first equine pregnancies obtained by artificial insemination (AI) technique (Heape, 1898). However, the development of ART in equine has been relatively slow compared to other domestic animals, namely ruminants and pigs. The low number of abattoirs (Choi *et al.* 1993), lack of interest from horse breeders and breed associations (Galli *et al.* 2007) along with the absence of standard *in vitro* fertilization (IVF) protocol (Hinrichs 2005) have been the main reasons for this delay. Besides AI and embryo transfer, which are now well developed in the equine industry (Squires 2005), other technologies based on *in vitro* procedures of horse embryo production have emerged, but the success rates are still low compared to other species such as human and bovine.

2.4.1 Oocyte collection and *in vitro* maturation

In equine, a very limited availability of the oocyte is the major obstacle so far for research in the field of *in vitro* maturation (IVM) and related oocyte/embryo-based

biotechnologies (Choi *et al.* 1993). Although collection of oocytes from mares using a transvaginal ultrasonically guided follicular aspiration technique (ovum pick-up: OPU) has been reported (Goudet *et al.* 1997; Meintjes *et al.* 1995; Scott *et al.* 2001), number of oocytes yielded are low because most mares only produce one preovulatory follicle per oestrus (Bézard *et al*. 1995). Moreover, the anatomy of oocyte attachment to the follicle wall in the horse interferes with the efficiency of recovery methods both *in vivo* and *in vitro*. The cumulus has a close and broad attachment to the follicular wall, however, with cumulus cell extensions into an underlying thecal pad; this results in the surrounding cells firmly attaching the oocyte to the follicular wall (Hawley *et al.* 1995). Consequently, collection rates from immature follicles are often less than 50% (Blanco *et al.* 2009; Carnevale and Maclellan 2006). The low availability of preovulatory follicles suitable for aspiration has not been compensated accurately by developing treatments to 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). However, collection of immature oocytes from slaughtered mares has allowed extensive research on ART in equine. In these cases, the mare's ovaries can be harvested post-mortem and after transportation to the laboratory. They can be processed for oocyte recovery (Figure 3. A) and oocytes can be removed from the ovaries by various methods. Oocytes can be collected from follicles either by aspiration (Okolski *et al.* 1987) or by incision of follicles and scraping of the follicle wall with a curette (Figure 3. B) followed by extensive flushing to detach the cumulus–oocyte complexes (COCs) (Choi *et al.* 1993). Comparative studies have shown that scraping method yields more good quality COCs than aspiration (Alm *et al.* 1997; Dell'Aquila *et al.* 2001; Okolski *et al.* 1987).

After retrieval from the follicles, immature equine oocytes are capable of resuming meiosis in the *in vitro* culture system and progress from germinal vesicle (GV) stage to metaphase of the second meiotic division, as they would do during final follicle maturation *in vivo*. The first successful report of *in vitro* maturation of equine oocytes was that of Fulka and Okolski (1981). At that time, research on *in vitro* maturation of abattoir oocytes was at its peak, especially in ruminants. Since then a variety of oocyte maturation conditions have been evaluated using different culture media comprising TCM199, B2, Ham's F10 and DMEM-F12, supplemented with different concentrations of serum, hormones, follicular fluid or serum replacement. These conditions have

resulted in maturation rates varying from 20 to 85% (Galli *et al.* 2007). So far, multiple factors have been reported to affect *in vitro* maturation of horse oocytes.

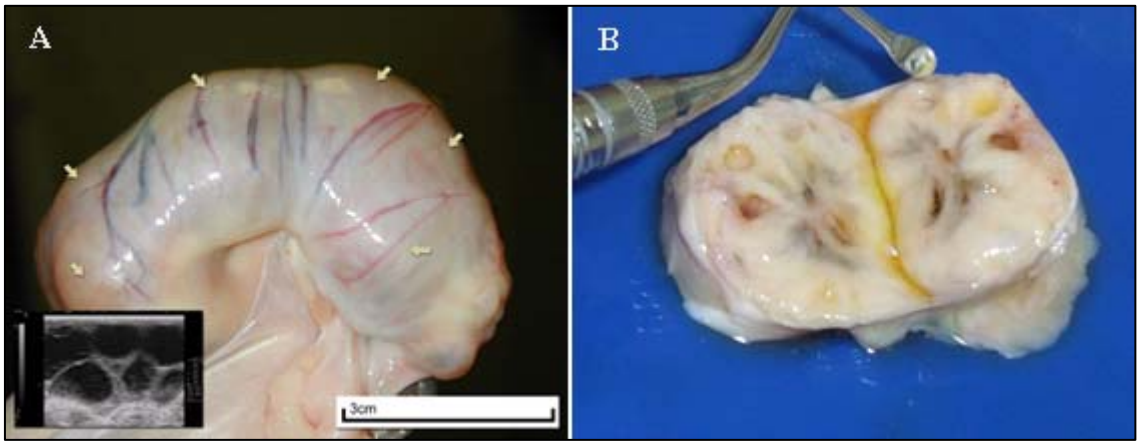

Figure 3: An equine ovary is shown with ultrasonographic image of the antral follicles, arrows representing the locations of the follicles (A). After aspiration of all visible follicles, the ovary was sliced with a scalpel blade and the granulosa layer of each follicle were scraped using a 0.5 cm curette (B).

The temperature at which ovaries were stored during transportation did not affect equine oocyte meiotic or developmental competence (Ribeiro *et al.* 2008) however, transportation time seems to play an important role in subsequent oocyte quality (Carnevale and Maclellan 2006; Matsukawa *et al.* 2007; Ribeiro *et al.* 2008). Moreover, the follicle size (Goudet *et al.* 1997) and the duration of maturation period (Hinrichs *et al.* 2005) are of major importance for the overall efficiency of *in vitro* maturation. There is little biochemical and morphological information concerning oocyte cytoplasmic maturation in equine. Nevertheless, the relationship of chromatin configuration (Hinrichs *et al.* 2005), mitochondrial aggregation and activity (Caillaud *et al.* 2005; Dell'Aquila *et al.* 2009; Torner *et al.* 2007) and cytoplasmic lipid droplets aggregation of the oocyte (Dell'Aquila *et al.* 2009) to its developmental competence has been described. Although, changes in nuclear and cytoplasmic maturation can be determined in details with advanced imaging procedures, the clinician relies primarily on gross morphology of the oocyte and cumulus cells to judge maturation and viability. As maturation is complete, cells of the corona complete their expansion, resulting in a 'sunburst' appearance and easier imaging of the oocyte (Carnevale and Maclellan 2006). However, the ultimate criterion for the cytoplasmic maturation and developmental

competence of an *in vitro* matured oocyte is its ability to be fertilized and to develop into a viable embryo.

2.4.2 Conventional *in vitro* fertilization

Efficient methods for oocyte recovery from live mares have engendered clinical interest in methods for fertilization *in vitro*. Interestingly, in the horse, in spite of the early success of *in vitro* maturation (Fulka and Okolski 1981), standard *in vitro* fertilization, is not efficient (Hinrichs 2005). To date, only two foals were reported as born from IVF and both were derived from *in vivo* matured oocytes collected by OPU from gonadotropin-stimulated donors (Bézard J 1992; Palmer *et al.* 1991). Until now, there is no published report of equine pregnancies derived from both *in vitro* maturation and conventional fertilization in vitro and as reported previously, the average fertilization rates are as low as 0-33% (Alm *et al.* 2001; Dell'aquila *et al.* 1996; Hinrichs *et al.* 2002; Zhang *et al.* 1990) and failure of IVF remains as a mystery yet. Inefficient sperm capacitation (Alm *et al.* 2001), changes in the zona pellucida (Dell'Aquila *et al.* 1999; Hinrichs *et al.* 2002) and incomplete *in vitro* maturation (Li *et al.* 2001) are among the most commonly proposed reasons for this failure. Although this latter aspect has been more recently addressed and resolved by using more suitable culture methods, but none of these has increased the efficiency of conventional IVF (Galli *et al.* 2007). The principal barrier to successful IVF in equine appears to be penetration of the zona pellucida by the sperm (Figure 4), since zona pellucida dissection (Choi *et al.* 1994) and drilling (Li *et al*. 1995) both markedly increase fertilization rates. Cumulus cell contributions to the zona pellucida have been documented for the equine oocyte, and interestingly it has been reported that during *in vitro* maturation the cumulus cells loose the ability to synthesize zona proteins. This results in alterations of the structure of the zona pellucida implying that in the horse the cumulus cells play a crucial role for zona integrity (Kolle *et al.* 2007). In turn, the most commonly proposed reasons for poor zona penetration are changes in the oocyte investments induced during IVM, and inadequate capacitation of stallion sperm *in vitro* (Tremoleda. 2003). Moreover, hardening of the zona pellucida during IVM, for example by premature cortical granule release, has been proposed as a possible barrier to sperm penetration, however preventing zona hardening does not improve penetration (Dell'Aquila *et al.* 1999).

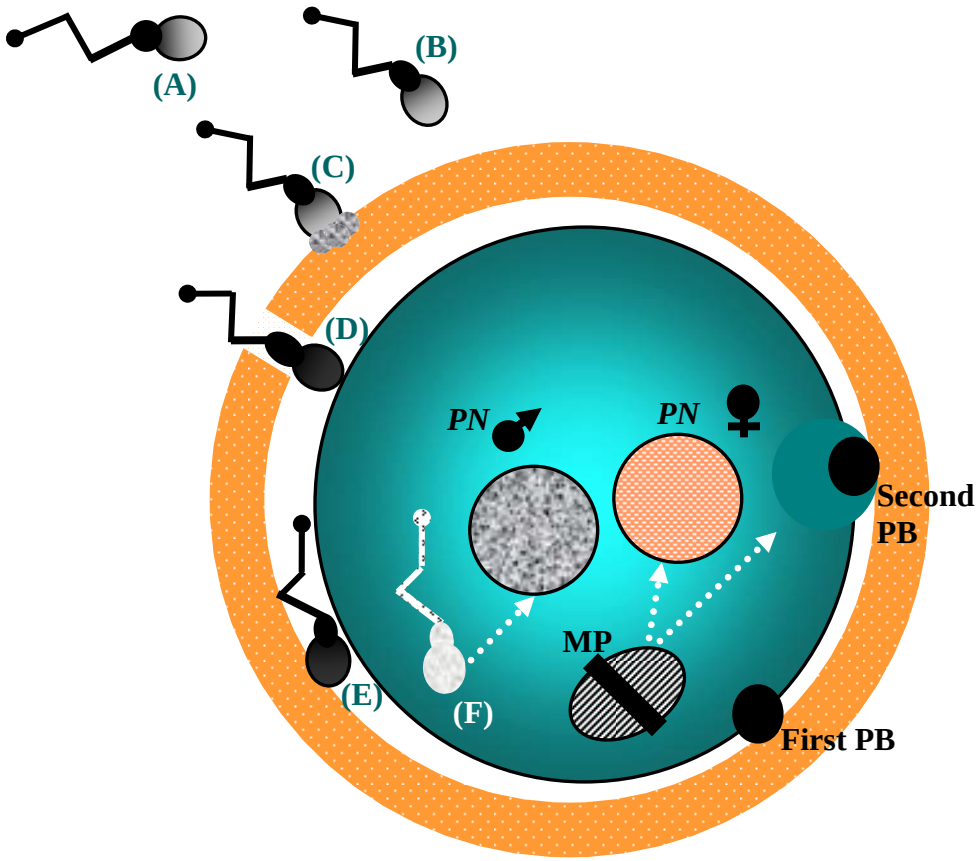

Figure 4: A schematic representation of the sequence of events that occur during mammalian fertilization. Inside the female genital tract, spermatozoa are activated during a process called capacitation (A). Capacitated sperm cells become hypermotile and able to bind to the zona pellucida (B) and thereby 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 (PB). Finally, the female and male pronuclei are formed as the final prelude to syngamy (Tremoleda. 2003).

With regard to capacitation of stallion sperm *in vitro*, compounds such as heparin, equine zona proteins, caffeine and lysophospholipids increase the percentages of capacitated and acrosome-reacted spermatozoa, but they do not facilitate sperm penetration into IVM oocytes (Graham 1996).

By contrast, calcium ionophore A23187 induces sperm capacitation, acrosome reaction and penetration of IVM oocytes (Alm *et al.* 2001; Hinrichs *et al.* 2002; Zhang *et al.* 1990) and was used in the production of the only two conventional IVF foals (Palmer *et al.* 1991). Overall, despite a long period of research into conventional IVF in equine, fertilization rates remain poor. There is little consistency in methodology and there is undoubtedly a need for a systematic investigation how a sperm binds to and penetrates an oocyte.

2.4.3 Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) consists of fertilizing a metaphase II (MII) oocyte by the direct injection of a single spermatozoon into the ooplasm with both the acrosome and sperm membrane intact (Figure 5). This technique was originally developed in the hamster (Uehara and Yanagimachi 1976). Thereafter in human assisted reproduction, Palermo and collaborators fine-tuned this technique in 1992 (Palermo *et al.* 1992); ICSI has been utilized mainly in order to solve male infertility specially in human. In laboratory or livestock species, ICSI is also used as a reproductive option to solve different problems but the focus mostly is not about low male fertility. The first successful report of ICSI in the horse was that of Squires *et al*. (1996); these workers injected four *in vitro* matured oocytes with sperm, and transferred them to the oviducts of recipient mares. They obtained one pregnancy that was carried to term.

By bypassing critical events such as zona binding and penetration, ICSI has proven to be a valuable and repeatable technique to produce equine embryos *in vitro*. Even early studies demonstrated relatively high fertilization rates after ICSI (Dell'Aquila *et al.* 1997a; Dell'Aquila *et al.* 1997b; Grondahl *et al.* 1997), although most of the resulting zygotes then arrested between the pronucleus and 4-cell stages and very few developed into blastocysts. The Piezo drill techniques for ICSI have been developed in the horse, which have resulted in high fertilization and cleavage rates (Choi *et al.* 2002; Hinrichs 2005). However, the successful application of ICSI to equine oocytes is hampered by the poor development of fertilized oocytes to the blastocyst stage (Galli *et al.* 2007). Therefore, the injected oocytes and early-cleaved embryos are used to be transferred into the oviducts of recipients to minimize any detrimental effect of embryo culture *in vitro*.

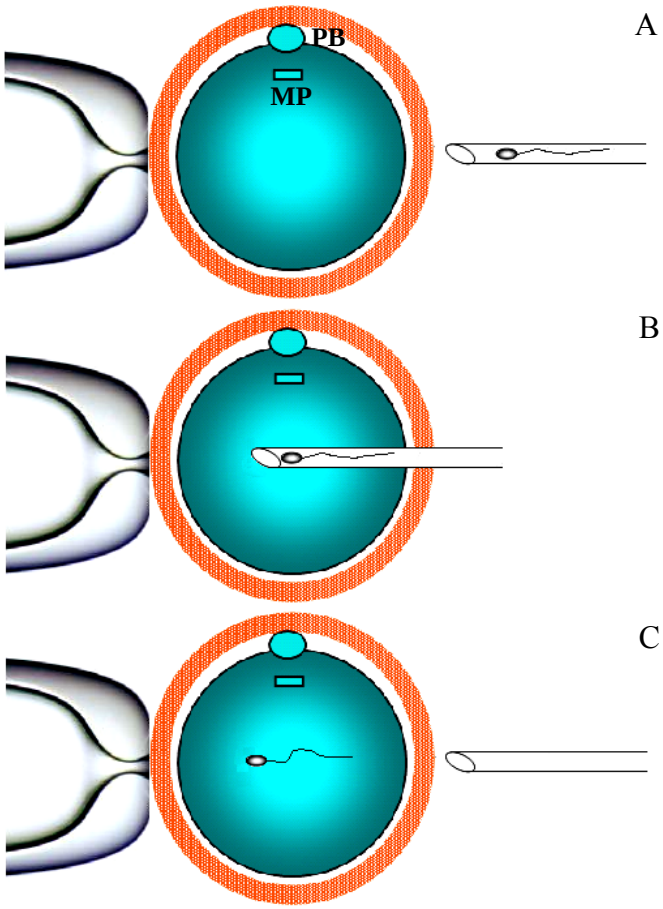

Figure 5: Schematic representation of ICSI of a MII oocyte. Initially, a single, motile spermatozoon is selected and immobilized by crushing its tail against the bottom of the petri dish using the injection pipette. The sperm is then aspirated tail-first into the injection pipette. The oocyte is held 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) (Tremoleda. 2003).

Early transfer of ICSI-derived embryos (produced from *in vitro* matured oocytes) to the mare oviduct immediately after injection resulted in a blastocyst recovery rate of 36%, indicating that ICSI can result in efficient embryo production if embryos are cultured in an optimal environment (Hinrichs 2005).

2.5 Factors affecting developmental competence of ICSI produced embryos

Amongst the multiple factors, which affect the developmental competence of ICSI-derived equine embryos, the two most important are technical parameters and parameters related to the oocyte.

2.5.1 Technical parameters and ICSI outcomes

Fertilization efficiency and blastocyst formation rate after ICSI can be affected by a variety of technical factors, including sperm injection techniques, necessity for assisted oocyte activation, and embryo culture conditions.

2.5.1.1 Piezo-assisted micromanipulation *vs.* conventional micromanipulation

Intracytoplasmic sperm injection (ICSI) has been used throughout the world as Palermo *et al.* (1992) introduced its clinical application. Since then, injection pipettes with a bevel and often a spike have been widely used for this procedure (Figure 6. A).
In 1995, a method using a Piezo-driven pipette was applied to mouse oocytes, which are easily disrupted after pricking, and a high success rate reported (Kimura and Yanagimachi 1995). In the Piezo-ICSI technique, a device is used to advance the injection of a needle into the oocyte by Piezo-electric movements (Figure 6. B). This method makes ICSI more efficient in comparison with conventional needle introduction, especially for species like the equine where the oolemma is difficult to penetrate. Increases in efficiency have been reported in mice (Yoshida and Perry 2007), humans (Yanagida *et al.* 1999) and cows (Devito *et al.* 2009) when ICSI using the Piezo drill was compared with conventional ICSI. Likewise, the Piezo application has resulted in higher rates of activation, fertilization and embryonic development after ICSI in horses and removed most of the inconsistency of the technique in this species (Hinrichs 2005).
During ICSI, it seems to be important that the sperm membrane is ruptured before injection, ensuring that sperm cytosolic factors, important for oocyte activation, are released (Devito *et al.* 2009; Hinrichs 2005). Potential reasons for higher efficiency of Piezo microinjection include both increased damage and permeabilization of the sperm

membrane, and more reliable breakage of the oolemma, ensuring that the spermatozoa is deposited in the cytoplasm (Choi *et al.* 2002).

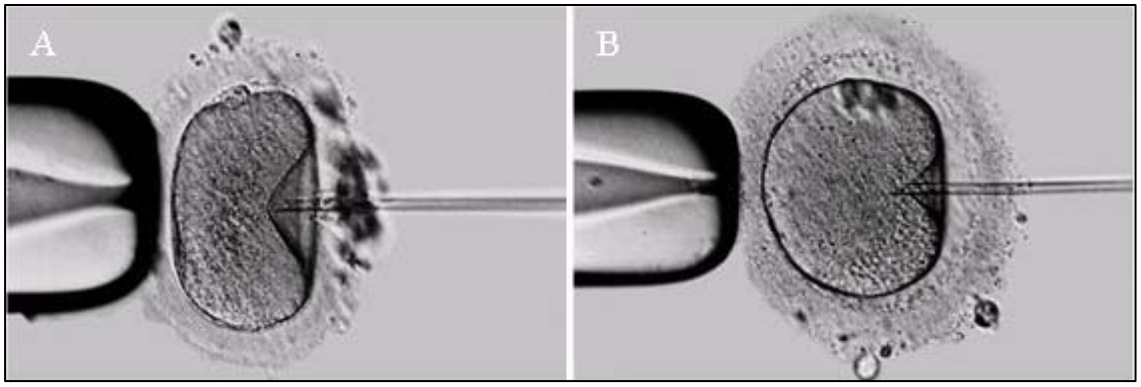

Figure 6: An injection needle is deeply inserted, but the zona pellucida and oolemma are not broken (conventional method). The oocyte is greatly deformed (A). After the tip of the injection pipette is brought into intimate contact with the zona pellucida at the 3 o'clock position, the sperm was moved carefully to the very tip of the pipette. Several Piezo pulses are administered to advance the pipette until its tip passes through the zona pellucida. (B) The pipette is advanced quickly, and manually to the opposite side of the cortex of the oocyte, and the oolemma is broken (as confirmed by rapid relaxation of the oolemma) using a light negative pressure without piezo-pulses (Takeuchi *et al.* 2001).

2.5.1.2 Assisted oocyte activation

A critical component of oocyte activation is the resumption of meiosis II that occurs during the time the sperm chromatin is decondensing in the oocyte cytoplasm; this resumption marks re-entry of the oocyte into the cell cycle. The cell cycle is controlled by a balance of the activities of kinases and phosphatases that modulate the activity of cellular proteins (Williams 2002). Prior to fertilization, the meiotic cell cycle of the mammalian oocyte is arrested at MII because of the presence of active maturation promoting factor (MPF) (Verlhac *et al.* 1993). Oocyte activation includes a large number of well defined morphological and biochemical endpoints, some of which occur within seconds or minutes of sperm–oocyte plasma membrane interaction, and some that occur over the course of several hours (Schultz and Kopf 1995; Yanagimachi 1994). One of the earliest events of oocyte activation is an increase in the level of intracellular calcium (Williams 2002). In oocytes from all mammalian species studied

to date, fertilization induces a series of species-specific intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations (Bedford *et al.* 2003) that are responsible for triggering the activation of MII oocytes (Figure 7).

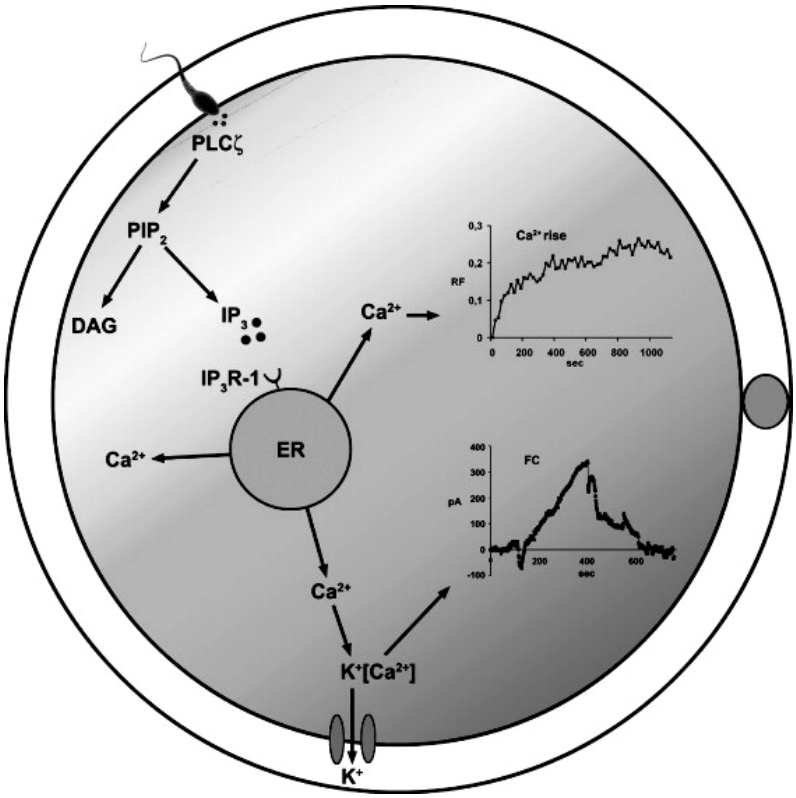

Figure 7: Scheme illustrating the most reliable mechanism of the first events, which occur at mammalian oocyte activation following fertilization. Sperm entry releases a sperm factor likely phospholipase C-zeta (PLCζ) that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) and produces 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 stimulates a specific receptor (IP_3R-1) which evokes a free Ca^{2+} $[Ca^{2+}]_i$ rise from the endoplasmic reticulum (ER). This Ca^{2+} activates Ca^{2+}-activated K^+ channels ($K^+[Ca^{2+}]$) causing a ion current (Fertilization Current: FC). RF = relative fluorescence pA = picoampere (Boni *et al.* 2007).

The primary role of calcium oscillations is to down-regulate the activity of the cell cycle regulatory MPF that leads to exit from the metaphase arrest (Lorca *et al.* 1993; Nanassy *et al.* 2008). Indeed, parthenogenetic activation can be induced through the elevation of cytoplasmic free calcium by several methods. Even, spontaneous activation by *in vitro* oocyte aging occurs (Nanassy *et al.* 2008). In several species such as human (Tesarik *et al.* 1994), rabbit (Perreault *et al.* 1988), hamster (Uehara and Yanagimachi 1976) and mice (Kuretake *et al.* 1996), the ICSI procedure itself is apparently sufficient to activate

the oocyte, as the sperm nucleus can undergo decondensation and formation of a pronucleus when injected into the oocytes. However, this technique has failed to produce physiological rates of embryonic or foetal development in the absence of exogenous activation stimulus in some domestic species such as bovine (Catt and Rhodes 1995), porcine (Garcia-Rosello *et al.* 2006), and ovine (Shirazi *et al.* 2009).

A great variety of procedures in different species have been used to induce artificial activation after ICSI such as ethanol (Horiuch *et al.* 2002), ionomycin (Rho *et al.* 1998a), calcium ionophore A23187 (Kolbe and Holtz 1999), electrical current (Lee *et al.* 2003), $CaCl_2$ (Probst and Rath 2003) and inositol-1-4-5-triphosphate (Garcia-Rosello *et al.* 2006), or a combination of protein synthesis inhibitors (for review see Garcia-Rosello *et al.* 2009). However, the most common assisted oocyte activation protocols in ICSI are activation with single agents such as ionomycin, calcium ionophore A23187, or activation with ionomycin or calcium ionophore A23187 in combination with 6-DMAP (Chung *et al.* 2000; Hou *et al.* 2009; Li *et al.* 1999; Pereyra-Bonnet *et al.* 2008; Rho *et al.* 1998b). In bovine, some authors have found that assisted activation of oocytes after injection is necessary to obtain cleavage (Abdalla *et al.* 2009; Devito *et al.* 2009). In pig, additional activation is beneficial to enhance the developmental competence of oocytes after ICSI (Garcia-Rosello *et al.* 2009). However, activation treatment after ICSI may induce abnormal development, and this may have a negative effect on embryo production *in vitro*. For example Rho *et al.* (1998a) reported that only 47-61% of ICSI-derived bovine blastocyst are diploid and that the rest have abnormalities such as mixoploidy and polyploidy. In human, as reported by Nasr-Esfahani *et al.* (2008), assisted oocye activation may be useful in selected patients when there is no or low fertilization potential.

Conflicting data have been reported for the horse concerning the necessity of activation treatment after ICSI. Although, it was shown previously that horse oocytes are capable of displaying [Ca2+]i transients in response to injection of sperm extracts, and, to a lesser degree, after ICSI (Bedford *et al.* 2004), Li *et al.* (2000) did not observe normal fertilization without assisted activation in their study. It has been reported that horse oocytes fertilized by ICSI inconsistently display $[Ca^{2+}]_i$ oscillations. This is not due to inadequate sperm factor release but to the inability of the oocyte to activate/process and/or provide the adequate substrate for the factor released by the sperm (Bedford *et al.* 2004). Accordingly, Matsukawa *et al.* (2007) suggested that activation treatment is not

essential for in vitro equine embryo production. Furthermore, the laboratories of Galli and Hinrichs reported high cleavage rates and blastocyst formation rates after ICSI without additional activation treatments (Galli *et al.* 2007; Hinrichs *et al.* 2005). In contrast, recently Pereyra-Bonnet *et al.* (2008) after conventional ICSI even using calcium ionophore A23187, followed by 3 h incubation prior to treatment with 6-dimethylaminopurine did not reach the blastocyst stage of development in their study.

2.5.1.3 Embryo culture conditions

Ideally, embryos resulting from ICSI could be allowed to develop *in vitro* until the late morula or blastocyst stage, when they would be transferred into recipient uteri, cryopreserved, or transferred back into the donor's uterus. However, development of equine embryos *in vitro* is not yet optimal (Carnevale and Maclellan 2006). Therefore, fertilized equine oocytes, produced by ICSI, are being used to develop effective equine embryo culture systems (Choi *et al.* 2003a). Many different culture conditions have been reported for preimplantation development of ICSI fertilized horse oocytes, including defined media such as G1/G2 (Choi *et al.* 2002), DMEM-F12 and CZB (Choi *et al.* 2004a) and modified SOF (Galli *et al.* 2007). Earlier work evaluated co-culture with somatic cells including Vero cells (Dell'Aquila *et al.* 1997b), epithelial cells of oviduct (Battut *et al.* 1991), cumulus cells (Li *et al.* 2001), granulosa cells (Rosati *et al.* 2002) or culture in conditioned media (Choi *et al.* 2001). In most of these systems, however, the blastocyst rate remained low, ranging from 4 to 16% of injected oocytes. In contrast, the culture of presumptive zygotes following ICSI *in vivo,* either in the mare oviduct or in the surrogate sheep oviduct, allowed much greater development to the blastocyst stage (reaching up to 36% of injected oocytes; Galli *et al.* 2007).

When cell number counts were compared among *in vivo* produced embryos and those produced by *in vitro* culture in a modified SOF medium, both on day 7 of development, the *in vitro* produced embryos had significantly fewer cell numbers, resembling a day 5 rather than a day 7 embryo (Matsukawa *et al.* 2007; Tremoleda *et al.* 2003).

Recently, an *in vitro* culture system has been developed using DMEM/F-12 medium under a mixed gas atmosphere that provides blastocyst development rates similar to those seen *in vivo* (Choi *et al.* 2006a; Choi *et al.* 2006c). The advantage of *in vitro*-

produced embryos is that it allows to freeze the embryos at the best stage, i.e., at the late morula or early blastocyst stage, to maximize survival post-thawing (Galli *et al.* 2007).

2.5.2 Oocyte parameters and ICSI outcomes

Multiple factors related to the female gamete are believed to be affecting the fertilization outcome and developmental competence of ICSI-produced embryos. Here some important factors that are involved in ICSI outcomes are reviewed.

2.5.2.1 Maternal age

Age of oocyte donor is a significant factor influencing oocyte competence and thus the efficiency of *in vitro* embryo production (Armstrong 2001). Aging is associated with physiological changes and eventual reproductive failure in the mare (Madill 2002). Oocyte quality has been implicated as a primary cause of reduced fertility in old mares (Altermatt *et al.* 2009), although aging results in multifactorial changes in the reproductive system (Carnevale 2008). Age-associated changes in equine fertility occur early in gestation, before the embryo enters the mare's uterus. Mare age did not affect fertilization rates, but embryos flushed from oviducts of old mares had delayed cleavage, fewer cells and more morphological abnormalities than those from young mares (Altermatt *et al.* 2009). To eliminate the confounding effects of age on the tubular reproductive tract, oocytes were collected from preovulatory follicles of young and old mares and transferred into oviducts of young, inseminated recipients. Significantly, fewer pregnancies resulted after the transfer of oocytes from old mares than from young, suggesting that oocytes from old mares were less competent for fertilization or embryo development (Carnevale and Ginther 1995). In that study, fertilization occurred *in vivo*, albeit in the reproductive tracts of young mares. Therefore, age-associated changes in oocyte investments, including the cumulus cells and zona pellucida of old mares' oocytes could negatively affect fertilization outcome (Altermatt *et al.* 2009). Delayed fertilization *in vivo* causes slower embryo development and higher embryo losses (Woods *et al.* 1990). Likewise, in human ART a definite correlation has been observed between maternal age and pregnancy outcomes with both naturally conceived and IVF-conceived children (Oehninger *et al.* 1995). The effects of maternal aging in

the horse have been compared with those in women (Altermatt *et al.* 2009; Carnevale 2008; Ginther *et al.* 2004). Transfer of oocytes from old donor mares into young recipient mares (Carnevale and Ginther 1995) and the use of young women as oocyte donors for older women (Navot *et al.* 1991; Sauer *et al.* 1992) have been used to elucidate oocyte competence. Although few studies have been conducted in mares to evaluate the effects of maternal age on oocyte quality (Altermatt *et al.* 2009; Carnevale 2008), negative effects on viability and subsequent developmental competence have been investigated well in women (Moffatt *et al.* 2002; Navot *et al.* 1991; Ng *et al.* 2003; Peluso *et al.* 1980; Wu *et al.* 2000) and other species (Lopes *et al.* 2009; Su *et al.* 2009).

2.5.2.2 Oocyte/cumulus morphology

Methods for oocyte classification and selection have not been standardized in the horse, and rates of embryo development of oocytes vary greatly among laboratories. To date, a number of attempts has been made to identify prognostic factors based on morphological characteristics of the equine oocyte that may allow a prediction of oocyte quality, fertilization rate and embryo development (Altermatt *et al.* 2009; Ambruosi *et al.* 2009; Carnevale and Maclellan 2006). Morphology of the COCs can be evaluated according to the expansion of the cumulus cells and/or appearance of the ooplasm, which reflect aspects of oocyte maturation and viability (Carnevale and Maclellan 2006).

At time of collection from the follicle, horse oocytes are usually classified as having an expanded (Ex) or compact (Cp) cumulus cells (Figure 8). Typically, half or greater than half of the recovered oocytes are with Ex cumulus cells and half or fewer are with Cp cumulus cells (Choi *et al.* 2006b). In other species such as ruminants and pigs, the presence of expanded cumulus is linked to the collection of oocytes from atretic follicles and these oocytes are generally discarded immediately because of their lower developmental capacity (de Loos *et al.* 1989).

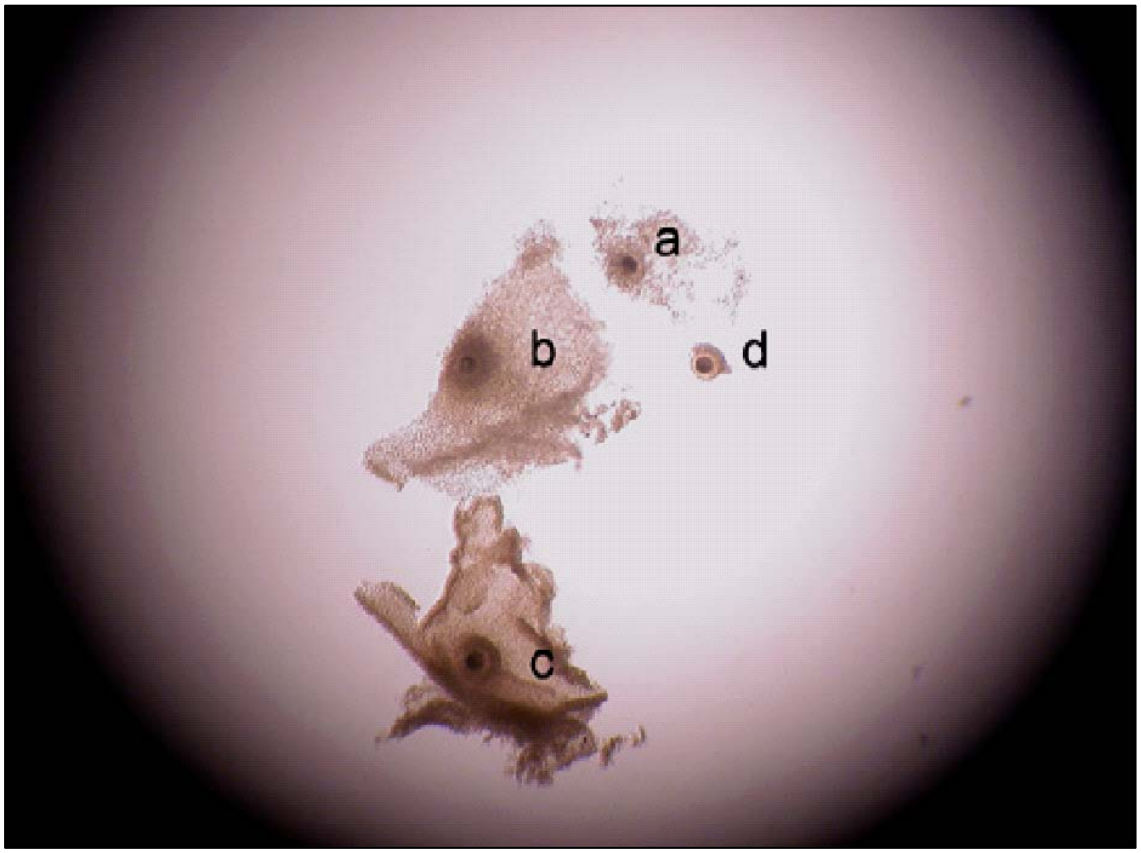

Figure 8: Oocytes collected from excised ovaries and classified as atretic/expanded (a), expanded (b), compact (c), and mostly denuded (d) (Carnevale and Maclellan 2006).

In horses, however, oocytes with expanded cumulus not only mature normally, but also are more capable of completing maturation than oocytes with a compact cumulus (Galli *et al.* 2007; Hinrichs *et al.* 2005; Torner *et al.* 2007). Accordingly, oocytes classified as Cp largely originate from small viable follicles 'juvenile', and have lower maturation rates (Galli *et al.* 2007), mature more slowly *in vitro* (Hinrichs *et al.* 1993), and take more time to produce proteins necessary for maturation than do Ex oocytes (Alm and Hinrichs 1996). Conflicting results have been published regarding the ability of these two oocyte categories to reach the blastocyst stage after ICSI (Galli *et al.* 2007; Matsukawa *et al.* 2007).

As maturation is complete, the ooplasm can be different shades of gray, often with a polarized appearance, caused by uneven distribution of lipid droplets (LDs) and organelles (Carnevale and Maclellan 2006). Accordingly, in a recent study Ambruosi *et al.* (2009) investigated the correlations between LDs polar aggregation and nuclear

maturation, fertilization and embryonic development after ICSI in equine oocytes recovered from slaughtered mares and matured *in vitro*. In that study, morphologically normal oocytes were selected after culture and categorized as having uniform (U) distribution (Figure 9. A) or polar (P) aggregation of LDs (Figure 9. B).

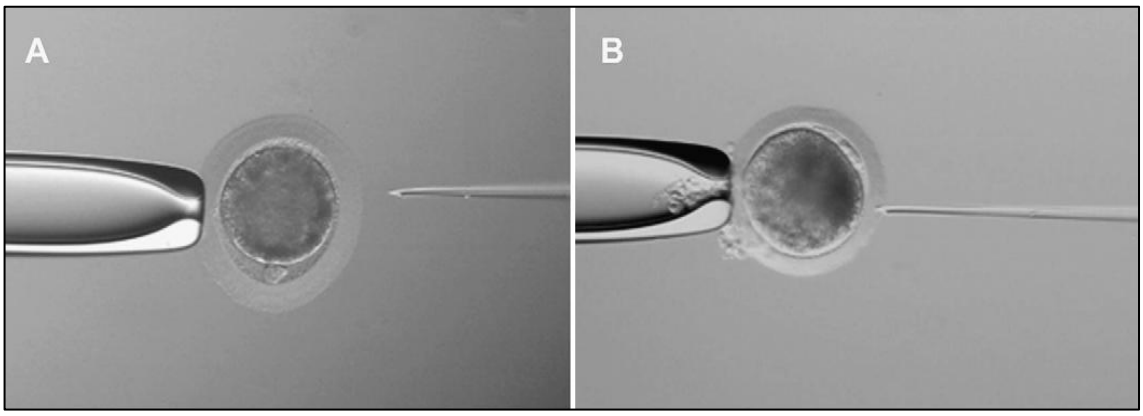

Figure 9: Equine oocytes evaluated for their lipid droplets (LDs) aggregation status while observed under an inverted microscope. (A) An oocyte with uniform (U) distribution of LDs within the cytoplasm is shown. (B) Show oocytes having polar (P) aggregation of cytoplasmic LDs (Ambruosi *et al.* 2009).

The maturation rate was significantly higher in P compared with U oocytes. However, no significant differences in the proportions of Day 3 embryos were seen between the two groups. Although cytoplasm appearance can provide valuable information regarding the oocyte's stage of maturation, detailed observation of the oocyte is always restricted by the presence of cumulus cells (Carnevale and Maclellan 2006). Taken together, COC morphology is a simple classification method that provides crude information for the assessment of oocyte quality and developmental competence for equine ART.

2.5.2.3 Follicle size, oocyte diameter and/or volume

Previous data supported the conclusion that oocytes derived from follicles with smaller diameter have lower developmental ability than those of larger-diameter follicles (Lonergan *et al.* 1994; Pavlok *et al.* 1992). In equine, for example, there is a significant effect of follicle size on maturation rate. The proportion of oocytes in MI or MII after 24-h culture increase with increasing follicle size (Hinrichs and Schmidt 2000).

However, follicles with the same diameter can be in very different physiological stages of development (Adams 1999; Hinrichs and Schmidt 2000). Several reports revealed that the developmental competence of an oocyte is related to the status of the follicle from which it is obtained, regardless of the dimensions of the follicle (Blondin and Sirard 1995; Seneda *et al.* 2001; Vassena *et al.* 2003). It appears, therefore, that follicular morphology including its size and/or status seems to be inadequate to predict oocyte developmental competence (Wang and Sun 2007).

A positive relationship has been found between oocyte diameter and developmental competence in cows (Arlotto *et al.* 1996), goats (Crozet *et al.* 1995), and pigs (Christmann *et al.* 1994). For instance, Hyttel *et al.* (1997) reported that bovine oocytes at diameter of about 100 µm shows full competence for the resumption of meiosis and at a diameter of about 110 µm full competence for the completion of meiotic maturation to MII is acquired. Full competence for sustaining embryonic development is acquired at an oocyte diameter of about 110 µm. Moreover, in their study, the percentage of blastocyst development was 20, 30, and 60% for oocytes of <100, 100–109, and 110–119 µm of diameter, respectively.

Oocyte volume is also an important indicator of oocyte competence. The relation between oocyte volume and the competence to fertilize (El Shourbagy *et al.* 2006) and cleave (St John 2002; Van Blerkom *et al.* 1998) has been well established in previous studies. Once the oocyte has acquired a critical amount of its final volume, it can reinitiate meiosis when it is retrieved from the follicle. This oocyte volume has been related to a certain follicular diameter in different species (Gardner *et al.* 2001). From all above mentioned, it is generally admitted that follicle size and oocyte diameter (or volume) are closely related, and as both increase, the developmental potential of the oocyte also increase, until reaching optimum size which differs within species (Albertini *et al.* 2003; Arlotto *et al.* 1996; Gandolfi *et al.* 2005).

2.5.2.4 Glucose-6-phosphate dehydrogenase activity

The rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PD) is a component of the pentose phosphate pathway (PPP). This pathway, which produces ribose-5-phosphate and NADPH, is considered as one of the major pathways for glucose metabolism. Ribose–5–phosphate is needed for the synthesis of nucleic acids, complex

sugar molecules, and other compounds called coenzymes that are essential for the functioning of various enzymes. NADPH provides hydrogen atoms for chemical reactions that result in the production of coenzymes, steroids, fatty acids, amino acids, and neurotransmitters (Figure 10). In addition, NADPH plays an important role in the synthesis of glutathione, a compound that is essential to the body's defense against damage from oxidative stress (Martin *et al.* 2003).

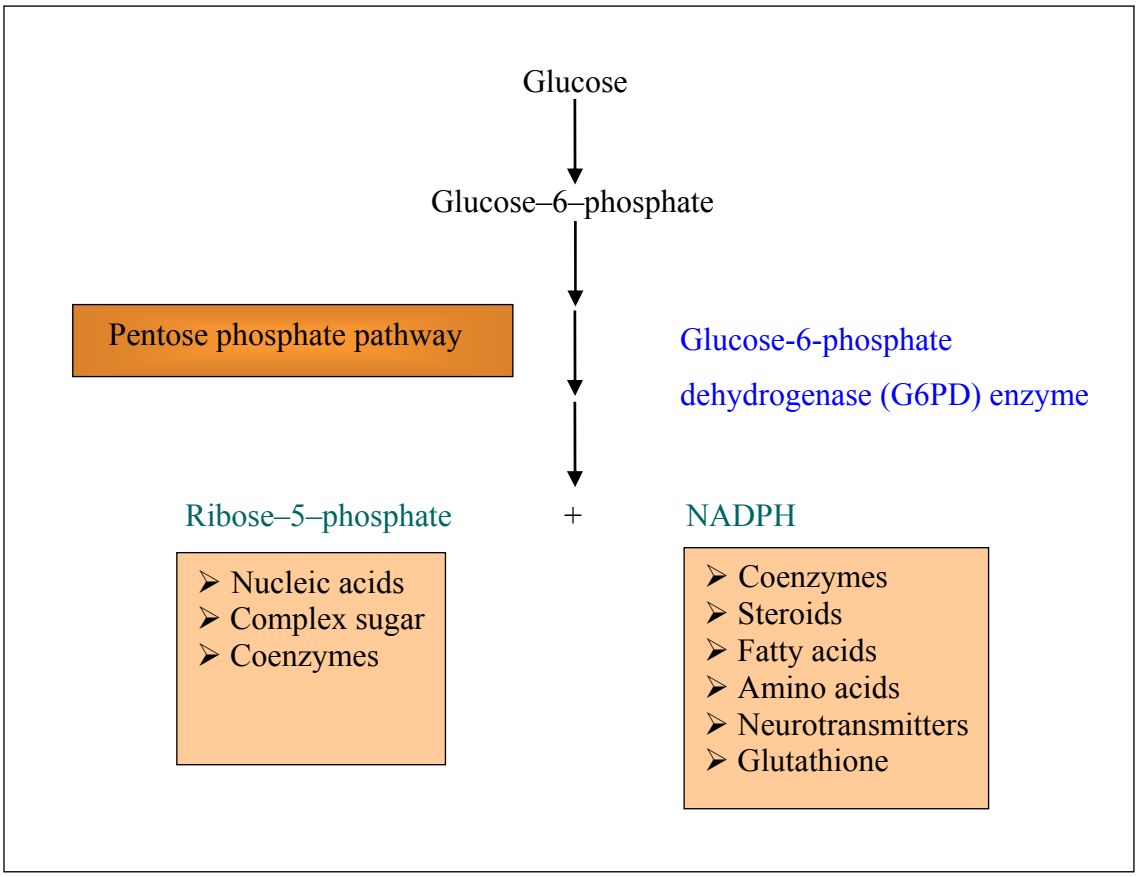

Figure 10 illustrates the Pentose Phosphate Pathway. Note the importance of G6PD in the production of ribose-5-phosphate and NADPH (Martin *et al.* 2003).

2.5.2.4.1 G6PD activity for prediction of oocyte quality

Immature oocytes are known to synthesize a variety of proteins, among them, G6PD (Wassarman 1988a). G6PD is synthesized and accumulates during oocyte growth phase (Mangia and Epstein 1975). The activity of this protein is decreased once growth phase has been completed and oocytes are then likely to have achieved developmental competence (Tian *et al.* 1998). Brilliant cresyl blue (BCB) is a dye that can be broken

down by G6PD (Tian *et al.* 1998). Thus, oocytes that have finished their growth phase show a decreased G6PD activity and exhibit a cytoplasm with a blue coloration (BCB+), while growing oocytes are expected to have a high level of active G6PD that resulted in colorless cytoplasm (BCB–), after incubation in media supplemented with BCB dye (Figure 11).

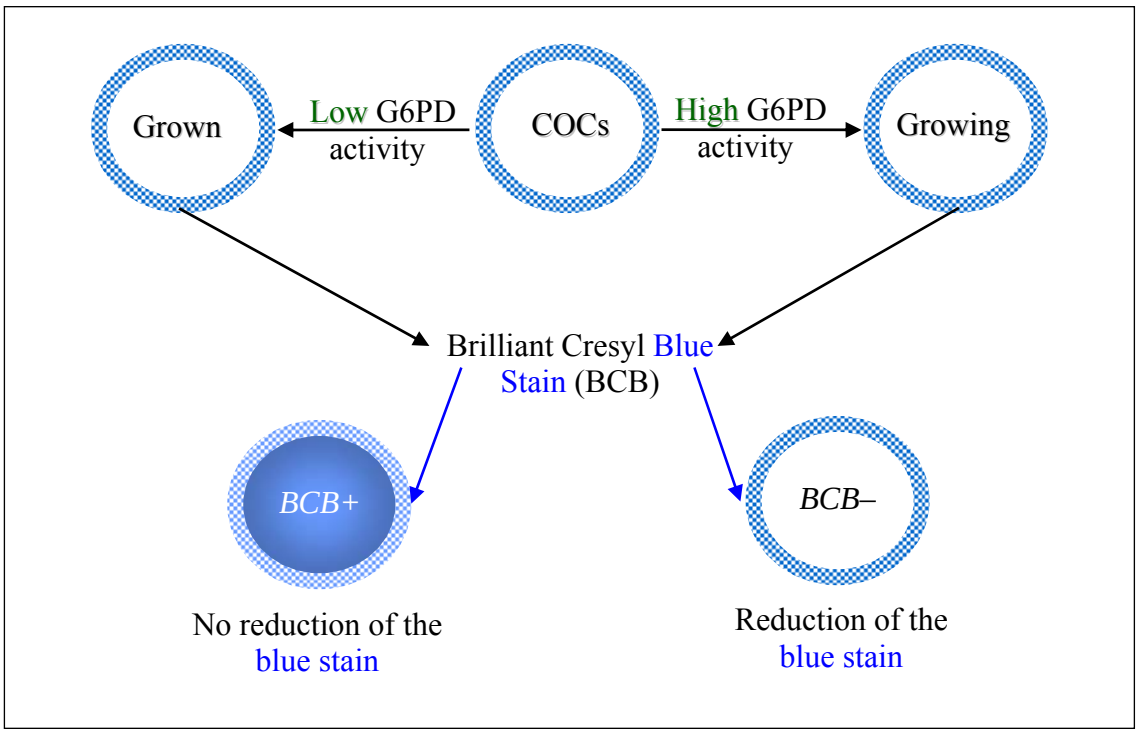

Figure 11: A schematic representation of the differentially stained cumulus-oocyte complexes COCs after exposure to BCB stain (BCB+, blue-colored; BCB–, unstained).

The BCB test has been successful in selecting pig oocytes for IVM–IVF (Ericsson *et al.* 1993). In prepubertal goat oocytes, Rodriguez-Gonzalez *et al.* (2002) showed that the BCB test permitted the selection of oocytes with larger diameters, higher percentages reaching Metaphase II, higher percentages of IVM–IVF oocytes with two pronuclei (normally fertilized) and higher embryo development up to the morula and blastocyst stage compared to oocytes selected exclusively by morphological criteria. The BCB test selects larger and more competent heifer oocytes for *in vitro* embryo production than conventional morphological criteria (Pujol *et al.* 2004). In addition, it has been shown that oocytes classified based on BCB test differ in their developmental potential to reach blastocyst stage (Alm *et al.* 2005) and efficiency in utilization for somatic cell nuclear transfer (Bhojwani *et al.* 2007) as well. The staining of buffalo oocytes with BCB stain

before IVM identified developmentally competent oocytes for *in vitro* production of embryos (Manjunatha *et al.* 2007). In mice, BCB staining was used efficiently for oocyte selection; however, the competence of the BCB+ oocytes may vary with oocyte diameter, animal sexual maturity and gonadotropin stimulation (Wu *et al.* 2007). Regarding the molecular and subcellular characterization of oocytes screened with BCB staining, it was reported that oocytes selected based on this method differ in various oocyte quality markers like cytoplasmic volume and mitochondria DNA copy number (El Shourbagy *et al.* 2006). In addition, in bovine less competent BCB– oocytes exhibited a delay in mtDNA replication due to the delayed onset of expression of their nuclear-encoded replication factors. For example, replication factor expression decreased during IVM in the BCB+ oocytes, and mtDNA copy number was higher in these oocytes than in BCB– oocytes on Days 1 and 2, suggesting that the major increase in mtDNA copy number had already occurred by this time. However, BCB– oocytes increased their expression of replication factors during oocyte maturation, and a sharp increase in mtDNA copy number was observed on Day 3, suggesting that the majority of mtDNA replication in these oocytes was occurring during the IVM period (May-Panloup *et al.* 2005; Opiela *et al.* 2009; Spikings *et al.* 2007)

2.5.2.5 Zona pellucida

The plasma membrane of all mammalian eggs is surrounded with an extracellular glycoprotein coat, the 'zona pellucida' that is synthesized and deposited around the oocytes during folliculogenesis. A variety of functions has been attributed to the mammalian zona pellucida including species-specific gamete recognition and binding, initiation of the acrosome reaction of the spermatozoon, blocking of polyspermy, and maintaining integrity of the early embryo during oviduct transition (Haines *et al.* 1999; Pastor *et al.* 2008; Qi *et al.* 2002; Wassarman *et al.* 2004). The composition of the zona pellucida matrix (Figure 12) has been elucidated for various species and was shown to be composed of 3−6 or more glycoproteins depending on the species (Izquierdo-Rico *et al.* 2009). Recent studies have revealed that some mammals present a zona pellucida formed by four glycoproteins, e.g., human (Lefievre *et al.* 2004), rat (Hoodbhoy *et al.* 2005), and bonnet monkey (Ganguly *et al.* 2008). These four glycoproteins have been designated ZP1, ZP2, ZP3, and ZP4 (Izquierdo-Rico *et al.* 2009).

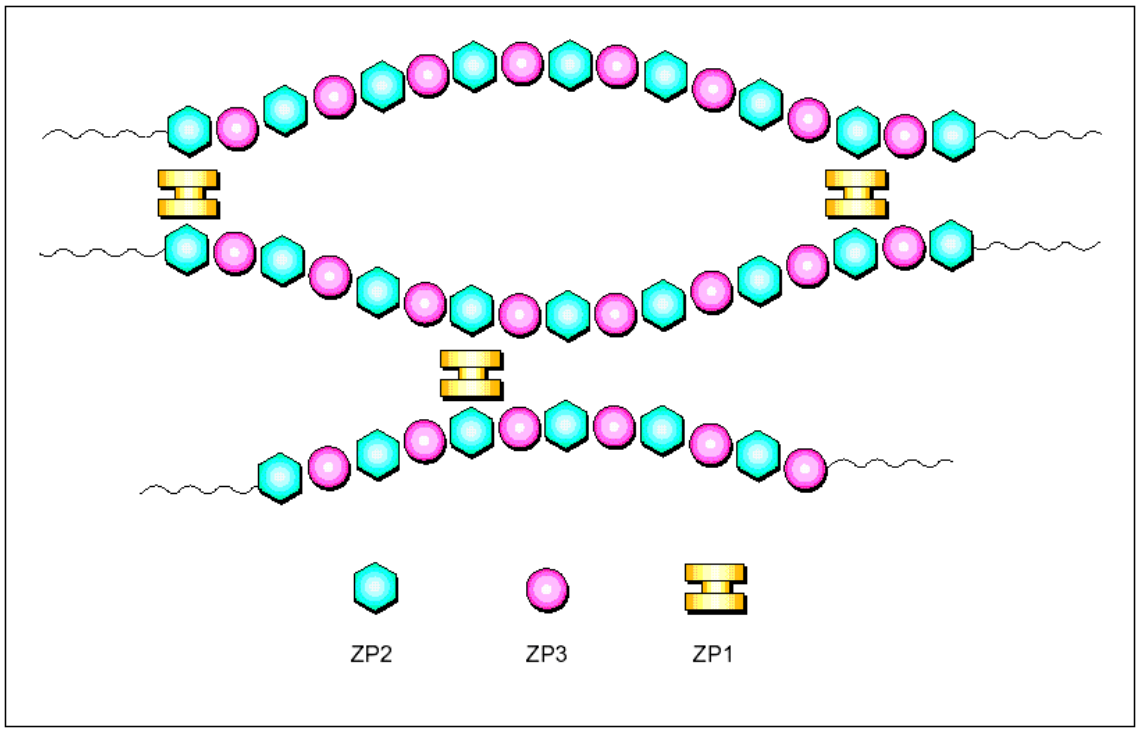

Figure 12: The current model of the zona pellucida as proposed by Wassarman and Redrawn from Wassarman (1988b). The major strands of the zona are composed of repeating dimers of proteins ZP2 and ZP3. These strands are occasionally crosslinked together by ZP1, forming a meshlike network (Green 1997).

Very scarce studies have been performed on the composition and structure of the equine zona pellucida. Nevertheless, up to now, three zona glycoproteins with apparent molecular masses of 60, 75, and 90 kDa have been identified (Miller *et al.* 1992). It has been demonstrated that equine zona glycoproteins share common antigens with bovine and porcine zona glycoproteins (Liu and Shivers 1982; Miller *et al.* 1992), which are named ZP2, ZP4, and ZP3 (Harris *et al.* 1994; Kolle *et al.* 2007). At the ultrastructural level, the three-dimensional highly ordered filament structure of the zona pellucida has been confirmed by studies in mammalian oocytes (Familiari *et al.* 1992; Green 1997; Oehninger 2003; Shen *et al.* 2005; Wassarman *et al.* 1999; Wassarman *et al.* 2004).

2.5.2.5.1 Polarized light microscopy technique for prediction of oocyte quality

The introduction of a newly developed microscopy technique based on detection of polarized light generated by birefringent cell structures has offered the possibility to visualize noninvasively the meiotic spindle and the zona pellucida, whose presence are

critical for fertilization and later developmental stages. The meiotic spindle is crucial for normal chromosome alignment and separation of maternal chromosomes during meiosis. It has also been used to examine spindle dynamics, detect spindle morphology, predict chromosome misalignment, monitor thermal control, and perform spindle transfer (Liu *et al.* 2000; Wang and Keefe 2002a; Wang and Keefe 2002b; Wang *et al.* 2002). Using polarization light microscopy the zona pellucida has been revealed as a three-layer structure, and arrangements of filaments within these layers are different from each other (Keefe *et al.* 1997; Pelletier *et al.* 2004). Due to the different orientation of these filaments, the three layers exhibit different birefringence patterns (Figure 13).

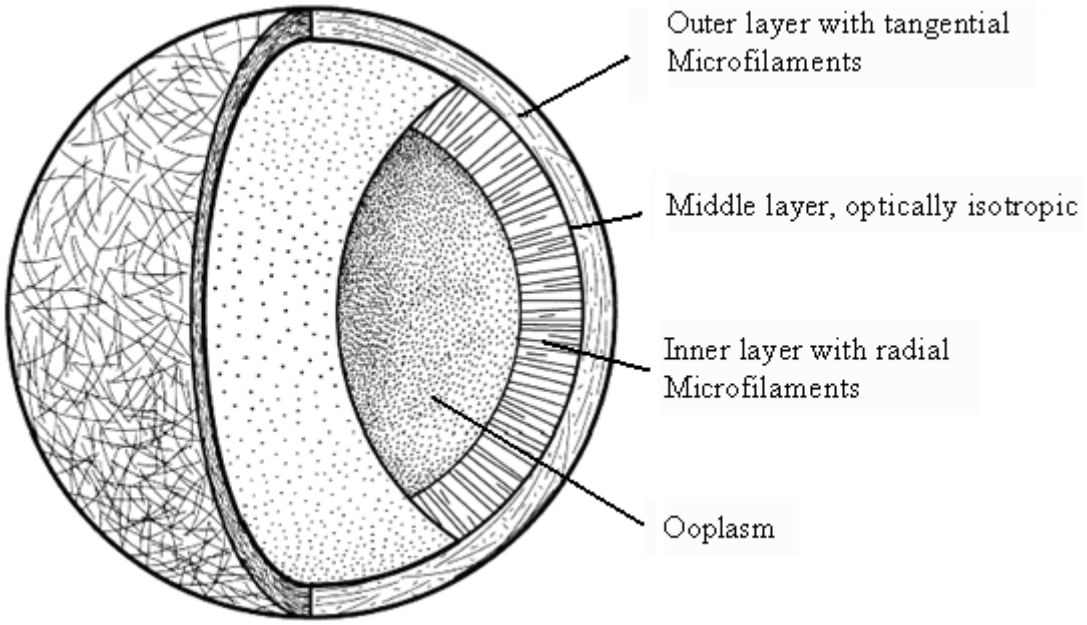

Figure 13: Schematic structure of the zona pellucida shows three-layer structure of the zona pellucida, and arrangements of filaments within these layers are different from each other (modified from Keefe *et al.* 1997).

The filaments in the inner layer of the zona pellucida are arranged radially and exhibit maximum birefringence, whereas filaments of the outer zona layer are oriented tangentially and retard the light to a lesser degree, yielding moderate birefringence (Oldenbourg 1996). The inner and outer layers are separated by the middle layer, which exhibits least birefringence due to random orientation of filaments (Silva *et al.* 1997).
Over the past few years, some authors have focused to establish a relationship between birefringent characteristics of the zona pellucida and subsequent embryonic

developmental competence of oocytes. Studies by Pelletier *et al.* (2004) suggested that the birefringence of the zona pellucida changes with different maturational stages of the oocyte and embryo. Shen *et al.* (2005) reported a variation in the mean birefringence of the inner layer zona of oocytes in conception and non-conception cycles with a higher mean birefringence of the inner layer zona in the oocytes of conception cycles. They also showed that the differences between mean birefringence of the middle and outer layer of the zona pellucida were not statistically significant. Hence, the birefringence of the inner layer of the zona pellucida has been proposed to have a positive predictive value as a marker for the selection of oocytes with higher developmental competence. In this respect, Rama Raju *et al.* (2007) found a correlation between zona birefringence and the potential of an embryo to develop to the blastocyst stage. Furthermore, results from recent studies demonstrated higher implantation, pregnancy (Madaschi *et al.* 2009; Montag *et al.* 2008) and live birth rates (Montag *et al.* 2008) when embryos derived from oocytes with high zona birefringence were transferred compare to those of low birefringence.

3 Material and methods

3.1 Material

3.1.1 Ovaries

For this study, equine ovaries were collected from mares of different breeds and unknown reproductive history, from a slaughterhouse in Brussels and transported to the laboratory, a distance of about 350 km.

3.1.2 Chemicals

Sigma-Aldrich Chemie GmbH (Munich)	Bovine Serum Albumin (BSA)
	Brilliant Cresyl Blue (BCB)
	Calcium chloride
	Calcium ionophore A23187
	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12)
	Dulbecco´s Phosphate Buffer Saline (DPBS)
	Fetal Calf Serum (FCS)
	Gentamycin
	Gentamicin sulfate
	Glycerol
	Heparin
	Hepes
	Hoechst 33258
	Hyaluronidase
	Magnesium chloride
	Medium 199
	Mineral oil
	Phenol red solution
	Polyvinylalcohol (PVA)
	Polyvinylpyrolidone (PVP)
	Potassium chloride

Sodium chloride
Sodium dihydrogen phosphate
Sodium hydrogen carbonate
Sodium lactate solution (60%)
Sodium pyruvate

3.1.3 Reagents and media

modified TALP	Sodium chloride	5.800 g
(1000 ml)	Potassium chloride	0.230 g
	Sodium hydrogen carbonate	2.100 g
	Sodium dihydrogen phosphate	0.034 g
	Hepes	2.380 g
	Magnesium chloride	0.310 g
	Calcium chloride	0.290 g
	Sodium lactate solution (60%)	3.86 ml
	Phenol red solution	2 ml
	Water add to	1000ml

Culture medium	DMEM/F-12	1000ml
(1000 ml)	Gentamycin	25 mg

Holding medium	HEPES-M199	15.00 g
(1000 ml)	Gentamycin sulfate	0.050 g
	Sodium pyruvate	0.350 g
	BSA	1.000 g
	Millipore water add to	1000 ml

Maturation medium	DMEM/F-12	1000 ml
(1000 ml)	Gentamycin	25 mg

3.1.4 Equipment

Bench scale (2007 MP)	Sartorius; Germany
Carbon dioxide incubator (Heracell 150)	Thermo Heraeus, Germany
Carbon dioxide incubator (Innova CO-48)	New Brounswick scientific, UK
CellTram® Air/Oil/vario Microinjectors	Eppendorf, Germany
Centrifuge	Hermle (Wehingen, Germany)
Flaming/Brown Micropipette Puller (P-97)	Sutter Instrument, USA
Four-well dish	Nunc (Roskilde, Denmark)
Heating Dri Block (DB.3)	Techne, USA
Inverted microscope (DM-IRB)	Leica, Germany
Inverted microscope (TS 100)	Nikon, Japan
LCD monitor 8.4 inch (Digital Sight DS-L2)	Nikon, Japan
Mechanical micromanipulator	Leica, Germany
Micro forge	Bachofer laboratoriumsgeräte, Germany
Microinjection pipette for sperm injection	Cook ® K – MPIP – 1035 – 5 (Australia)
Osmometer (OSMOMAT 030)	GONOTEC GmbH, Germany
pH meter (MP230)	Labomedic, Germany
Small incubator (Brutschrank incubat)	Melag, Germany
Stereoscopic microscope (SMZ 2B)	Nikon, Japan
Vortex	Vortex-2 Genie, USA

3.1.5 Used softwares

EndNote X1	EndNote® and Reference Manager®
OCTAX Eyeware™	OCTAX Microscience GmbH, Altdorf, Germany
OCTAX ICSI Guard™	OCTAX Microscience GmbH, Altdorf, Germany
SAS® 9.2 Software	SAS Institute Inc., NC, USA

3.2 Methods

3.2.1 Oocyte collection

Ovaries were transported to the laboratory in saline solution at a temperature ranging from 26°C to 30°C within 3 h after collection. Adnexa were trimmed from the ovaries with scissors, and the ovaries were cleaned with sterilized gauze. The COCs were collected by follicle aspiration, followed by slicing the ovary and scraping the granulosa layer from opened follicles with a 0.5 cm bone curette. The contents of the curette were washed into individual petri dishes with Holding medium (TCM199, Sigma) supplemented with 4.43 mM HEPES, 33.9 mM NaCHO3, 2 mM pyruvate, 2.92 mM calcium lactate, 55 μg/ml gentamycin and 10% FCS. The contents of the dishes were examined using a dissection microscope at 10–20×.

3.2.2 Morphological evaluation of the cumulus-oocyte complexes

Classification of cumulus-oocyte complexes (COCs) was conducted as described previously (Hinrichs and Williams 1997). Briefly, COCs were classified as

Expanded (Ex) or Compact (Cp), depending on the expansion of both mural granulosa and cumulus cells. Oocytes with any sign of expansion of either the cumulus or the mural granulosa led to the classification of expanded (Figure 14. A). Oocytes with both compact cumulus and compact mural granulosa were classified as compact (Figure 14. B).

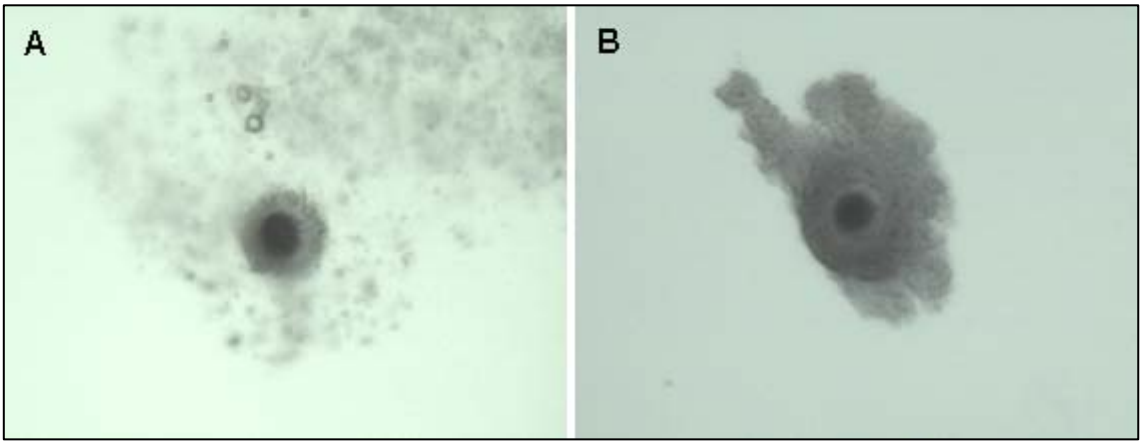

Figure 14: Photomicrographs of horse cumulus–oocyte complexes immediately after recovery from the follicle: (A) expanded (Ex), (B) compact (Cp).

3.2.3 Brilliant cresyl blue staining

The procedure performed for BCB staining was described elsewhere (Alm *et al.* 2005). Immediately after oocyte collection and morphological classification according to their initial cumulus morphology (Ex and Cp), equine COCs were washed 3 times in modified Dulbecco PBS (mDPBS, Dulbecco PBS supplemented with 0.4% (w/v) BSA (fraction V) 0.34 mM pyruvate, 5.5 mM glucose and 50 mg/ml gentamycin). Then the oocytes were subjected to 26 µM BCB (Sigma, B-5388) diluted in mDPBS for 90 min at 38.2 ºC in a humidified air atmosphere.

Following BCB exposure, the oocytes were transferred to mDPBS and washed 3 times. During the washing procedure, the oocytes were examined under a stereomicroscope and according to their cytoplasm coloration categorized into the two groups: BCB+ oocytes showed a blue cytoplasm coloration (Figure 15. A) and BCB– oocytes were those without blue coloration in cytoplasm (Figure 15. B). The percentage of selected oocytes by BCB test was recorded for each COC category.

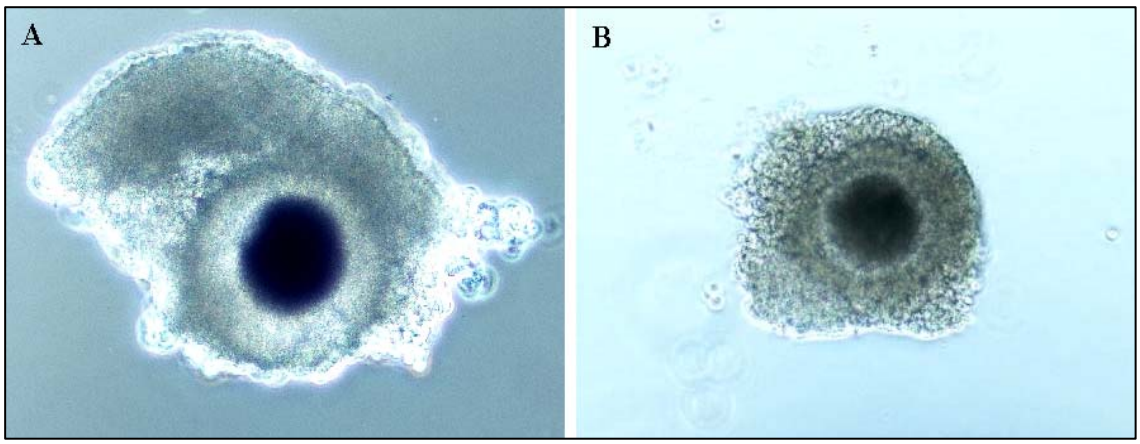

Figure 15: Differentially stained oocytes after exposure to BCB stain. (A) BCB+ (blue cytoplasm), (B) BCB– (colorless cytoplasm).

3.2.4 Measurement of oocyte diameter, volume and zona thickness

Oocyte morphological measurements were obtained for Ex and Cp oocytes soon after retrieval from the follicles, and immediately after BCB incubation for BCB+ and BCB– oocytes. Five to ten oocytes from each culture dish were individually pipetted into 5 µl droplets of IVM media, and examined at 400× on an inverted microscope after removal of cumulus cells enzymatically (by 600 IU/mL hyaluronidase; Type I-S, from bovine testes; Sigma) and hand-drawn glass-pipettes. Images of blastocysts were prepared with a Leica DM-IRB microscope (Leica CameraAG, Solms, Germany) with a digital camera (WV-BP 334; Panasonic, Neumünster, Germany), transferred to a personal computer and analysed with Image Tool (version 1.27, The University of Texas Health Science Center, San Antonio, TX, USA). The values for oocyte diameter (D) were the average of two measurements from the outer surface of the zona pellucida at 90° angles at the widest and narrowest oocyte axes. Oocyte volume was then calculated using the standard formula: $V=4/3\ \pi\ (D/2)^3$. The value for zona thickness, were the average of two measurements at 90° to 180° apart and at the most distinct outer borders of the zona pellucida.

3.2.5 Live zona imaging

The technical set-up for zona imaging of individual gametes resembled the one recently published by Montag *et al.* (2008) and more recently by Ebner *et al.* (2009). In brief,

live zona imaging of individual oocytes was performed non-invasively on a Leika DM-IRB inverted microscope equipped with ×10, ×20 and ×40 Hoffmann interference optics, a circular polarization filter, and liquid crystal analyzer optics (Figure 16. A). The birefringence analysis including autocalibration was fully controlled by a polarization imaging software module (OCTAX ICSI Guard™, OCTAX Microscience GmbH, Altdorf, Germany) implemented in an imaging software system (OCTAX Eyeware™). Individual images combining dark field (black) and birefringence (red) views were recorded online by the imaging software (Figure 16. B).

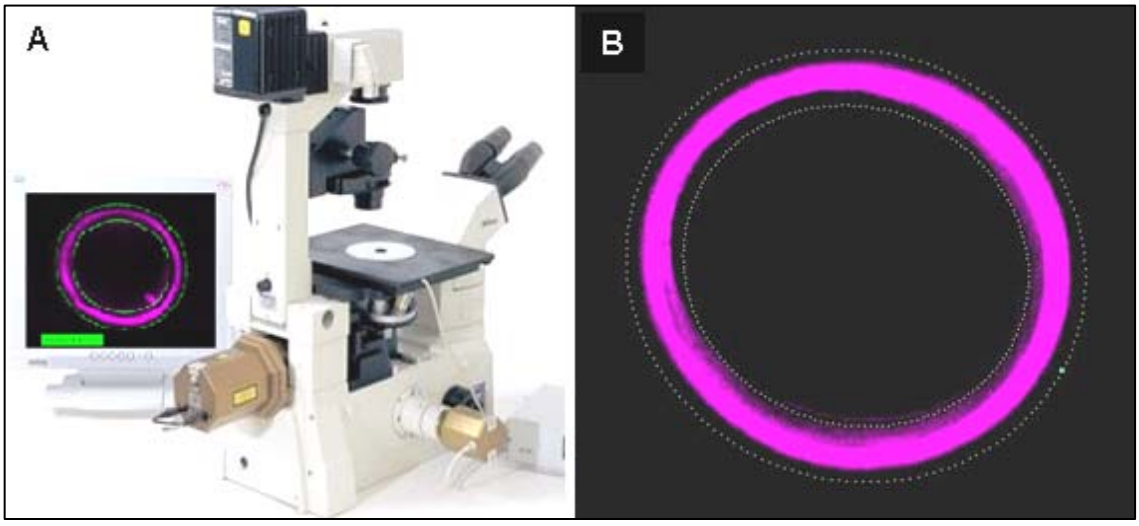

Figure 16: A Leika DM-IRB inverted microscope equipped with Hoffmann interference optics, a circular polarization filter, and liquid crystal analyzer optics (A). Polarization light microscopy reveals birefringence of the zona pellicia. Automatic user-independent zona pellucida imaging (zona extension matches the green frame) of an oocyte (B). The oocyte was placed in the center of the field of vision.

3.2.6 *In vitro* maturation

DMEM-F12 (D8062, Sigma) was used as basic media for *in vitro* maturation and was supplemented with 10% FCS, 5 mU/mL FSH, and 25 µg/ml gentamycin. Selected oocytes were washed twice in maturation medium then a group of 10 to 15 COCs were placed in 400 µl of medium covered with light white mineral oil, in four-well plates (Nunc, Roskilde, Denmark) and cultured at 38.5 °C under 5% CO2 in air for 28–30 h. After IVM culture, cumulus and corona cells were removed from COCs and those oocytes extruding a first polar body were classified as MII stage and were subjected to

ICSI. Oocytes not having a polar body were fixed to determine the chromatin configuration.

3.2.7 Nuclear chromatin evaluation

Only those oocytes without a polar body were stained with 2.5 µg/mL Hoechst 33258 (Sigma B2883) in 3:1 (v/v) glycerol/PBS, mounted on microscope slides, covered with cover slips, sealed with nail polish and kept at 4 °C in the dark until observation. Oocytes were evaluated in relation to their meiotic stage under a fluorescence microscope with a 365-nm exciter filter. Chromatin configuration was classified as previously described in detail elsewhere by Hinrichs *et al.* (2005). Briefly nuclear chromatin status was classified as follows: (i) germinal vesicle to telohase I (GV-TI), when a fluorescent nucleus with diffused, fibrillar, compact (Figure 17. A) or all stages between metaphase I and telophase I observed; (ii) degenerated, when no chromatin (Figure 17. B), chromatin spread throughout the cell (Figure 17. C), or abnormal chromatin configurations detected.

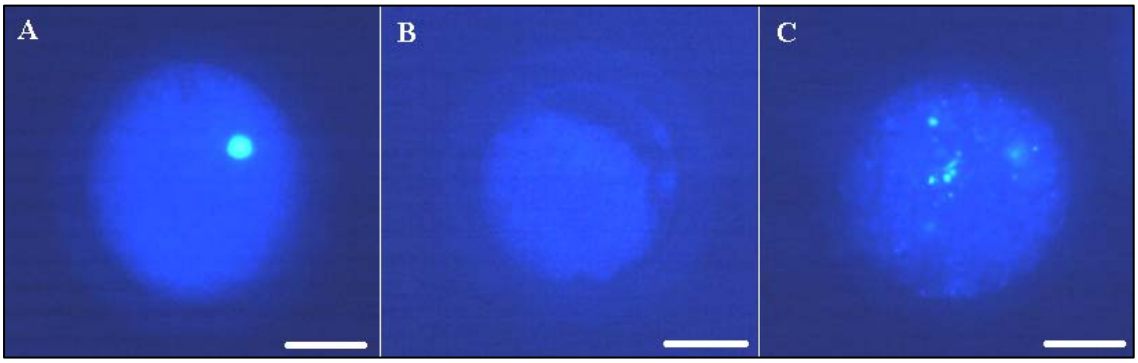

Figure 17: Photomicrographs of horse oocytes stained with Hoechst 33258 and evaluated under fluorescence microscopy, demonstrating; GV-TI (e.g., tightly condensed fluorescent) nucleus chromatin configuration (A), degenerated oocytes with no chromatin (B), and chromatin spread throughout the cell (C). Scale bar represents 50 µm.

3.2.8 Semen preparation for ICSI

Straws of frozen semen were thawed at 37 °C in a water bath for 30 s, and 200 µl of semen was placed at the bottom of a 5-ml tube containing 1 ml of modified TALP (Sp-TALP, Parrish *et al.* 1988) for swim up. After 20 min incubation in an atmosphere of

5% CO2 in air, the top 0.6 ml of medium was collected and washed by centrifugation at 300×g for 5 min at room temperature. The supernatant was removed, and the pellet resuspended for ICSI.

3.2.9 ICSI procedure

Conventional ICSI was performed (Figure 18) as previously described (Palermo *et al.* 1992). Briefly, immediate before injection, 1 µl of sperm suspension was placed in a 3 µl droplet of modified TALP containing 10% polyvinylpyrrolidone under oil for manipulation.

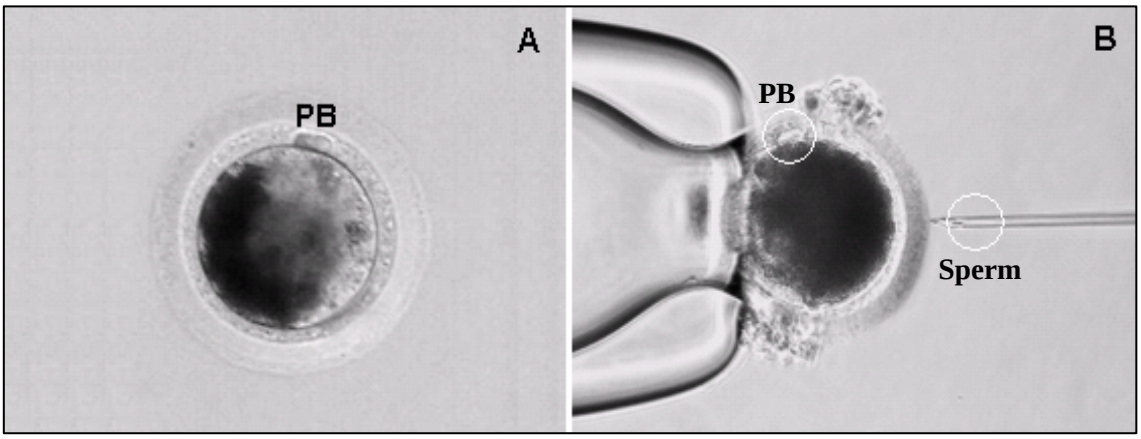

Figure 18: After maturation, MII-stage oocytes with first polar body (PB) visible (A) were subjected to intracytoplasmic sperm injection. An oocyte was fixed using a holding pipette, with the first polar body at 12 o'clock. An immobilized spermatozoon aspirated into the pipette is positioned very close to the tip of the injection pipette (B).

A group of ten to fifteen MII-stage (Figure 18. A) oocytes were placed in a 50 µl drop of DMEM-F12 (D8437, Sigma) containing 10% FCS. Spermatozoa were immobilized by breaking their tail and then aspirated tail-first into the injection pipette. Oocytes were held under gentle negative pressure onto the holding pipette such that the first polar body was orientated at ~90° (6 o'clock or at 12 o'clock) relative to the injection pipette, as appropriate. The spermatozoon was positioned very close to the open tip of the injection pipette before penetration of the zona pellucida and oolemma (Figure 18. B).
Negative pressure was then applied to the injection pipette until some free-flowing inward movement of ooplasm was detected. The negative pressure was then rapidly neutralized and then slowly reversed and the spermatozoon deposited into the

approximate geometrical centre of the oocyte along a 3 towards 9 o'clock track. The manipulations were performed at room temperature.

3.2.10 Assisted oocyte activation after ICSI

For chemical activation, approximately one hour after ICSI, the injected oocytes were washed twice in modified DPBS and exposed to 5 μM calcium ionophore A23187 (Sigma) in mDPBS at 37 °C for ten min. After activation treatment, oocytes were rinsed three times in DMEM-F12 containing 20% FCS and cultured as described below.

3.2.11 *In vitro* culture and data collection

Injected oocytes were cultured in DMEM/F-12 (D8062, Sigma) containing 10% FCS and 25 μg/ml gentamycin under light white mineral oil at 38.2 °C in a humidified atmosphere of 5% CO2, 5% O2 and 90 N2. Whole medium was changed 2 days after injection and retarded or degenerating embryos were removed at that time. Embryos were cultured for up to 8 days. Morphologically normal cleavage was evaluated 2 days after ICSI, and blastocyst rate was assessed after 6-8 days of in vitro culture.

3.3 Experimental design

3.3.1 Effect of additional activation by calcium ionophore A23187 on developmental competence of equine oocytes after ICSI

To determine whether additional activation by calcium ionophore A23187 has a positive influence on preimplantation embryo development of equine oocytes, MII oocytes were fertilized using ICSI technique and injected oocytes were either immediately placed in culture media (Non treated), or 1 hour after injection incubated with 5 μM of calcium ionophore A23187 for 10 min followed by culture. *In vitro* culture was carried out for 8 days and data for subsequent developmental competence in terms of cleavage and development to the blastocyst stage were recorded (Figure 19).

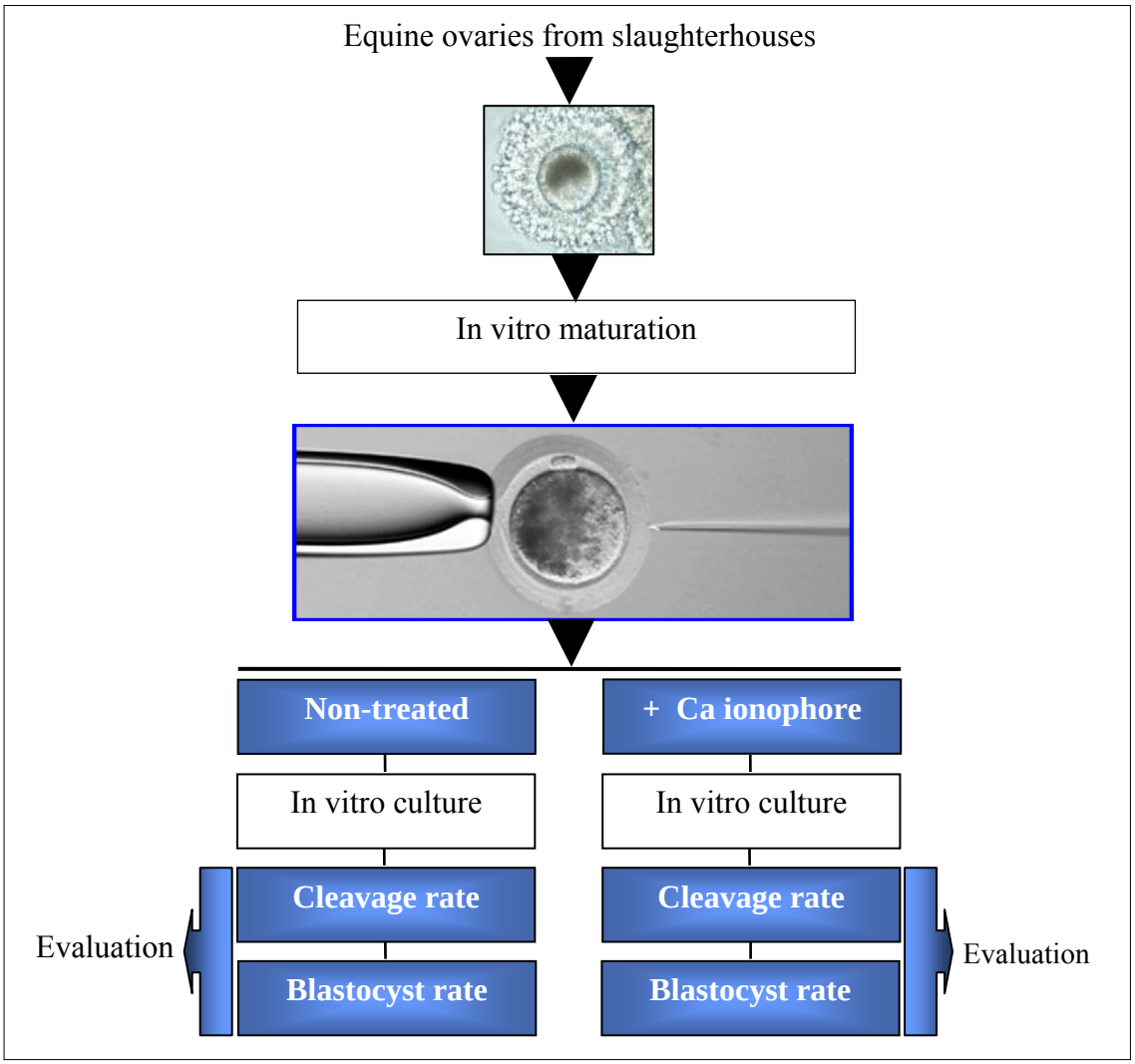

Figure 19: An outline of the experiment conducted to improve the efficiency of equine *in vitro* embryo production through the assisted oocyte activation with calcium ionophore A23187 after ICSI.

3.3.2 Effect of cumulus morphology on meiotic competence and preimplantation embryo development after ICSI

The effect of cumulus morphology on meiotic competence of equine oocytes was evaluated after IVM. Nuclear maturation after culture was determined by extrusion of the first polar body without fixing and staining because these oocytes were subjected to ICSI. Nuclear status of those oocytes without a polar body was determined for each Ex and Cp group after fixing and staining. To determine whether cumulus morphology of oocytes after retrieval from the follicles has a positive predictive value for preimplantation embryo development, MII oocytes obtained from both groups (i.e. Ex

vs. Cp) were fertilized using ICSI technique, and subsequent developmental competence in terms of cleavage and development to the blastocyst stage were analyzed (Figure 20).

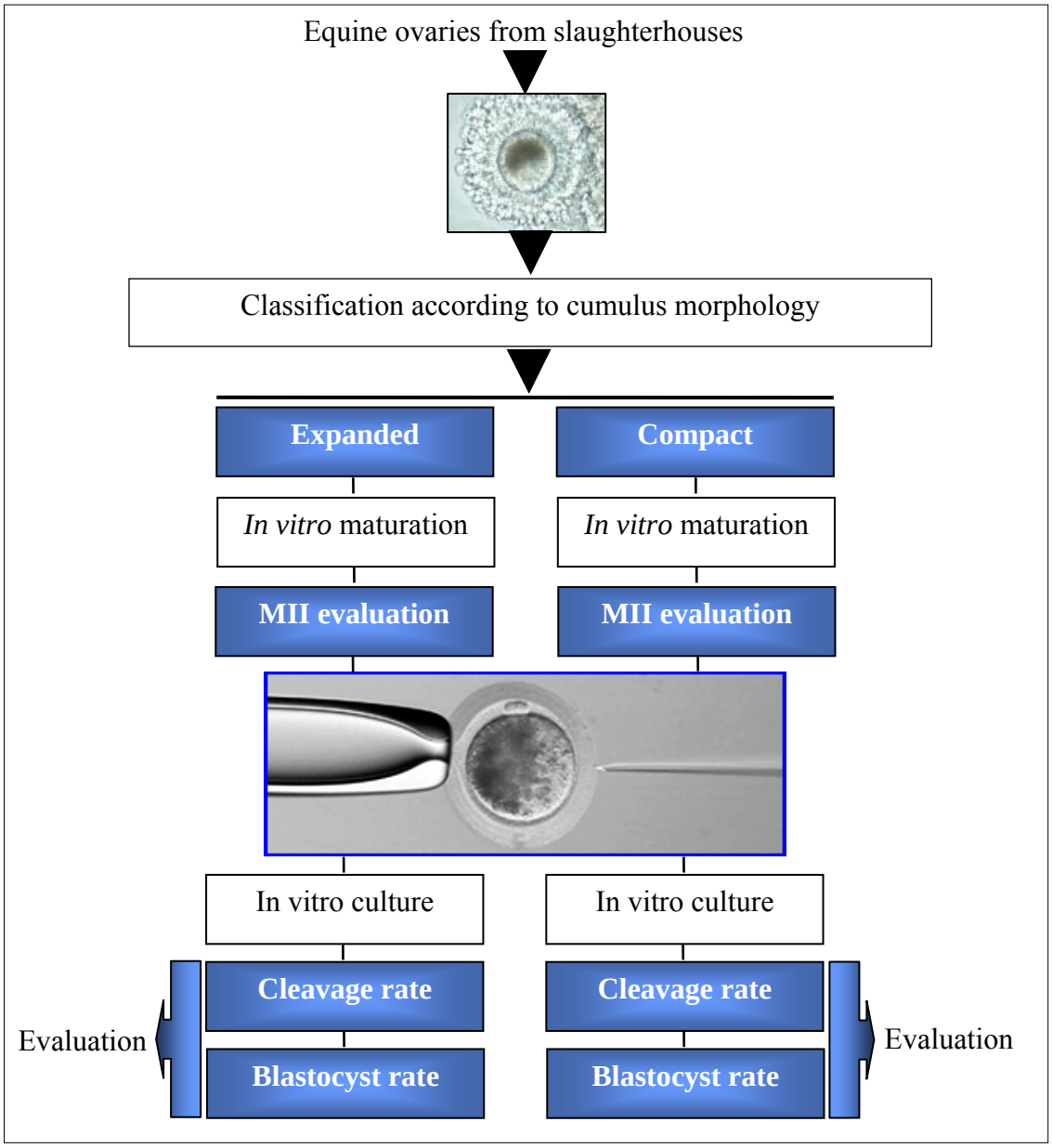

Figure 20: An outline of the experiment conducted to evaluate the effect of cumulus morphology on equine oocytes meiotic competence and subsequent preimplantation embryo development after ICSI.

3.3.3 Preliminary experiment to set up the BCB test using bovine oocytes

Preliminary experiment was initiated using bovine oocytes with a precisely large sample size to optimize the BCB test. Ovaries were obtained from a slaughterhouse and

transported to the laboratory; COCs were recovered by aspiration of the antral follicles. Only oocytes with a compact cumulus investment were used. Oocytes were placed into two groups: (i) control – placed immediately into culture; and (ii) treatment – incubation with BCB dye for 90 min at 38.5°C before culture. Treated oocytes were then divided into BCB+ and BCB– groups. In order to correlate the activity of G6PD with oocyte volume at retrieval time, a number of oocyte from BCB+ and BCB– groups were measured for their diameter after removal of cumulus cells enzymatically by vortexing. To evaluate the ability of the BCB test to predict the developmental competence of the COCs, after IVM, oocytes of each group (i.e. Control, BCB+ and BCB–) were fertilized *in vitro*. Presumable zygotes were cultured until day 9 and subsequent developmental characteristics in terms of cleavage and development to the blastocyst stage were analyzed.

3.3.4 G6PD activity in correlation with cumulus morphology and oocyte volume and its effect on meiotic competence and preimplantation embryo development after ICSI

The relationship between activity of G6PD and cumulus morphology was investigated after exposing the Ex and Cp oocytes to BCB test. The percentage of selected oocytes by BCB test was recorded for each categories of cumulus morphology. Moreover, in order to associate the activity of G6PD with oocyte volume at retrieval time, diameter of oocytes were measured immediately after BCB incubation for each BCB+ and BCB– group, considering their cumulus morphology. Moreover, the effect of G6PD activity on meiotic competence of equine oocytes was evaluated after IVM. Nuclear maturation after culture was determined by extrusion of the first polar body. Nuclear status of those oocytes without a polar body was analyzed for each BCB+ and BCB– group. To determine whether oocyte G6PD activity has a positive predictive value for preimplantation embryo development, MII oocytes obtained from both groups (i.e. BCB+ *vs.* BCB–) were fertilized using ICSI technique, and subsequent developmental characteristics in terms of cleavage and development to the blastocyst stage were analyzed (Figure 21).

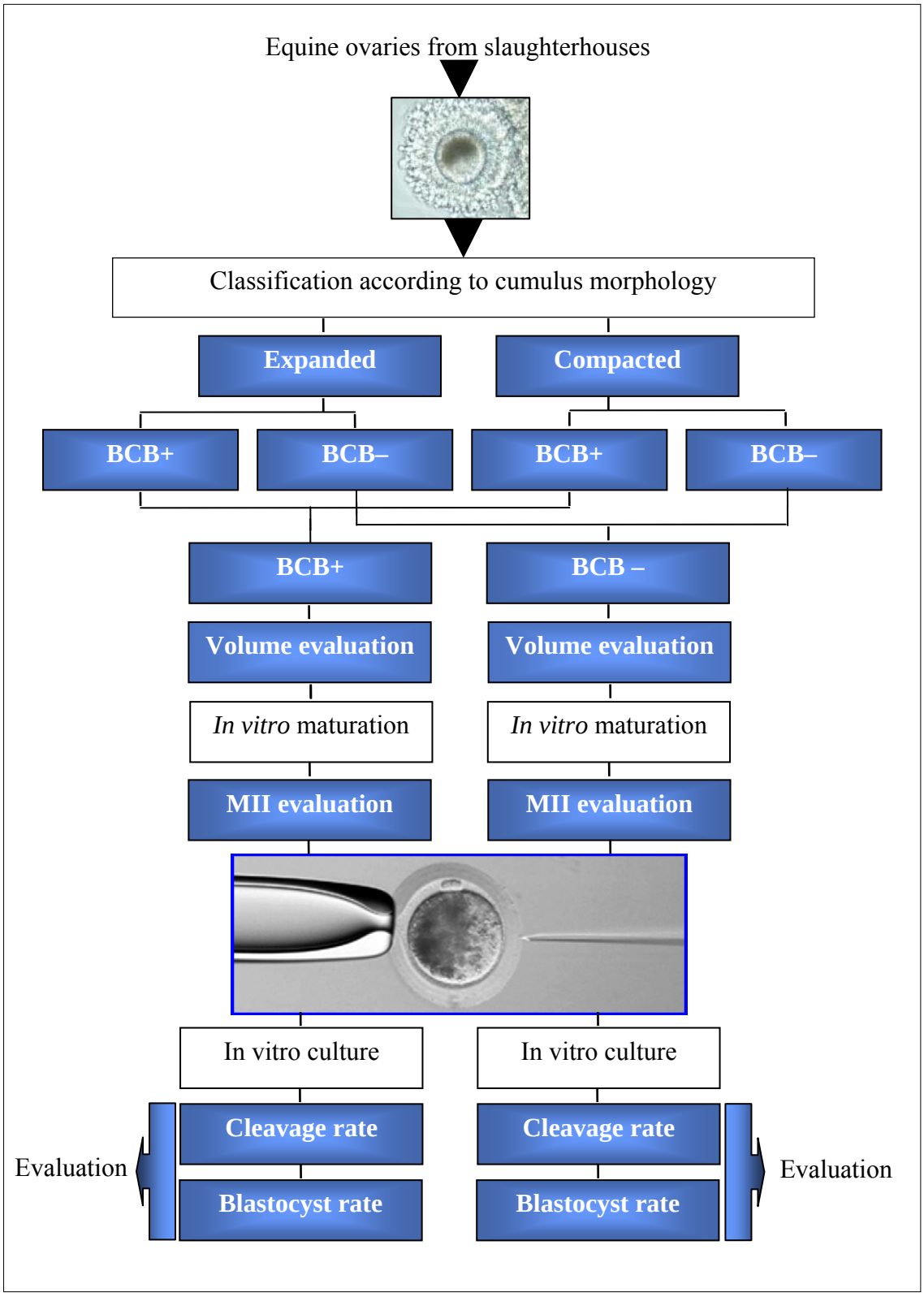

Figure 21: An outline of the experiment conducted to evaluate the effect of G6PD activity on equine oocyte meiotic competence and subsequent preimplantation embryo development after ICSI.

3.3.5 Correlation of the oocyte diameter, zona thickness and zona birefringence

To investigate the correlation of oocyte diameter with zona thickness and zona birefringence, diameter of the oocytes and thickness of the zona pellucida were measured in immature oocytes soon after removal of cumulus cells and corona cells. Then the zona birefringence was analyzed quantitatively for the same oocytes. The correlation of oocyte diameter with zona thickness and oocyte diameter with zona birefringence was determined. In addition, we studied the correlation between zona thickness and zona birefringence.

3.3.6 Correlation of cumulus morphology, G6PD activity and maturational status with zona thickness and birefringence

The experiment was performed to determine whether the quality and the maturational status of the equine oocytes could be correlated with the zona pellucida properties; particularly its thickness and birefringence.

Soon after retrieval from the follicles, the COCs were classified according to their cumulus morphology or were classified based on their G6PD activity levels immediately after BCB staining. To compare mean and variance of zona thickness and birefringence in correlation with oocyte quality, immature oocytes from each treatment groups (Ex *vs.* Cp and BCB+ *vs.* BCB–) were individually imaged after denudation of cumulus cells and corona cells.

To compare mean and variance of zona thickness and birefringence in correlation with maturational stages, irrespective of oocyte quality a group of immature oocytes were individually imaged immediately after retrieval from the follicles and a number of oocytes were imaged after IVM according to their meiotic competence, i.e. with polar body (PB+) or without polar body (PB–) extrusion. The quantitative analysis of zona birefringence was performed by polarization light microscopy and OCTAX polarAIDE-software (Figure 22).

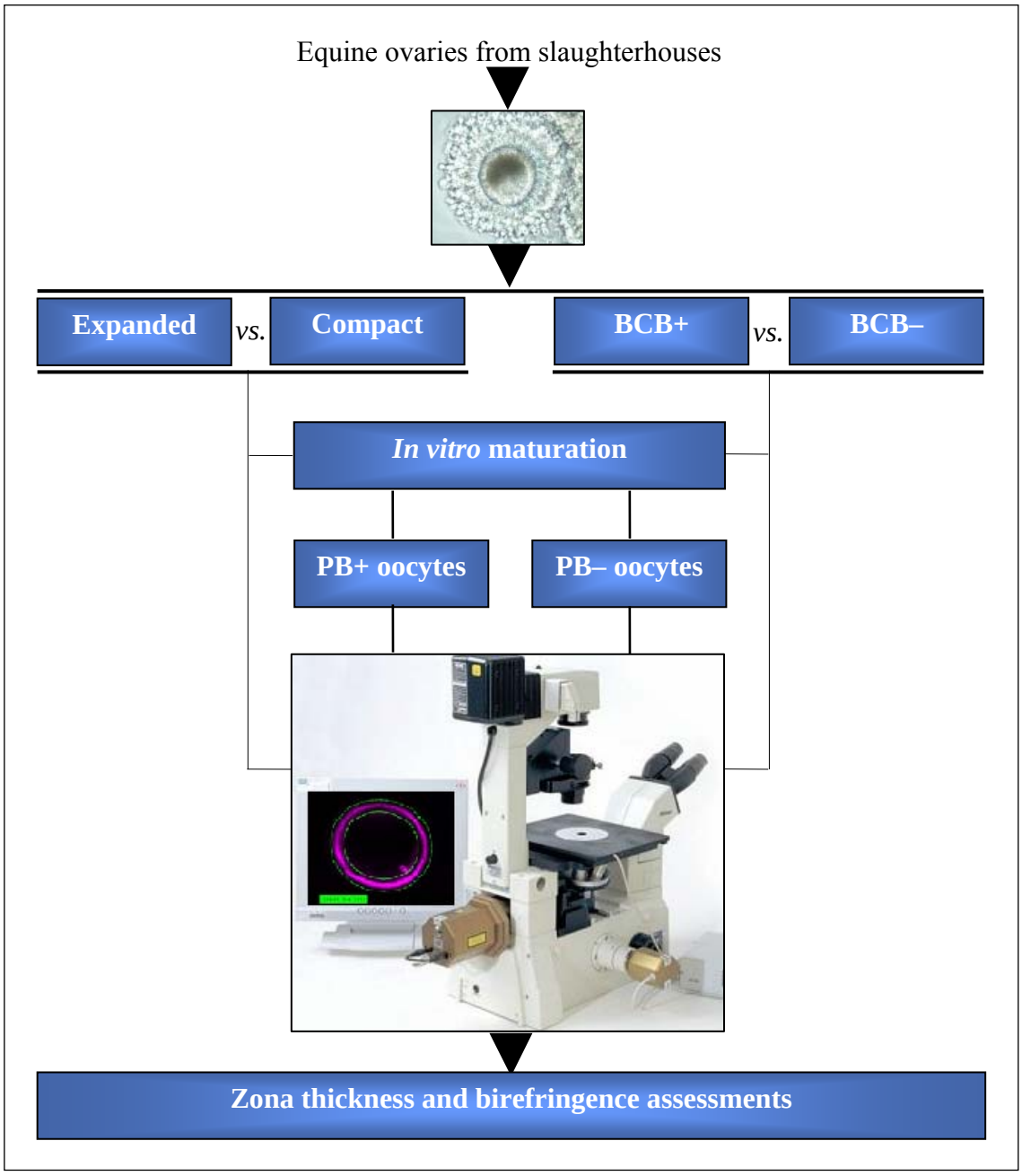

Figure 22: An outline of the experiment conducted to analyze the zona thickness and zona birefringence of equine oocyte in correlation with cumulus morphology, G6PD activity and maturational statues.

3.4 Statistical analysis

The proportion of selected oocytes by BCB for Ex and Cp oocytes, nucleus status of the oocytes after IVM, cleavage and blastocyst rate after IVC were compared between groups of oocytes (i.e. Ex *vs.* Cp and BCB + *vs.* BCB–) by Chi-square test. Fisher's exact probability test was used when the expected value for any parameter was less than

five. The oocytes volume, thickness and birefringence of zona pellucida between different groups of oocytes were analyzed by one-way ANOVA followed by Tukey's pairwise comparisons. The relationships between oocyte diameter, zona thickness, and zona birefringence were tested using linear correlation analysis (least square method).

4 Results

After aspiration, slicing and scraping follicles more than 5 mm in diameter, a total of 2492 cumulus-oocytes complexes (COCs) were recovered from 550 ovaries (average 4.5 COCs/ovary). Overall, 1548 (62.1%) COCs were morphologically classified as expanded (Ex) group and 944 (37.9%) COCs were classified as compact (Cp) group.

4.1 Effect of additional activation by calcium ionophore A23187 on developmental competence of equine oocytes after ICSI

The results for the effect of additional activation on subsequent developmental competence (Figure 23) of equine oocytes after ICSI are shown in Table 1. The rate of cleavage in calcium ionophore A23187 treated (ICSI with calcium ionophore A23187) group was significantly higher compared to that of the non-treated (ICSI without calcium ionophore A23187) group (50.0% *vs*. 28.4%; $P<0.05$). In addition, the calcium ionophore A23187 activated oocytes showed a significantly higher blastocyst rate than the non-treated group (8.6% *vs*. 1.9%; $P<0.05$) at day 8 after ICSI.

Table 1: Effect of assisted oocyte activation by calcium ionophore A23187 on developmental competence of equine oocyte after ICSI

Treatments	No. of oocytes	Cleavage rate n (%)	Blastocyst rate n (%)
ICSI *without* Ca ionophore	155	44 (28.4) [a]	3 (1.9) [a]
ICSI *with* Ca ionophore	174	87 (50.0) [b]	15 (8.6) [b]

Values with different superscripts within columns differ significantly ([a, b] $P<0.05$).

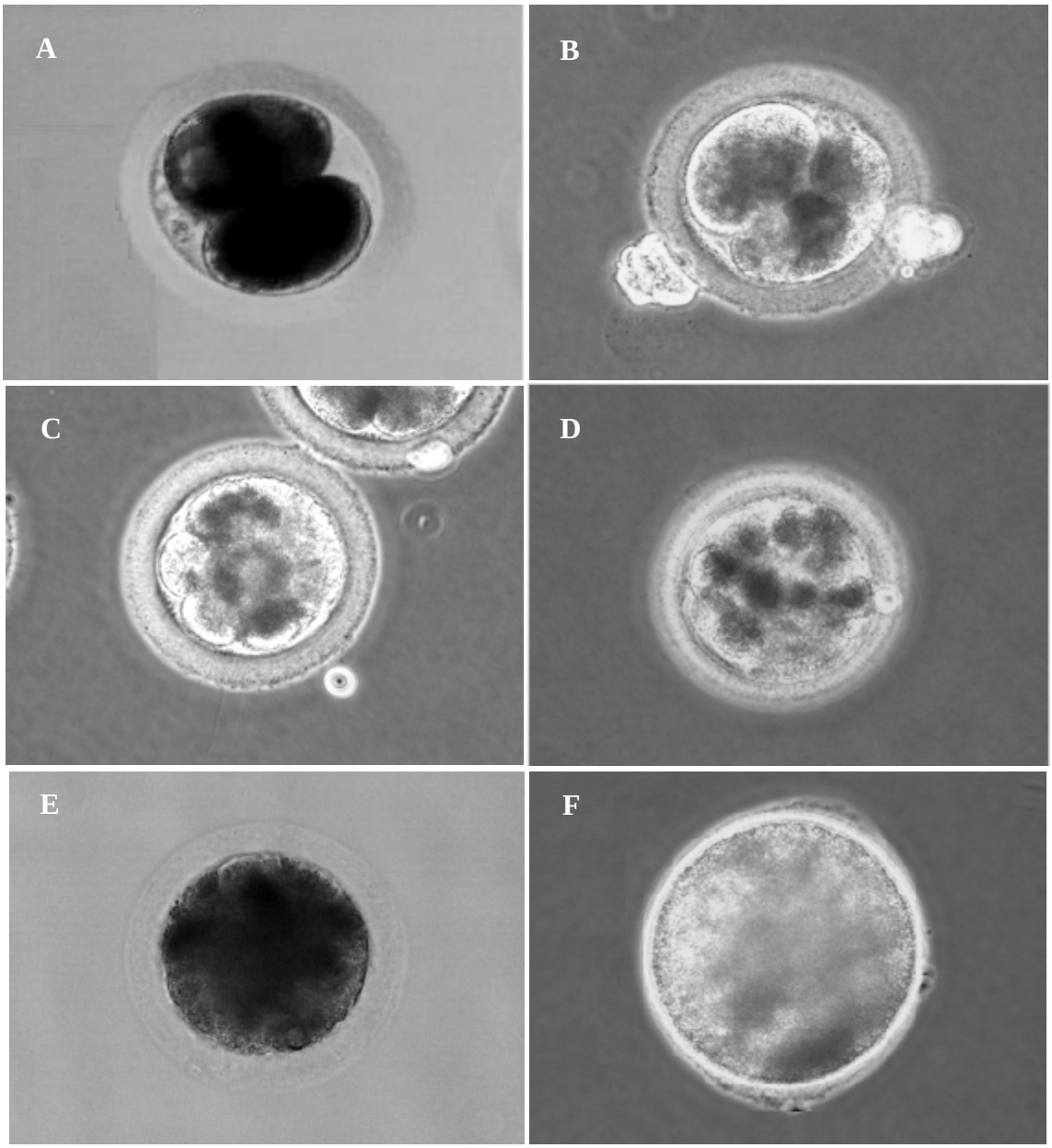

Figure 23: Progression to the blastocyst stage of an embryo derived from an *in vitro* matured oocyte, fertilized by intracytoplasmic sperm injection. (A) Two-cell embryo, (B) four-cell embryo, (C) eight-cell embryo, (D) 16-cell embryo, (E) morula and (F) expanded blastocyst.

4.2 Effect of cumulus morphology on meiotic competence and preimplantation embryo development after ICSI

The effect of cumulus morphology on nuclear progression of equine oocytes after in vitro maturation is presented in Table 2. The maturation rate was significantly higher in Ex oocytes compared to Cp oocytes (59.1% *vs.* 42.2%; $P<0.01$).

The proportion of oocyte with nucleus status between germinal vesicle to telophase I (GV-TI) observed after in vitro maturation culture was significantly lower in Ex oocytes compared to Cp oocytes (15.4% *vs.* 34.3 %; $P<0.01$). There was no significant difference in the proportion of degenerated oocytes that were classified as Ex or Cp at the time of recovery from the follicles (25.5% *vs.* 22.7%; respectively).

Table 2: Effect of cumulus morphology on nuclear progression of equine oocytes after *in vitro* maturation

Cumulus morphology	No. of oocytes	GV-TI n (%)	MII n (%)	Degenerated n (%)
Expanded	247	38 (15.4) [a]	146 (59.1) [a]	63 (25.5) [a]
Compact	198	68 (34.3) [b]	84 (42.2) [b]	46 (22.7) [a]

Values with different superscripts within columns differ significantly ([a, b] $P<0.01$).

After maturation, denuded oocytes with first polar body from both groups (i.e., Ex and Cp) were subjected to intracytoplasmic sperm injection followed by in vitro culture for 8 days. The effect of cumulus morphology on developmental competence of equine oocytes after ICSI is shown in Table 3.

There was no significant difference in the cleavage rate between the Ex and Cp oocytes (50.7% *vs.* 47.5%; respectively). However, the proportion of Ex oocytes that developed into blastocyst was significantly higher than that of the Cp oocytes (11.9% *vs.* 3.8%; $P<0.05$).

Table 3: Effect of cumulus morphology on developmental competence of equine oocytes after ICSI

Groups	No. of oocytes	Cleavage rate n (%)	Blastocyst rate n (%)
Expanded	134	68 (50.7) [a]	16 (11.9) [a]
Compact	80	38 (47.5) [a]	3 (3.8) [b]

Values with different superscripts within columns differ significantly ([a, b] $P<0.05$).

4.3 Preliminary experiment to set up the BCB test using bovine oocytes

A total of 971 bovine COCs were recovered and used for the experiment. Of these, 197 were used for the production of control embryos while 774 were subjected to BCB staining procedure out of which 416 (53.7%) were selected as BCB+ (blue stained oocytes) and 358 (46.3%) were selected as BCB– (colorless oocytes).

With respect to the proportion of cleaved embryos recorded two days after IVF, significant differences were found among the BCB+ and the BCB– groups (75.5 vs. 65.1%; $P<0.05$), and between the control group and the BCB– group (73.6 *vs.* 65.1%; $P<0.05$), whereas no significant differences were observed between the control group and the BCB+ group (73.6 *vs.* 75.5%).

The control group yielded a significantly higher blastocyst rate on day 9 after IVF than the BCB– group (23.9% *vs.* 11.5%; $P<0.05$). Moreover, the proportion of blastocysts from the BCB+ selected COCs was significantly higher than both the control and the BCB– groups (32.0% *vs.* 23.9% *vs.* 11.5%; respectively; $P<0.05$) as shown in Table 4.

Table 4: Effect of selection for G6PD activity via BCB staining on embryonic development of *in vitro* matured and *in vitro* fertilized bovine oocytes

Groups	No. of oocytes	Cleavage rate n (%)	Blastocyst rate n (%)
Control	197	145 (73.6) [a]	47 (23.9) [a]
BCB+	416	314 (75.5) [a]	133 (32.0) [b]
BCB–	358	233 (65.1) [b]	41 (11.5) [c]

Within columns, values with different superscripts differ significantly ($P<0.05$).

The diameter of BCB+ oocytes was significantly greater than diameter of BCB– oocytes (151.9 ± 3.9 µm *vs.* 143.0 ± 4.3 µm; $P<0.05$). Similarly, the volume of BCB+ oocytes was significantly larger compared to BCB– oocytes (1.84 x 10^6 μm^3 *vs.* 1.53 x 10^6 μm^3; $P<0.05$) as shown in Table 5.

Table 5: Effect of selection for G6PD activity via BCB staining on diameter and volume of the bovine oocytes

Groups	No. of oocytes	Mean oocyte diameter (μm)	Mean oocyte volume (μm^3)
BCB+	100	151.9 ± 3.9 [a]	1.84 x 10^{6} [a]
BCB–	91	143.0 ± 4.3 [b]	1.54 x 10^{6} [b]

Within columns, values with different superscripts differ significantly ($P<0.05$).

4.4 G6PD activity in correlation with cumulus morphology and oocyte volume and its effect on meiotic competence and preimplantation embryo development after ICSI

Overall, 1209 COCs were morphologically classified, 755 (62.4%) oocytes were classified as Ex group, and 454 (37.6%) as Cp group. After staining with BCB, 710 (58.7%) oocytes were classified as BCB+ (blue cytoplasm; low G6PD activity) and 499 (41.3%) oocytes were classified as BCB– (colorless cytoplasm, high G6PD activity). The percentage of BCB+ oocytes for Ex group was significantly higher than that of Cp group (67.0% *vs.* 44.9%; $P<0.01$). Contrarily, oocytes that classified as Cp group showed a colorless cytoplasm (BCB–) at a significantly ($P<0.01$) higher percentage (55.1%) than that of Ex group (33.0%) as shown in Table 6.

Table 6: Relationship between G6PD activity via BCB staining and cumulus morphology

Groups	Expanded n (%)	Compact n (%)	Total n (%)
BCB+	506 (67.0) [a]	204 (44.9) [a]	710 (58.7%)
BCB–	249 (33.0) [b]	250 (55.1) [b]	499 (41.3%)

Values with different superscripts within columns differ significantly ([a, d] $P<0.01$).

The mean volume of BCB+ oocytes was significantly higher than the mean volume of BCB– oocytes (2.1 x 10^6 μm^3 *vs.*1.75 x 10^6 μm^3; $P<0.01$). Likewise, mean volume of the BCB+ oocytes for each categories of cumulus morphology was significantly higher ($P<0.01$) than mean volume of the BCB– oocytes for each categories of cumulus morphology as shown in Table 7. Neither BCB+ nor BCB– oocytes showed a significant difference in oocyte volume between the different cumulus morphologies (Table 7).

Table 7: Relationship between G6PD activity via BCB staining and the volume of oocytes selected according to the cumulus morphology

groups	Expanded		Compact		Total	
	No	Mean oocyte volume (μm^3)	No	Mean oocyte volume (μm^3)	No	Mean oocyte volume (μm^3)
BCB+	68	2.08 x $10^{6\,a}$	21	2.16 x $10^{6\,a}$	89	2.10 x $10^{6\,a}$
BCB–	22	1.77 x $10^{6\,b}$	19	1.74 x $10^{6\,b}$	41	1.75 x $10^{6\,b}$

Values with different superscripts within columns differ significantly ($^{a, b}$ $P<0.01$).

A significantly higher proportion of oocytes from BCB+ oocytes reached MII-stage after IVM compared to BCB– oocytes (59.5% *vs.* 28.7%; $P<0.01$). The proportion of oocyte in GV-TI stage after maturation was significantly lower for BCB+ oocytes compared to BCB– oocytes (9.2% *vs.* 37.7%; $P<0.01$). However, the number of degenerated oocyte characterized by multiple morphological aspects was not statistically different between BCB+ and BCB– oocytes matured *in vitro* (Table 8).

Table 8: Effect of G6PD activity on nuclear progression of equine oocytes after *in vitro* maturation

Groups	No. of oocytes	GV-TI n (%)	MII n (%)	Degenerated n (%)
BCB+	284	26 (9.2) ^{a}	169 (59.5) ^{a}	89 (31.3) ^{a}
BCB–	247	93 (37.7) ^{b}	71 (28.7) ^{b}	83 (33.6) ^{a}

Values with different superscripts within columns differ significantly ($^{a, b}$ $P<0.05$).

The proportion of oocytes that cleaved by day 2 after ICSI differed by group, and was significantly higher for BCB+ oocytes than for BCB– oocytes (45.9% *vs.* 29.0%; $P<0.05$). Furthermore, the number of blastocyst obtained at day 8 was significantly higher in the BCB+ group compared to the BCB– group (9.2% *vs.* 1.4%; $P<0.05$) as shown in Table 9. Only one of sixty-nine MII-injected oocytes from BCB– group was progressed to the blastocyst stage; this oocyte had an expanded cumulus at the retrieval time.

Table 9: Effect of G6PD activity on preimplantation embryo development of equine oocytes after ICSI

Groups	No. of oocytes	Cleavage rate n (%)	Blastocyst rate n (%)
BCB+	98	45 (45.9) [a]	9 (9.2) [a]
BCB–	69	20 (29.0) [b]	1 (1.4) [b]

Values with different superscripts within columns differ significantly ([a, b] $P<0.05$).

4.5 Correlation of the oocyte diameter, zona thickness and zona birefringence

The thickness of the zona appeared almost normally distributed and varied between 13 and 25 µm. The correlation coefficient of the oocyte diameter and zona thickness is represented in Figure 24. As the zona pellucida plays a part in the measure of the oocyte diameter (mean diameter of all retrieved oocytes, 150.0 ± 7.9 µm), a thicker zona confers a larger diameter to the oocyte (correlation between oocyte diameter and zona thickness: r = 0.76, n = 308; P<0.0001).

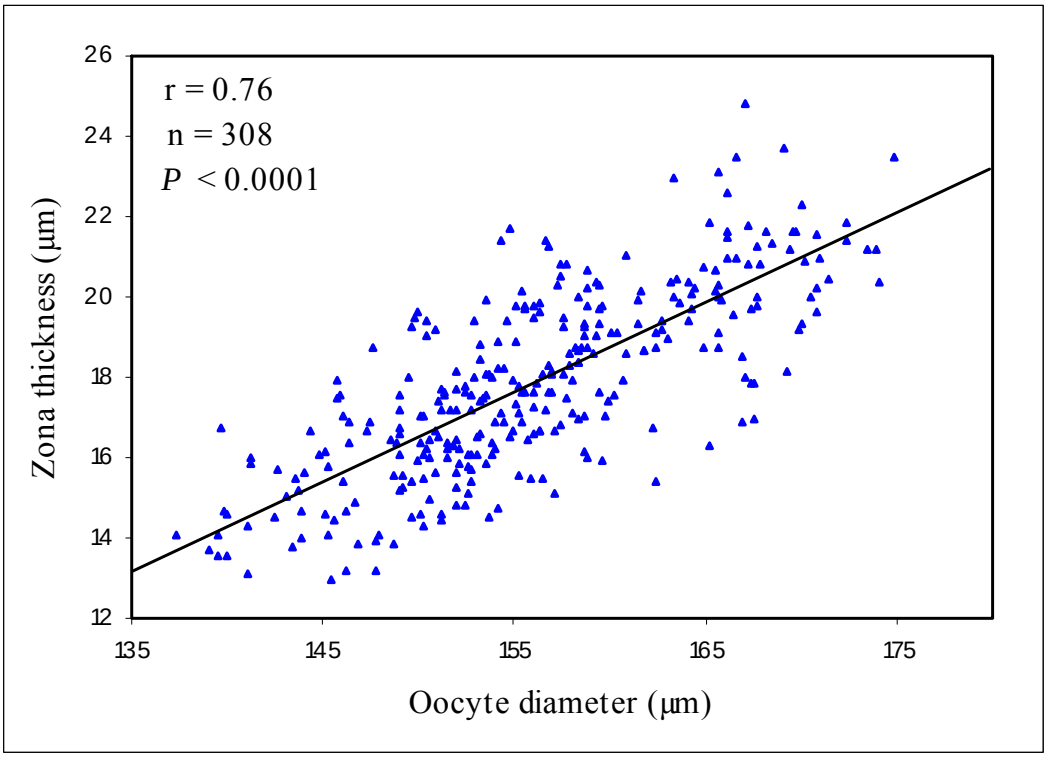

Figure 24: Correlation between diameter of the oocyte and thickness of the zona pellucida in equine.

Likewise, there was a significant (P<0.0001) correlation (r = 0.62, n = 308) between diameter of oocyte and birefringence of the zona (Figure 25); as oocyte diameter increases, there is a corresponding increase in zona birefringence.

The correlation between thickness and birefringence of the zona in equine oocytes, which is statistically significant (r = 0.44, n= 308; P<0.0001) is represented in Figure 26.

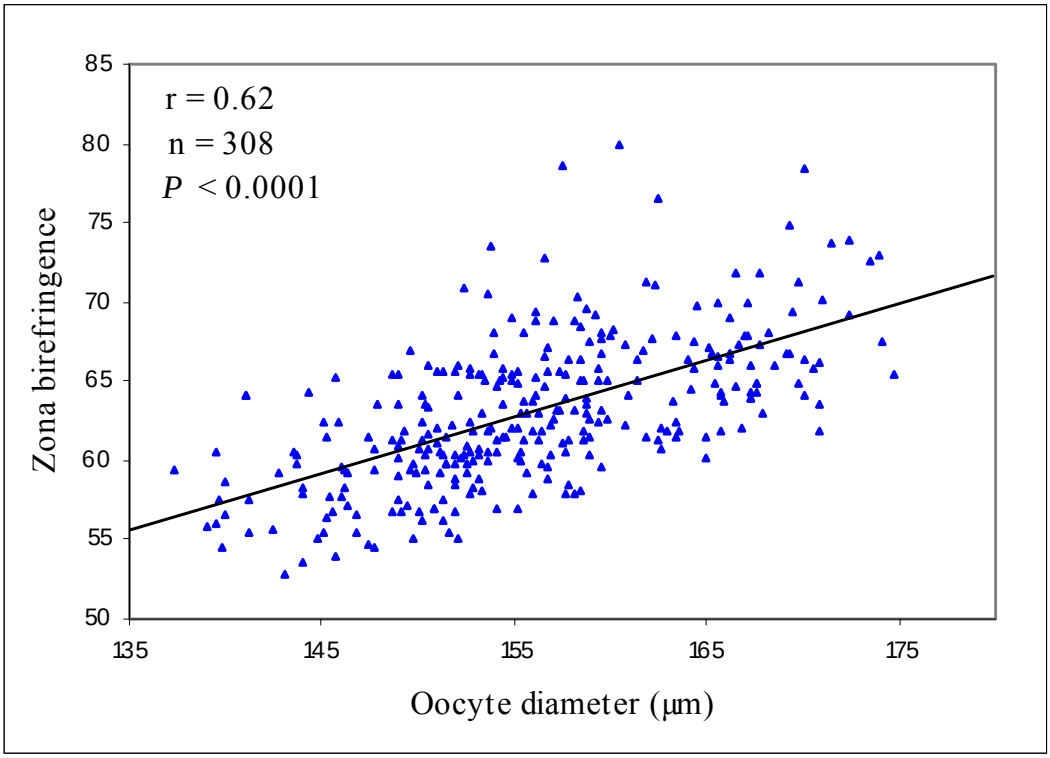

Figure 25: Correlation between diameter of the oocyte and birefringence of the zona pellucida in equine.

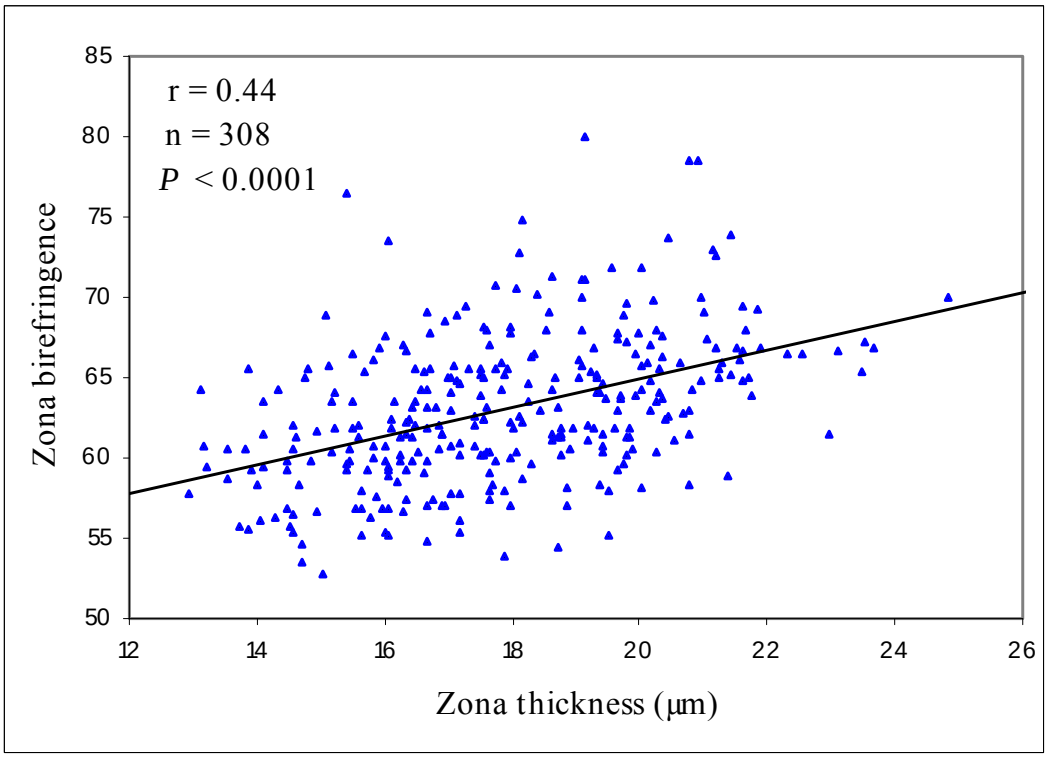

Figure 26: Correlation between thickness and birefringence of the zona pellucida in equine.

4.6 Correlation of cumulus morphology, G6PD activity and maturational status with zona thickness and birefringence

The quantitative analysis of the zona pellucida revealed that the average thickness and birefringence of the zona differed considerably not only between oocytes within a cohort but also between cohorts of oocytes with different quality (Figure 27).

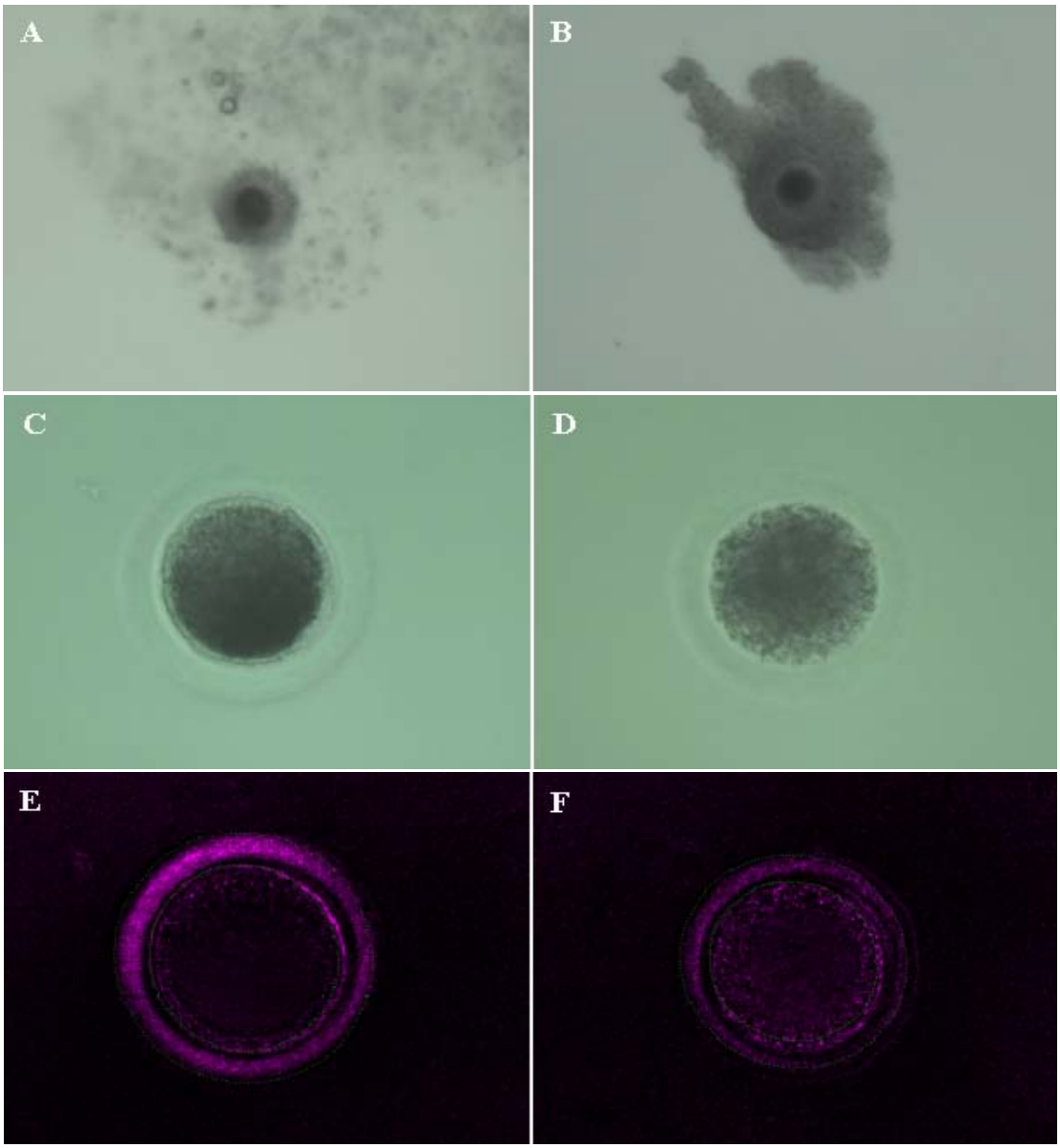

Figure 27: Equine oocytes with Expanded (A), and Compact (B) cumulus investment. After denudation, zona pellucida of the Ex (C) and Cp (D) oocytes observed by light microscopy appears transparent. Polarization light microscopy, however, reveals differences in birefringence of the zona layer (E and F).

When oocytes classified based on their cumulus morphology (Ex, n=93 *vs.* Cp, n=86) were compared, the Ex oocytes had a significant thicker zona than the Cp oocytes (18.2 ± 2.2 µm *vs.* 17.3 ± 2.1 µm; $P<0.05$). In addition, mean birefringence of the zona was significantly higher for Ex oocytes compared to Cp oocytes (64.6 ± 5.2 *vs.* 62.1 ± 4.2; $P<0.05$) as shown in Figure 28 and Table 10.

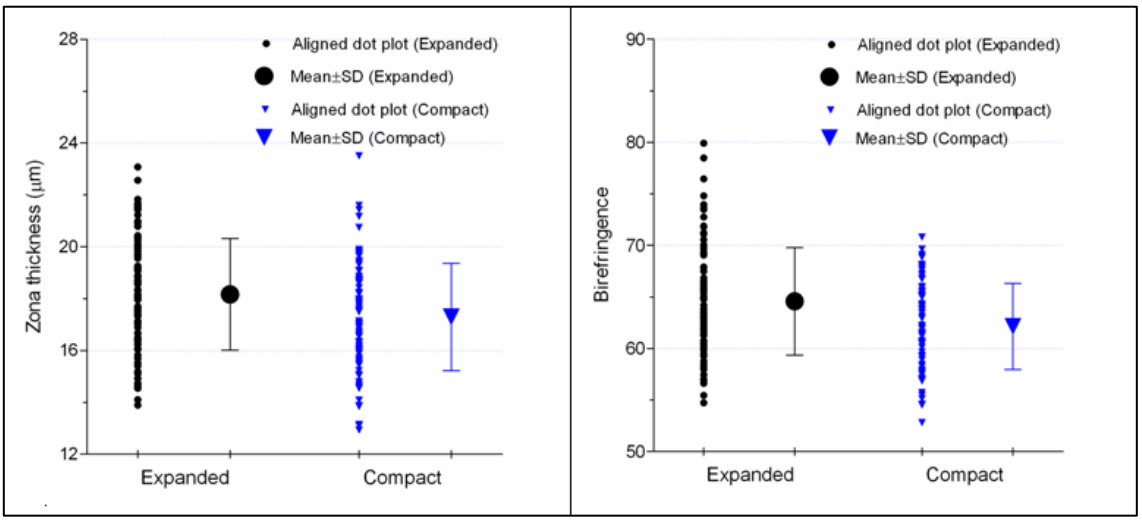

Figure 28: The zona pellucida is thicker in Ex oocytes than the Cp oocytes. The birefringence of zona pellucida is also greater in Ex oocytes than the Cp oocytes. *Small dots* are all measurements from individual oocytes; *large dots* and *bars* represent means and SDs.

Table 10: Cumulus morphology in correlation with thickness and birefringence of the zona pellucida

Cumulus morphology	No. of oocytes	Mean zona thickness (µm)	Mean birefringence
Expanded	93	18.2 ± 2.2 [a]	64.6 ± 5.2 [a]
Compact	86	17.3 ± 2.1 [b]	62.1 ± 4.2 [b]

Values with different superscripts within columns differ significantly ([a, b] $P<0.05$).

Of all oocytes stained, those classified as BCB+ (n=89) had a significant thicker zona in comparison to BCB– (n=41) oocytes (18.8 ± 2.4 µm *vs.* 16.1 ± 2.0 µm; $P<0.05$). We also found that BCB+ oocytes had a significantly greater mean of birefringence compared to those of BCB– oocytes (63.1 ± 4.5 *vs.* 61.3 ± 3.3; $P<0.05$) as shown in Figure 29 and Table 11.

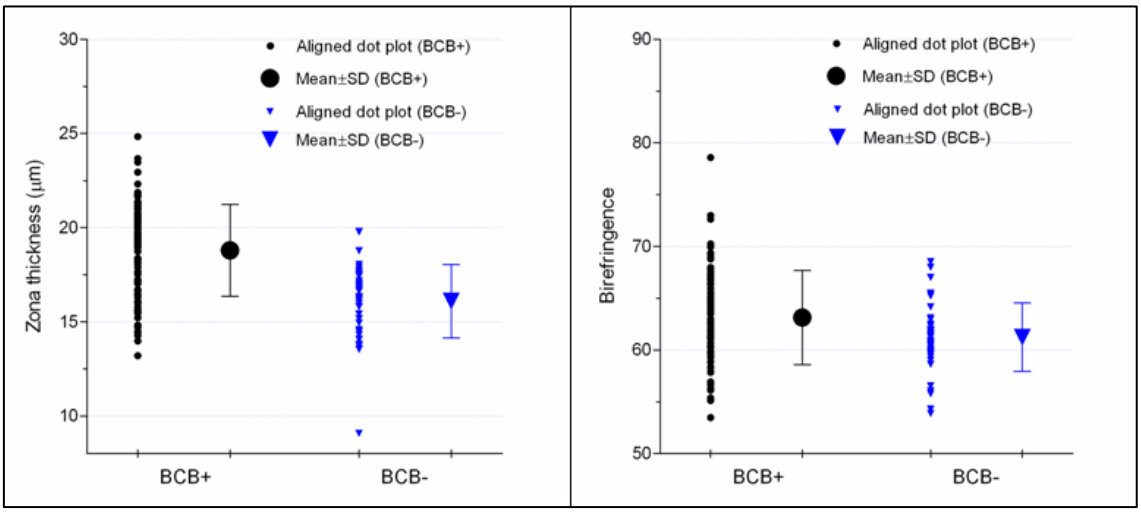

Figure 29: The zona pellucida is thicker in BCB+ oocytes than the BCB– oocytes. The birefringence of zona pellucida is also greater in BCB+ oocytes than the BCB– oocytes. *Small dots* are all measurements from individual oocytes; *large dots* and *bars* represent means and SDs.

Table 11: G6PD activity in correlation with thickness and birefringence of the zona pellucida

Groups	No. of oocytes	Mean zona thickness (µm)	Mean birefringence
BCB+	89	18.8 ± 2.4 [a]	63.1 ± 4.5 [a]
BCB–	41	16.1 ± 2.0 [b]	61.2 ± 3.3 [b]

Values with different superscripts within columns differ significantly ([a, b] $P<0.05$).

There was no significant difference in mean zona thickness between immature oocytes and mature oocytes with polar body (PB+ oocytes) after maturation culture (17.8 ± 2.4 *vs.* 17.5 ± 2.2 µm; $P<0.05$). However, the mean zona thickness of the mature oocytes without polar body (PB– oocytes) after in vitro maturation culture was significantly higher (18.8 ± 2.4 µm) than both the immature oocytes (17.8 ± 2.4 µm) and PB+ oocytes (17.5 ± 2.2 µm; $P<0.05$). Similar to the thickness our results show that there is no significant change in the zona pellucida birefringence of immature oocytes and PB+ oocytes (63.0 ± 4.6 *vs.* 62.7 ± 4.1) after in vitro maturation. Nevertheless, mean zona birefringence in the PB– oocytes (64.8 ± 5.5) was significantly higher ($P<0.05$) that that for immature oocytes (63.0 ± 4.6) and PB– oocytes (62.7 ± 4.1) after in vitro maturation (Figure 30 and Table 12).

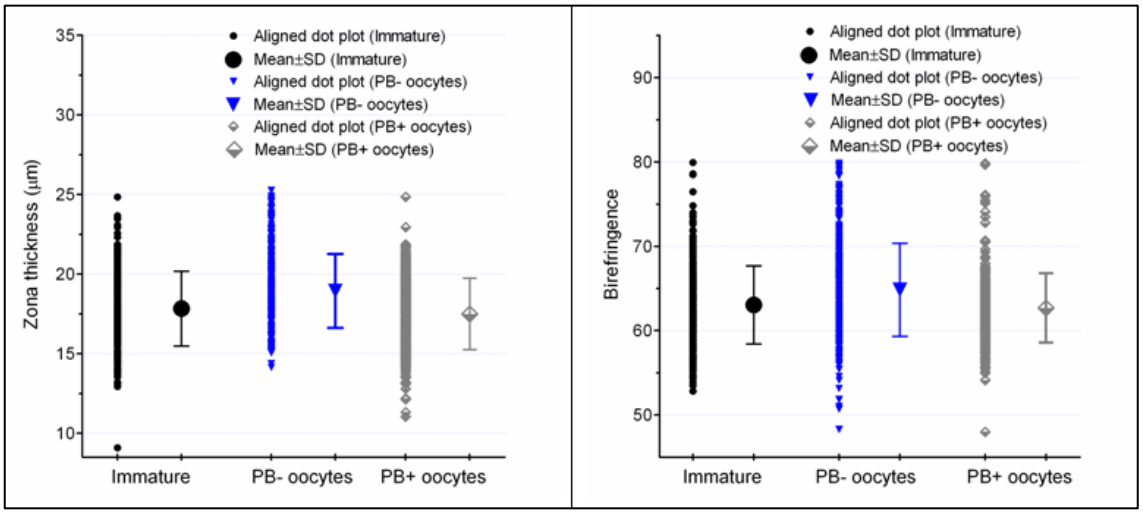

Figure 30: The thickness of zona pellucida is thicker in PB– oocytes than both immature and PB+ oocytes. The birefringence of zona pellucida also is higher in PB- oocytes than immature and PB+ oocytes. *Small dots* are all measurements from individual oocytes; *large dots* and *bars* represent means and SDs.

Table 12: Maturational status in correlation with thickness and birefringence of the zona pellucida

Groups	No. of oocytes	Mean zona thickness (µm)	Mean birefringence
Immature	309	17.8 ± 2.4 [a]	63.0 ± 4.6 [a]
PB^- oocytes	424	18.9 ± 2.3 [b]	64.8 ± 5.5 [b]
PB^+ oocytes	298	17.5 ± 2.2 [a]	62.7 ± 4.1 [a]

Values with different superscripts within columns differ significantly ([a, b] $P<0.05$).
PB^+; polar body extruded.
PB^-; polar body not extruded.

5 Discussion

Little work has been reported on the basic research concerning *in vitro* oocyte/embryo based biotechnologies in horses compare to ruminants and pigs. The following can be mentioned as main reasons for this delay: (i) the limited interest from horse breeders and breed associations, (ii) the very scarce availability of abattoir ovaries, (iii) the much poorer oocyte collection rate compared to other species, (iv) and the absence of standard IVF protocols (Galli *et al.* 2007).

Despite a great deal of research effort put into trying to develop an effective method for conventional IVF during the 1980s and 1990s, the results have been extremely disappointing and only two live foals have been born to date following IVF carried out on *in vivo* matured oocytes recovered by transvaginal ultrasound-guided pick-up (OPU) (For review see Allen 2005). Fortunately, the block to conventional IVF in the horse has been largely overcome by the development and application of the technique of ICSI. Several groups have reported live foals produced by ICSI (Allen 2005; Cochran *et al.* 1988; Galli *et al.* 2002; Grondhal *et al.* 1997; Li *et al.* 2001; McKinnon *et al.* 2000; Squires *et al.* 1996). Advantages of the method include the fact that the spermatozoon used to fertilize the oocyte can have been frozen and thawed, may have been sex-sorted and may even be totally immotile or tail-less before it is injected into the oocyte to cause fertilization and initiate embryonic development.

During the past 10 years, procedures for assisted reproduction have rapidly developed for clinical use. However, many of the procedures require special expertise and equipment, resulting in a relatively slow movement from academic to clinical settings. In addition, development of equine embryos *in vitro* is far away from being optimal (Carnevale and Maclellan 2006) and in most studies *in vitro* blastocyst rate is below of 16% (ranging from 4 to 16%; Galli *et al.* 2007).

Any significant improvement in the *in vitro* embryo production system and/or establishment of an efficient technique to predict the equine oocyte developmental competence would be valuable for horse breed associations and for the aid to salvage gametes after death as well. Therefore, in the present study a number of assumptions

were made to improve and/or to predict developmental competence of the equine oocytes following ICSI.

The availability of large numbers of mature, meiotically competent oocytes is a prerequisite to conduct studies involving any assisted reproductive technology. In this respect, recovery of oocytes from horse ovaries presents some technical problems: the collection of oocytes requires incision of follicles and scraping of the follicle wall with a curette and extensive flushing to detach the COCs. These difficulties are reflected in an increased length of time and number of personnel required for collecting enough oocytes to carry out even the simplest experiment. Galli *et al.* (2007) reported that the difference between species is over 10-fold both for time and for personnel: for example in their laboratory, to collect 100 horse oocytes requires 4 technicians working for 3–4 h while for the same number of bovine oocytes only 2 technicians working for 30–40 min are sufficient. Our data indicate that after aspiration, slicing and scraping the follicles with a diameter of more than 5 mm, the recovery rate was 4.5 oocytes per ovary that is in a range similar to previous studies (Galli *et al.* 2007; Wirtu *et al.* 2004). The need to scrape the follicles derives from the tight connections between the cumulus and the membrana granulosa and between the latter and the follicle wall (Hawley *et al.* 1995). In horses, it is not unusual to recover either oocytes surrounded only by the corona cells or, conversely, oocytes surrounded by large sheets of membrana granulosa cells. This indicates in the first instance most probably a COC detached from the membrana granulosa by the extensive washing that follows the scraping of the follicle, and in the second instance the effect of the scraping itself (Galli *et al.* 2007).

As the oocytes were retrieved from the ovary, they may be classified as expanded (Ex) or compact (Cp), based on cumulus morphology. In our laboratory, repeated collection of horse oocytes with Ex cumulus cells accounts for 62.1% of all recovered oocytes when compared to 37.9% of oocytes with Cp cumulus cells. This is in accordance with previous studies in which Cp oocytes composed the same or a fewer number of retrieval oocytes (40–50%) compared to Ex oocytes (50–60%) (Choi *et al.* 2003b; Choi *et al.* 2006b; Choi *et al.* 2004b). However, Galli *et al.* (2007) reported that only a third of the recovered COCs had Ex cumulus cells whereas the rest was classified as Cp cumulus cells. One possible factor, which may account for these disparate results from different laboratories, could be the fact that morphological classification of cumulus cells depends on a visual subjective appraisal by laboratory personal and as such can vary

with the individual and the laboratory. In addition, the effect of ovary holding temperature and duration of the holding period on equine cumulus morphology has been reported previously (Pedersen *et al.* 2004; Ribeiro *et al.* 2008), which may explain the difference observed among different laboratories as well.
In other species such as ruminants and pigs, the presence of Ex cumulus is linked to the collection of oocytes from atretic follicles and these oocytes are generally discarded immediately because of their extremely low developmental capacity. In horses, however, oocytes with Ex cumulus mature normally and have normal developmental competence (Galli *et al.* 2007; Hinrichs and Schmidt 2000).

5.1 Effect of additional activation by calcium ionophore A23187 on developmental competence of equine oocytes after ICSI

In ICSI-assisted fertilization, the oocyte is activated by a sperm-derived oocyte activation factor (also known as sperm factor) after the injection (Swann 1990; Tesarik *et al.* 1994). A decrease or loss in activity of this factor is a possible cause of fertilization failure after ICSI. When this factor is completely absent, oocyte activation does not occur and the oocyte remains in MII-stage even if ICSI is performed.
An immobilized sperm injected into the oocyte only undergoes decondensation if the sperm nucleoprotein and the oocyte reduction mechanism are normal. When the activity level of the sperm factor is reduced, the oocyte activation mechanism is stimulated at a poor level, resulting in insufficient reduction of maturation-promoting factor (MPF) in the oocyte and restraining the release of the second polar body (Yanagida *et al.* 2008).
Dozortsev *et al.* (1995a) postulated that each step in the human ICSI procedure (such as puncture of the oocyte with a pipette, the technique of injection, injection of Ca^{2}+ containing medium, and the spermatozoon itself) might potentially contribute to oocyte activation and subsequent cleavage. In hamster, rabbit, and mouse oocytes, puncture of the oolemma and aspiration of cytoplasm is sufficient for oocyte activation during ICSI (Sansinena *et al.* 2007; Tesarik *et al.* 1994). Contrarily, pig, cattle, and sheep oocytes have not been activated solely by mechanical treatment with the injection pipette or by an injected spermatozoon, and additional oocyte activation after ICSI is necessary to obtain cleavage and subsequent development (Asada *et al.* 2001; Sansinena *et al.* 2007; Shirazi *et al.* 2009). Conflicting data have been reported for the horse concerning the

effect of activation treatment after ICSI. Therefore, in this study we investigated the effect of additional activation by calcium ionophore A23187 on the developmental competence of equine oocytes after ICSI.

Our results demonstrate that the ability of the oocytes to cleave and to develop to the blastocyst stage after ICSI is enhanced by assisted activation by calcium ionophore A23187. This is in accordance with the results from Li *et al.* (2000 and 2001) but in contrast with previously reported data (Choi *et al.* 2006b; Hinrichs *et al.* 2005; Matsukawa *et al.* 2007). In this respect, Matsukawa *et al.* (2007) reported that although the efficiency of equine ICSI can be improved by sperm pretreatment, activation treatment is not essential for equine embryo production after ICSI. On the other hand, Li *et al.* (2000) reported that they did not observe normal fertilization without artificial activation after ICSI. Horse oocytes fertilized by ICSI inconsistently display $[Ca^{2+}]_i$ oscillations, however this failure is not due to inadequate sperm factor release but to inability of the oocyte to respond to the activation process and/or to provide the adequate substrate for the factor released by the sperm. This may partially explain the inconsistencies reported for oocyte activation and embryonic development for this technique in equine and suggest that improving the ability of horse gametes to initiate $[Ca^{2+}]_i$ responses after ICSI may improve the success of this technique in the horse (Bedford *et al.* 2004).

Accordingly, additional activation with calcium ionophore A23187 helps to activate the oocyte by increasing the Ca^{2+} permeability of the cell membrane, resulting in an influx of extra cellular Ca^{2+}. After treatment with A23187, the Ca^{2+} concentration in the oocyte peaks after approximately 1 min, and then gradually decreases. The treatment causes a single transient increase in Ca ion concentration, but no Ca^{2}+ oscillation (Yanagida *et al.* 2008).

Another possible explanation for the observed difference may be due to the technique of ICSI (i.e., conventional *vs.* Piezo methods), which have been used for fertilization *in vitro*. The apparent requirement for additional activation of oocyte after conventional ICSI has been reported previously for equine (Choi *et al.* 2002; Guignot *et al.* 1998; Kato *et al.* 1997; Li *et al.* 2001; Liu *et al.* 2000). It has been suggested that it may be related to a lesser degree of sperm damage induced by scoring the sperm tail with the injection pipette or rolling the spermatozoa against the bottom of the dish before conventional injection (Catt and O'Neill 1995; Choi *et al.* 2002; Dozortsev *et al.*

1995b). On the other hand, in piezo-ICSI, a motile sperm is immobilized by applying piezo pulses through the injection pipette. Potential reasons for higher efficiency of piezo microinjection include both increased damage and permeabilization of the sperm membrane, and a more reliable breakage of the oolemma, ensuring that the spermatozoa is deposited in the cytoplasm (Choi *et al.* 2002). Increases in efficiency have been reported in mouse (Kimura and Yanagimachi 1995), human (Yanagida *et al.* 1999), pig (Katayama *et al.* 2005), and cattle (Devito *et al.* 2009) when ICSI using the Piezo drill was compared with conventional ICSI. However, further studies comparing conventional and Piezo-ICSI are required to confirm this in equine.

5.2 Effect of cumulus morphology on nuclear progression of equine oocytes after *in vitro* maturation

Numerous studies using in vitro embryo production system have demonstrated that, from her total pool of retrieved gametes, a mare has only a limited number of oocytes that are capable of supporting preimplantation embryo development as well as full development to term (for review see Galli *et al.* 2007). Convenient criteria to predict the oocyte viability are currently among the most important topics in equine assisted reproduction. The ability to reliably and non-invasively predict which oocytes bear the ability to develop to full-term would constitute a major breakthrough in equine oocyte-based biotechnologies. This would not only obviate the need for extended periods of *in vitro* culture, which may be associated with gradual reduction in developmental capacity (Hinrichs 2005), but could, in a major way, compensate the low efficiency of embryo production following assisted reproductive technologies (ART) and the associated high rates of early embryo mortality in equine.

The quality of oocytes can be assessed based on various morphological, cellular and molecular parameters (Wang and Sun 2007), including cytoplasmic appearance and morphology of the cumulus investments by the time of retrieval from the follicle. Previous studies have shown that equine oocyte with Ex cumulus cells are more competent to resume meiosis (Galli *et al.* 2007; Hinrichs *et al.* 2005) and to promote pronuclear formation after fertilization (Alm *et al.* 2001; Torner *et al.* 2007) compared to oocytes with Cp cumulus cells. Nonetheless, whether oocytes of these two different cumulus morphologies are equivalent for their ability to progress to the blastocyst stage

is still under debate. With this in mind, we first re-evaluated the effect of morphological assessment of cumulus cells on the meiotic competence of equine oocyte after *in vitro* maturation.

Our results showed that the maturation rate was significantly higher in Ex oocytes compared to Cp oocytes, which confirmed the results of previous studies (Galli *et al.* 2007; Hinrichs *et al.* 2005; Torner *et al.* 2007). Contradictorily, lower proportion of Ex oocytes showed GV-TI nucleus configuration after IVM in comparison with Cp oocytes; indicating insufficient meiotic competence for the later group. Further evidence for this claim arose from a study by Hinrichs *et al.* (2005) showing that oocytes with Cp cumulus cells mature more slowly than oocytes with Ex cumulus cells. The reason for this delay in oocyte meiotic maturation and different developmental competence might be due to the cytoplasmic quality of the oocyte. This could be explained by the higher amount of oocytes with diplotene configuration before GVBD in the Cp COC group at recovery. The majority of oocytes with Ex cumulus cells before IVM were at meiotic stages ≥MI which indicates that the final meiotic maturation has already started in these group of oocytes before recovery and IVM (Hinrichs *et al.* 2005; Torner *et al.* 2007). Further, studies performed by Torner *et al.* (2007) indicated a significant difference in mitochondrial distribution and mitochondrial activity during IVM related to the initial cumulus morphology in equine COCs. These observation along with our results, suggest that Cp oocytes might not mature completely in the present IVM system; further investigation on the IVM conditions for equine oocyte is required.

5.3 Effect of cumulus morphology on developmental competence of equine oocytes after ICSI

To validate if preimplantation embryo development of equine oocytes is attributed to the cumulus morphology at retrieval time, oocytes were fertilized using ICSI technique followed by culture for 8 days. Our results showed that, although the proportion of cleaved embryos recorded two days after ICSI was not different between Ex and Cp oocytes, the blastocyst rate was significantly higher for Ex oocytes than for Cp oocytes. This is in accordance with a previous study (Matsukawa *et al.* 2007) where the type of initial cumulus morphology affected developmental competence of equine oocytes after ICSI. Matsukawa *et al.* (2007) indicated that oocytes with Ex cumulus cells had a

higher competence to develop to the blastocyst stage compared to oocytes with Cp cumulus cells. Oocytes from ovaries preserved for one day were used for evaluation of developmental competence in that study. The observed differences in that study are probably due to fact that Cp oocytes are juvenile and their chromatin is labile to damage during post-mortem storage within the ovary (Galli *et al.* 2007; Hinrichs *et al.* 2005). In agreement with this idea, Ribeiro *et al.* (2008) concluded that ovary storage appeared to decrease developmental competence to the blastocyst stage after 7 h, and decreases both meiotic and developmental competence after 20 h.

Another explanation for the differences in developmental competence that we observed among Ex and Cp oocytes appears to be attributed to the follicular origin of the oocytes. Equine oocytes with Cp cumulus cells are largely recovered from histologically viable follicles, whereas oocytes with Ex cumulus cells are largely recovered from atretic follicles (Choi *et al.* 2006b; Torner *et al.* 2007). Atretic follicles are presumably among those competing for dominance and as such an environment may provide a better conditions for proper cytoplasmic maturation, giving the oocyte greater capacity for preimplantation embryo development (Lonergan *et al.* 1994). Such follicles, like preovulatory follicles, could signal the oocytes via certain molecules and confer them the greater developmental competence. In other words, the greater developmental competence may be gained late in the follicular phase so that a final differentiation is required, and this differentiation is similar with the beginning of atresia (Blondin and Sirard 1995). It was shown that developmental competence of Cp oocytes was improved when the oocytes were maintained at GV stage for 24 h using rescovitine (Choi *et al.* 2006b; Franz *et al.* 2003). The result from this experiment reveals that in our IVEP system, selection of oocytes based on morphology of cumulus cells at retrieval time has a positive predictive value in terms of meiotic competence and preimplantation in vitro embryo development to the blastocyst stage.

5.4 Relationship between G6PD activity via BCB staining and cumulus morphology

With the aim of establishing a convenient criterion of evaluating oocyte viability, the BCB staining test has been successfully used in various species. However, to our knowledge, there have been no reports of equine ICSI using oocytes that were screened based on BCB test. Therefore, an experiment was conducted to investigate the

correlation of G6PD activity with initial cumulus morphology as well as oocyte volume, and its effect on meiotic competence and preimplantation embryo development after ICSI. As a result, low G6PD activity was strongly correlated with cumulus expansion. In fact, the proportion of Ex oocytes with low activity of G6PD (blue cytoplasm) was significantly higher than that of Cp oocytes. This finding is in agreement with a previous study that reports a relationship between G6PD activity and COCs morphology in heifers (Pujol *et al.* 2004). The higher proportion of Cp oocytes with high activity of G6PD (colorless cytoplasm) could be related to the follicular status of these oocytes at retrieval time. A lower proportion of Cp oocytes may have achieved their intraovarian growth phase, since they did not convert the BCB dye from blue to colorless (Alm *et al.* 2005; Bhojwani *et al.* 2007).

5.5 Relationship between G6PD activity via BCB staining and the volume of oocytes according to the cumulus morphology

We have also analyzed the relation between G6PD activity and oocyte volume at retrieval time. Our results showed that, BCB+ oocytes had a greater volume than BCB– oocytes, which was irrespective to their cumulus morphology. This is in accordance with our preliminary experiment using bovine oocytes and with previous reports in other species where diameter (Manjunatha *et al.* 2007; Pujol *et al.* 2004; Rodriguez-Gonzalez *et al.* 2002), volume and essential component of volume such as mitochondrial DNA copy number (El Shourbagy *et al.* 2006) have been associated with G6PD activity. Of great interest was the finding that supplementation of BCB– pig oocytes with mitochondria from maternal relatives was believed to have a positive effect on fertilization outcomes following both IVF and ICSI. Nevertheless, this could be supported by the earlier report in human that oocyte with low cytoplasmic volume would simply not have enough cytoplasmic content to sustain meiotic maturation, normal fertilization and further embryonic development (El Shourbagy *et al.* 2006; St John 2002; Van Blerkom *et al.* 1998).

5.6 Effect of G6PD activity on nuclear progression of equine oocytes after *in vitro* maturation

Our data demonstrate that low G6PD activity of equine oocytes is correlated with increased meiotic competence after IVM. The rate of maturation to MII stage was significantly higher in BCB+ compared to BCB– oocytes. In addition, the proportion of oocytes with GV-TI nucleus configuration was significantly lower in BCB+ compared with BCB– oocytes suggesting insufficient meiotic competence for the later group. This finding is in accordance with previous reports in which BCB– pig oocytes were found to be less competent than the BCB+ oocytes (Ericsson *et al.* 1993) which might be due to inadequate cytoplasmic maturation of these oocytes. Further evidence for this idea was later provided from a study in mice showing that BCB+ oocytes gained better cytoplasmic maturity (determined as the intracellular content of glutathione level and pattern of mitochondrial distribution) and higher developmental competence after IVM than the BCB− oocytes (Wu *et al.* 2007). In that study, the better cytoplasmic maturity and higher developmental competence of the BCB+ oocytes were correlated with their larger sizes, higher percentages of surrounded nucleoli (SN) chromatin configuration and higher frequencies of early atresia in comparison with the BCB– oocytes.

5.7 Effect of G6PD activity on preimplantation embryo development of equine oocytes after ICSI

Assuming that the lower activity of G6PD is an important determinant for oocyte competence, then screening of oocytes based on G6PD activity could be proposed as another valuable marker of developmental competence. Hence, to evaluate this hypothesis, oocytes were screened based on their G6PD activity via BCB test and after in vitro maturation, MII stage oocytes were fertilized using ICSI technique followed by culture to the blastocyst stage. Consequently, low activity of G6PD was markedly correlated with improved preimplantation embryo development. Our data demonstrate that the proportion of cleaved embryos in BCB+ oocytes was significantly higher compared to BCB– oocytes, which ultimately led to a significantly higher blastocyst rate at 8 days after ICSI. This result is consistent with earlier reports in which BCB+ goat (Rodriguez-Gonzalez *et al.* 2003), heifer (Pujol *et al.* 2004), bovine (Alm *et al.*

2005), and buffalo (Manjunatha *et al.* 2007) oocytes have been found to be significantly more capable of supporting preimplantation embryo development than the BCB– oocytes.

In our preliminary experiment using bovine oocytes as model for optimizing the BCB test, we also found that a higher blastocyst rate was observed in the BCB+ group than both control and BCB– groups. The lower developmental competence of BCB– oocytes can be attributed to their follicular origin. Since these oocytes are largely retrieved from follicles, which might be in earlier stages of follicular development after antrum formation (Bhojwani *et al.* 2007), and thereby intrinsically less competent to complete the cytoplasmic maturation process i.e. the molecular and structural changes that provide the matured oocyte the capacity to support fertilization and early embryonic development (Bevers and Izadyar 2002). Contrarily, the better performance of BCB+ oocytes, which are largely originating from fully-grown follicles, can be associated to the better cytoplasmic maturation of these oocytes during the final phases of folliculogenesis. Accordingly, the relationship between G6PD activity and cytoplasmic maturation in terms of the incidence of apoptotic granulosa cells (Wu *et al.* 2007), pattern of mitochondrial distribution (Torner *et al.* 2008; Wu *et al.* 2007), and intracellular GSH level (Wu *et al.* 2007) has been well addressed in the literatures for other species. Moreover, our previous study demonstrated that bovine oocytes screened based on BCB test differ in various oocyte quality markers such as chromatin configuration, mitochondrial activity and expression of distinct set of genes; mainly regulating transcription, translation, cell cycle, chromatin remodeling and mitochondrial machineries (Ghanem *et al.* 2007; Torner *et al.* 2007).

Taken together, results of this experiment show that selection of oocytes based on G6PD activity via BCB test is an efficient tool to predict the preimplantation embryo development in equine. This useful predictive parameter for oocyte quality may be used as a unique tool for further clarification of underlying mechanisms related to developmental competence. Studies are currently underway in our laboratory to characterize BCB screened oocytes at the cellular and molecular level. Relevant results from ongoing research will be presented in the near future.

5.8 Correlation of the oocyte diameter, zona thickness and zona birefringence

A set of experimental and clinical applications of a newly developed microscopy technique have been postulated as breakthrough in the field of assisted reproductive techniques (ART). Using this microscopic technique, new prognostic parameters of oocyte quality based on non-invasive analysis of polarized light generated by birefringent cell structures (i.e., zona pellucida and meiotic spindle) have been proposed (Pelletier *et al.* 2004; Rienzi *et al.* 2005; Rienzi *et al.* 2003; Wang *et al.* 2001). The underlying concept is that polarization light microscopy allows the distinction of three layers within the zona pellucida of oocytes and among them; the inner layer exhibits the highest amount of birefringence (Montag *et al.* 2008; Pelletier *et al.* 2004). A previous study showed a correlation between between zona thickness and birefringence in human (Shen *et al.* 2005). However, the birefringent properties of the equine zona pellucida and its correlation with oocyte diameter and zona thickness have not been reported so far and needs to be determined.

Irrespective of oocyte quality, a significant correlation was found between oocyte diameter and zona thickness. The oocyte diameter increased significantly with a corresponding increase in the zona thickness. This relation has already been documented in mice (Wassarman and Albertini 1994), and human (Wolf *et al.* 1995), where increase in diameter of the oocyte lead to an increase in thickness of the zona pellucida. Moreover, our results indicated that both the diameter of the oocyte and the thickness of the zona were significantly correlated with zona birefringence. The zona pellucida increased relatively in birefringence, when the oocytes increased in size and/or zona thickness. The optimal molecular organization of the filaments composing the zona pellucida is probably acquired gradually and this might elucidate the high birefringence observed in zona pellucida of oocytes with lager diameter and elevated thickness (Oldenbourg and Mei 1995; Pelletier *et al.* 2004; Vanroose *et al.* 2000). Alternatively, a longer light path through the paracrestline structure of zona might be able to explain the elevated birefringence (Pelletier *et al.* 2004), which has been observed in oocyte with the thicker zona layers. To our knowledge, this is the first report demonstrating that there is a positive correlation between the diameter of the equine oocyte, thickness of the zona pellucida and zona birefringence as quantitatively assessed by polarization light microscopy.

5.9 Cumulus morphology in correlation with thickness and birefringence of the zona pellucida

Over the last few years, several authors have focused on establishing correlations between the birefringent characteristic of the zona and the oocyte quality (Jelinkova *et al.* 2008; Madaschi *et al.* 2009; Montag *et al.* 2008; Rama Raju *et al.* 2007; Shen *et al.* 2005). However, the birefringent properties of the zona pellucida and its correlation with quality-related parameters of equine oocytes have not been reported so far and needs to be determined. Therefore, in the light of this evidence, first we aimed to compare the thickness and birefringence of zona pellucida in oocytes of different developmental competence screened based on cumulus morphology at retrieval time. Quantitative analysis of zona birefringence might be an advantage for the prediction of oocyte's developmental competence in equine ART, markedly because of simple implementation and an incredibly fast performance as well.

Our results suggest that there is a clear correlation between cumulus morphology of the oocytes at retrieval time and the zona properties (i.e., thickness and birefringence). The zona pellucida was thicker in oocytes with Ex cumulus cells than in oocytes with Cp cumulus cells. Likewise, the zona pellucida of oocytes with Ex cumulus cells exhibited greater birefringence than the oocytes with Cp cumulus cells. This result is consistent with earlier human studies in which both thickness and birefringence of the zona has been reported to differ within a cohort (Pelletier *et al.* 2004), and between cohorts of oocytes (Shen *et al.* 2005). The birefringent characteristic of the zona revealed by polarization light microscopy is directly linked to the paracrystalline network structure of the zona glycoprotein (Montag *et al.* 2008; Wassarman *et al.* 2004), which is formed by the oocyte and the granulosa cells during follicular growth, cooperatively (Sinowatz *et al.* 2001). Indeed, cumulus cells in equine not only contribute to zona synthesis, but also overtake total glycoprotein synthesis in the tertiary follicle (Kolle *et al.* 2007). In this respect, probably the oocytes with Cp cumulus cells are not as competent as oocytes with Ex cumulus cells to accomplish their functional roles in zona formation and this might lead to the observed differences.

As it has been shown in our experiments, not only higher developmental competence, but also the elevated zona thickness and birefringence were correlated with cumulus expansion at retrieval time from the follicles. Consequently, these results clearly

demonstrate that oocytes with Ex cumulus cells had a higher developmental competence and were thicker in zona and greater in zona birefringence in comparison with Cp cumulus cells. The greater zona thickness and birefringence of equine Ex oocytes may be attributed to their recovery from atretic follicles; oocyte changes associated with follicle atresia mimic those of oocytes in viable follicles approaching ovulatory size (Choi *et al.* 2006b; Hinrichs and Schmidt 2000). The zona pellucida changes during atresia have already been studied using light microscopy techniques (Centola 1982), and ultrastructurally (Pastor *et al.* 2008). Such follicles, like preovulatory follicles, could signal the oocytes via certain molecules and confer to them the greater developmental competence and probably the associated zona integrity. In other words, it might be that a fine structure of zona pellucida is gained late in the follicular phase so that a final differentiation is required, and this differentiation is similar to the beginning of atresia (Blondin and Sirard 1995).

5.10 G6PD activity in correlation with thickness and birefringence of the zona pellucida

It has been shown that BCB selected oocytes differ in various oocyte quality markers at the cellular (El Shourbagy *et al.* 2006; Torner *et al.* 2008; Wu *et al.* 2007) and molecular level (Ghanem *et al.* 2007; Torner *et al.* 2008). An explanation for the better performance of BCB+ oocytes can be related to better cytoplasmic maturation of these oocytes during the final phases of folliculogenesis. Since the zona pellucida deposits around the oocytes during folliculogenesis, we proposed that different oocytes qualities based on BCB screened oocytes are reflected in their zona properties.

Our results indicate that the zona pellucida of BCB+ oocytes differed in thickness and birefringence compared with BCB– oocytes. The zona was thicker in BCB+ oocytes than the BCB– oocytes. Similar to the thickness, birefringence of the zona was greater for BCB+ oocytes compared to BCB– oocytes; providing more evidence that zona properties is more likely related to the oocyte quality. This could be explained by the fact that the BCB+ oocytes are largely recovered from those follicles, which completed their growth phase and thus acquired full developmental competence in terms of integrity of cytoplasmic organelles (El Shourbagy *et al.* 2006; Torner *et al.* 2008; Wu *et al.* 2007) and zona pellucida structure. On the other hand, BCB– oocytes are largely originated from smaller antral follicles, which may be in earlier stages of follicular

development after antrum formation (Bhojwani *et al.* 2007). Therefore, due to inadequate cytoplasmic maturation (Wu *et al.* 2007), they might be intrinsically less competent to form a regular structure of zona pellucida as indicated in our study by elevated thickness and greater birefringence. Generally, the ability for synthesizing zona pellucida proteins is strongly correlated to the developmental capacity of the COC (Kolle *et al.* 2007; Kolle *et al.* 1998). This is corroborated by findings that the mean thickness of the zona is greater in ovulated oocytes than in oocytes matured *in vitro* (Funahashi *et al.* 2000; Kolle *et al.* 2007).

5.11 Maturational status in correlation with thickness and birefringence of the zona pellucida

We compared mean and variance of zona thickness and birefringence at different maturational stages. We did not observed any significant differences in mean zona thickness and birefringence between immature oocytes and oocytes with polar body (PB+ oocytes) after in vitro maturation, which confirmed previous findings (Pelletier *et al.* 2004). Nevertheless, the zona pellucida of oocytes, which failed to extrude the polar body (PB– oocytes), was thicker than of oocytes immature and PB+ oocytes.

Similarly, the mean zona birefringence in PB– oocytes was significantly greater than that of immature oocytes and PB+ oocytes as well. This is in line with a previous report (Jelinkova *et al.* 2007) where the extremely high zona birefringence has been associated with lower developmental competence in human. Goudet *et al.* (1997) found that overall oocyte diameter is decreased as nuclear maturation progressed: oocytes which remained in germinal vesicle stage were larger in diameter than oocytes that resumed meiosis and the metaphase I oocytes were larger than MII oocytes. This also may explain why the thickness of zona pellucida and related birefringence was greater in PB– oocytes than that of immature and PB+ oocytes. The two parameter for oocyte quality, as well as oocyte diameter/volume, which has been postulated as predictor have been correlated with birefringence and zona thickness.

Results of this study show that activation of equine oocytes by calcium ionophore A23187 is beneficial following ICSI procedure, in particular when conventional ICSI is the method of sperm injection. Our data reveals that selection of oocytes based on morphology of cumulus cells affects meiotic competence and subsequent

preimplantation embryo development. In addition, selection of oocytes based on activity of G6PD enzyme also affects meiotic competence and subsequent preimplantation embryo development. These two parameters for quality, as well as oocyte diameter/volume which has been postulated as marker for quality have been correlated with zona thickness and birefringence. Oocytes presented with a higher developmental competence were thicker in zona and exhibited greater birefringence. The prediction of oocyte/embryo quality through the extent and pattern of zona birefringence may obviate the need for extended periods of culture *in vitro*, which is considered as the main cause of early embryo mortality in equine. The positive predictive parameters of oocyte quality, which have been reported in this study, may be used as a unique model to further clarify the underlying mechanisms of developmental competence acquisition. Studies are currently underway in our laboratory to characterize BCB classified oocytes at the cellular and molecular level. Relevant results from ongoing research will be presented in the near future.

6 Summary

The present study was performed, (i) to study the effect of additional activation by calcium ionophore A23187 on developmental competence of equine oocytes after intracytooplasmic sperm injection (ICSI), (ii) to re-evaluate the effect of cumulus morphology on meiotic competence and preimplantation embryo development after ICSI, (iii) to study glucose-6-phosphate dehydrogenase (G6PD) activity in correlation with cumulus morphology and oocyte volume and its effect on meiotic competence and preimplantation embryo development after ICSI, (iv) to analyze the zona pellucida of equine oocytes of different quality and maturational status, quantitatively by polarization light microscopy.

To determine whether additional activation by calcium ionophore A23187 has a positive influence on preimplantation embryo development of equine oocytes, metaphase II (MII) oocytes were fertilized using ICSI technique and injected oocytes were either immediately placed in culture media, or 1 hour after injection incubated with 5 μM of calcium ionophore A23187 for 10 min followed by culture. *In vitro* culture was carried out for 8 days and data for subsequent developmental competence in terms of cleavage and development to the blastocyst stage were recorded. The rate of cleavage in calcium ionophore A23187 treated (ICSI with calcium ionophore A23187) group was significantly increased in comparison with that of the non-treated (ICSI without calcium ionophore A23187) group (50.0% *vs*. 28.4%; $P<0.05$). In addition, the calcium ionophore A23187 activated oocytes showed a significantly higher ($P<0.05$) blastocyst rate than the non-treated group (8.6% *vs*. 1.9%; $P<0.05$).

A second experiment was performed to evaluate the effect of cumulus morphology on meiotic competence and preimplantation embryo development after ICSI. Oocytes were categorized into expanded (Ex) and compact (Cp) group, depending on expansion of cumulus cells. Nuclear maturation after culture was determined by extrusion of the first polar body and nuclear status of those oocytes without a polar body was determined for each Ex and Cp group after fixing and staining. The maturation rate was significantly higher in Ex oocytes compared to Cp oocytes (59.1% *vs.* 42.2%; $P<0.01$). The proportion of oocyte with GV-TI nucleus status observed after IVM culture was significantly lower in Ex oocytes compared to Cp oocyets (15.4% *vs.* 34.3 %; $P<0.01$). There was no significant difference in proportion of degenerated oocytes that were

classified as Ex or Cp cumulus cells. There was no significant difference in cleavage rate between the two oocyte categories. However, the proportion of the Ex oocytes that developed into blastocyst was significantly higher than that of the Cp oocytes (11.9% *vs.* 3.8%; $P<0.05$).

In an attempt to study G6PD activity in correlation with cumulus morphology and oocyte volume and its effect on meiotic competence and preimplantation embryo development after ICSI, the Ex and Cp oocytes were exposed to brilliant cresyl blue (BCB) test. Overall, 1209 COCs were morphologically classified, 755 (62.4%) oocytes as Ex group, and 454 (37.6%) as Cp group. After staining with BCB, 710 (58.7%) oocytes were classified as BCB+ (blue cytoplasm; low G6PD activity) and 499 (41.3%) oocytes were classified as BCB– (colorless cytoplasm, high G6PD activity). The percentage of BCB+ oocytes of Ex group was significantly higher than that of Cp group (67.0% *vs.* 44.9%; $P<0.01$). The mean volume of BCB+ oocytes was significantly higher than the mean volume of BCB– oocytes (2.1×10^6 μm^3 *vs.*1.75×10^6 μm^3; $P<0.01$). Likewise, mean volume of the BCB+ oocytes for each categories of cumulus morphology was significantly higher ($P<0.01$) than mean volume of the BCB– oocytes for each categories of cumulus morphology. Neither BCB+ nor BCB– oocytes showed a significant difference in oocyte volume between the different cumulus morphologies. A significantly higher proportion of oocytes from BCB+ group had reached MII-stage after IVM than was observed in BCB– group (59.5% *vs.* 28.7%; $P<0.01$). The proportion of oocyte with GV-TI nucleus status observed after culture was significantly lower in BCB+ group compared to BCB– group (9.2% *vs.* 37.7%; $P<0.01$). However, the number of degenerated oocyte was not statistically different between two groups. The proportion of oocytes that cleaved by day 2 after ICSI was significantly higher for BCB+ oocytes than the BCB– oocytes (45.9% *vs.* 29.0%; $P<0.05$). Furthermore, the number of blastocyst obtained by day 8 was significantly higher in BCB+ group compared with BCB– group (9.2% *vs.* 1.4%; $P<0.05$).

A further experiment was performed to study the correlation of oocyte diameter with zona thickness and oocyte diameter with zona birefringence. In addition, we studied the correlation between zona thickness and zona birefringence. As the zona pellucida plays a part in the measure of the oocyte diameter, a thicker zona confers a larger diameter to the oocyte ($r=0.76$, $n=308$; $P<0.0001$). Likewise, there was a positive correlation ($r=0.62$, $n= 308$; $P<0.0001$) between diameter of oocyte and birefringence of the zona;

as oocyte diameter increases, there is a corresponding increase in zona birefringence. The correlation between thickness and birefringence of the zona in equine oocytes was also statistically significant (r=0.44, n=308; $P<0.0001$); supporting that the birefringence of the zona is positively correlated with zona thickness.

In addition, we investigated whether cumulus morphology, G6PD activity and maturational status could be correlated with zona thickness and birefringence. The quantitative analysis of zona birefringence was performed by polarization light microscopy and OCTAX polarAIDE-software. The Ex (n=93) oocytes had a significant thicker zona than the Cp (n=86) oocytes (18.2 ± 2.2 µm *vs.* 17.3 ± 2.1 µm; $P<0.05$). In addition, mean birefringence of the zona was significantly higher for Ex oocytes compared to Cp oocytes (64.6 ± 5.2 *vs.* 62.1 ± 4.2; $P<0.05$). Of all oocytes stained, those classified as BCB+ (n=89) had a significant thicker zona in comparison to BCB– (n=41) oocytes (18.8 ± 2.4 µm *vs.* 16.1 ± 2.0 µm; $P<0.05$). We also found that BCB+ oocytes had a significantly greater mean of zona birefringence compared to those of BCB– oocytes (63.1 ± 4.5 *vs.* 61.3 ± 3.3; $P<0.05$). There was no significant difference in mean zona thickness between immature oocytes and PB+ oocytes after IVM. However, the mean zona thickness of PB– oocytes after IVM was significantly higher (18.8 ± 2.4 µm) than both immature (17.8 ± 2.4 µm) and PB+ oocytes (17.5 ± 2.2 µm; $P<0.05$). Similar to the thickness our results showed that there was no significant change in the zona pellucida birefringence of immature oocytes and PB+ oocytes after maturation. Nevertheless, the mean zona birefringence in the PB– oocytes (64.8 ± 5.5) was significantly higher than that for immature (63.0 ± 4.6) and PB– oocytes (62.7 ± 4.1) after IVM.

In conclusion, the data of this study showed that activation of equine oocytes by calcium ionophore A23187 is effectively improved development to the blastocyst stage. In addition, our results confirmed that G6PD activity of oocyte before maturation as well as cumulus morphology is a reliable predictor for subsequent development of equine oocytes. Likewise, the extent and pattern of ZP birefringence may constitute a new marker for oocyte quality assessment in equine in vitro embryo production system.

Zusammenfassung

Die vorliegende Studie untersucht den Effekt der assistierten Eizellaktivierung mit Calcium Ionophor A23187 equiner Embryonen nach der intrazytoplasmatischen Spermainjektion (ICSI) auf die Entwicklungskompetenz. Es wurde eine Re-evaluierung der Einflusses der Kumulusmorphologie, der Glucose-6-Phosphat-Dehydrogenase-Aktivität und der Beschaffenheit der Zona pellucida mit Hilfe der Polarisationslichtmikroskopie im Hinblick auf die meiotische und embryonale Entwicklungskompetenz durchgeführt.

Die gereiften Eizellen wurden entweder direkt nach ICSI in Kultur gesetzt oder eine Stunde nach ICSI für 10 Minuten in 5 µM Calcium Ionophor A23187 inkubiert und anschließend kultiviert. Die Dauer der in vitro Kultivierung betrug acht Tage. Eizellen, die mit Calcium Ionophor A23187 aktiviert wurden, erreichten eine höhere Teilungsrate als die nicht aktivierten Eizellen (50,0% vs. 28,4%; $P<0.05$) und eine höhere Blastozystenrate (8,6% vs. 1,9%; $P<0,05$).

In einem zweiten Experiment wurde der Einfluss der Kumulusmorphologie auf die präimplantative Entwicklungskompetenz untersucht. Verglichen wurden Eizellen mit expandiertem (Ex) und kompaktiertem (Cp) Kumulus. Die Maturationsraten der Ex Eizellen war im Vergleich zu den Cp Eizellen signifikant höher (59,1% vs. 42,2%; $P<0,01$). Der Anteil der Eizellen die sich nach der in vitro Maturation im GV-TI Stadium befanden war in der Gruppe der Ex Eizellen signifikant geringer als in der Gruppe der Cp Eizellen (15,4% vs. 34,3%; $P<0,01$). Der Anteil der degenerierten Eizellen war in beiden Gruppen ähnlich. Bezüglich der Teilungsraten nach ICSI konnten keine signifikanten Unterschiede festgestellt werden. Die Ex Eizellen wiesen ein signifikant höheres Entwicklungspotential zur Blastozyste auf als die Cp Eizellen (11,9% vs. 3,8%; $P<0,05$).

In einem Versuch zur Bestimmung der G6PD-Aktivität in Korrelation zur Kumulusmorphologie und dem Volumen der Eizellen sowie dessen Einfluss auf die meiotische Kompetenz und das präimplantive Entwicklungspotential nach ICSI wurden die Eizellen dem Brilliant Cresyl Blue (BCB) Test unterzogen. Insgesamt wurden 1209 Kumulus-Oozyten-Komplexe (COC) morphologisch klassifiziert, wobei 755 Eizellen (62,4%) expandierte Kumuluszellen und 454 Eizellen (37,6%) kompaktierte Kumuluszellen aufwiesen. Nach Färbung mit BCB wurden insgesamt 710 Eizellen

(58,7%) als BCB+ (blaues Cytoplasma, geringe G6PD Aktivität) und 499 (41,3%) als BCB- (farbloses Cytoplasma, hohe G6PD Aktivität) eingestuft. Der prozentuale Anteil der BCB+ Eizellen war in der Gruppe der Eizellen mit expandiertem Kumulus signifikant höher als in der Gruppe mit kompaktiertem Kumulus (67,0% vs. 44,9%; P<0,01). Des Weiteren wurde das Volumen der Eizellen gemessen, wo das durchschnittliche Volumen der BCB+ Eizellen signifikant höher war als das der BCB- Eizellen (2.1×10^6 µm^3 *vs.* 1.75×10^6 µm^3; *P*<0.01).

Weder BCB+ noch BCB- Eizellen zeigten bezüglich des Volumens der Eizellen signifikante Unterschiede zwischen den beiden Gruppen Ex und Cp.

Hinsichtlich der Maturationsraten konnten zwischen BCB+ und BCB- signifikante Unterschiede festgestellt werden (59.5% *vs.* 28.7%; *P*<0.01). Der Anteil der Eizellen, die sich nach der in vitro Maturation im GV-TI Stadium befanden, war bei den BCB+ Eizellen signifikant geringer als bei den BCB- Eizellen (9.2% *vs.* 37.7%; *P*<0.01). Jedoch konnten in der Anzahl der degenerierten Eizellen zwischen den Gruppen keine signifikanten Unterschiede beobachtet werden.

Des Weiteren wurden die Eizellen auf ihr Entwicklungspotential nach ICSI untersucht. Hier waren sowohl in den Teilungsraten als auch in den Blastozystenraten signifikante Unterschiede zu erkennen. Tag zwei nach ICSI wiesen die BCB+ Eizellen eine höhere Teilungsrate auf als die BCB- Eizellen (45.9% *vs.* 29.0%; *P*<0.05). Gleichermaßen verhielt es sich am Tag acht, 9,2% der BCB+ Eizellen entwickelten sich zur Blastozyste, wohingegen nur 1,4% der BCB- Eizellen das Blastozystenstadium erreichten (P<0,05).

In einem weiteren Experiment wurde zum einen die Korrelation zwischen dem Durchmesser der Eizellen und der Dicke der Zona pellucida gemessen und zum anderen der Durchmesser der Eizellen in Korrelation zur Doppellichtbrechungsintensität. Zusätzlich wurde die Korrelation zwischen der Dicke der Zona pellucida und ihrer Doppellichtbrechungsintensität untersucht.

Die Zona pellucida spielt eine Rolle in der Vermessung des Eizelldurchmessers, wobei eine dickere Zona auf einen größeren Durchmesser der Eizelle schließen lässt (r=0.76, n=308; *P*<0.0001). Gleichermaßen war eine positive Korrelation zwischen dem Durchmesser der Eizellen und der Doppellichtbrechungsintensität der Zona pellucida zu beobachten (r=0.62, n= 308; *P*<0.0001); je größer der Durchmesser der Eizelle desto höher ist auch die Doppellichtbrechungsintensität der Zona pellucida. Ebenfalls

korreliert die Dicke der Zona pellucida positiv mit ihrer Doppellichtbrechungsintensität (r=0.44, n=308; *P*<0.0001).

Zusätzlich untersuchten wir, ob es einen Zusammenhang zwischen den Parametern Kumulusmorphologie, G6PD Aktivität und meiotischer Kompetenz und den Eigenschaften der Zona pellucida wie Dicke und Doppellichtbrechungsintensität gibt. Die quantitative Analyse der Doppellichtbrechungsintensität wurde mit Hilfe eines Polarisationslichtmikroskops und der OCTAX polarAIDE-software durchgeführt.

Sowohl die Dicke der Zona war bei den Ex Eizellen (n=93) signifikant höher als bei den Cp Eizellen (n=86) (18.2 ± 2.2 µm *vs.* 17.3 ± 2.1 µm; *P*<0.05) als auch die Doppellichtbrechungsintensität (64.6 ± 5.2 *vs.* 62.1 ± 4.2; *P*<0.05). Bei den mit BCB gefärbten Eizellen zeigten jene, die als BCB+ (n=89) eingestuft wurden, eine signifikant dickere Zona pellucida als die BCB- Eizellen (n=41) (18.8 ± 2.4 µm *vs.* 16.1 ± 2.0 µm; *P*<0.05). Ebenso konnten wir bei den BCB+ Eizellen eine signifikant höhere Doppellichtbrechungsintensität feststellen (63.1 ± 4.5 *vs.* 61.3 ± 3.3; *P*<0.05). Zwischen den immaturen Eizellen und denen, die nach der in vitro Maturation einen Polkörper ausschleusten, konnten keine signifikanten Unterschiede in der Dicke der Zona pellucida beobachtet werden. Jedoch unterschieden sich die Eizellen, die nach der in vitro Maturation keinen Polkörper ausschleusten, signifikant in der Dicke der Zona (18.8 ± 2.4 µm) von den immaturen Eizellen (17.8 ± 2.4 µm) und den Eizellen mit Polkörper (17.5 ± 2.2 µm; *P*<0.05). Ähnlich fielen unsere Untersuchungen zur Doppellichtbrechungsintensität aus. Die immaturen Eizellen (63.0 ± 4.6) und die Eizellen mit Polkörper (62.7 ± 4.1) unterschieden sich signifikant von den Eizellen, die nach der in vitro Maturation keinen Polkörper ausschleusten. Diese zeigten eine signifikant höhere Doppellichtbrechungsintensität (64.8 ± 5.5).

Zusammenfassend zeigt diese Studie, dass die Aktivierung der equinen Eizellen nach ICSI mit Calcium Ionophor A23187 das präimplantive Entwicklungspotential effektiv steigert. Des Weiteren bestätigten unsere Ergebnisse, dass sowohl die Kumulusmorphologie als auch die G6PD Aktivität verlässliche Vorhersagen über die Entwicklungskompetenz der Eizellen geben. Ebenfalls könnten die Dicke und die Struktur der Zona pellucida, gemessen an Hand der Doppellichtbrechungsintensität, als neue Methode zur Bestimmung des Entwicklungspotentials equiner Eizellen dienen.

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4. Publications

4.1 Peer reviewed publication

Salilew-Wondim D., Hoelker M., Rings F., Phatsara C., **Mohammadi-Sangcheshmeh A.,** Tholen E., Schellander K and Tesfaye D. (2010). Depletion of Birc6 leads to retarded bovine early embryonic development and blastocyst formation in vitro. *Reprod Fertil Dev*. 22(3) 564-579

Mohammadi-Sangcheshmeh A., Held E., Rings F., Tholen E., Schellander K., Tesfaye D., Hoelker M. Glucose-6-phosphate dehydrogenase (G6PD) activity in equine oocyte is associated with meiotic competence and preimplantation embryo development after ICSI. **(*In preparation*)**

Mohammadi-Sangcheshmeh A., Held E., Rings F., Tholen E., Schellander K., Tesfaye D., Hoelker M. Quantitative analysis of zona birefringence in equine oocytes: Association with cumulus morphology, G6PD activity and maturational stages. **(*In preparation*)**

4.2 Abstracts / posters / theatre presentations

Mohammadi-Sangcheshmeh A, Naumann A, Rings F, Koester M, Schimming T, Ghanem N, Phatsara C, Tholen E, Tesfaye D, Montag M, Schellander K, Hölker M (2008): Zona pellucida birefringence as a prognostic tool for developmental potential of bovine oocytes and zygotes: A study using poloscope. 35. Jahrestagung der Arbeitsgemeinschaft Embryotransfer deutsprachiger Länger (AET-d), 19./20.6.2008, Dipperz/Friesenhausen, Germany (Proc)

Mohammadi-Sangcheshmeh A, Koester M, Naumann A, Rings F, Ghanem N, Schimming T, Phatsara C, Tholen E, Tesfaye D, Schellander K, Montag M, Hoelker M (2008): Zona birefringence intensity as a prognostic tool for

embryonic development of bovine oocyte and zygote using polarization light microscopy. Vortragstagung der DGfZ und GfT, 17./18.9.2008, Bonn, Deutschland, Tagungsband R1 (Proc)

Mohammadi-Sangcheshmeh A, Naumann A, Rings F, Koester M, Schimming T, Ghanem N, Phatsara C, Tholen E, Tesfaye D, Montag M, Schellander K, Hoelker M (2008): Bovine oocyte quality is reflected in zona pellucida architecture when assessed based on G6PDH activity. 24. Scientific Meeting Association Europeenne de Transfert Embryonnaire/ European Embryo Transfer Association (AETE), 12./13.9.2008, Pau, France, 210 (Abstr)

Salilew-Wondim D, Rings F, Hölker M, Phatsara C, **Mohammadi-Sangcheshmeh A,** Tesfaye D Schellander K, (2008): BIRC6/apollon is involved in bovine preimplantation embryo development. Third International Symposium on Animal Functional Genomics (ISAFG), 7.-9.4.2008, Edinburgh, Scotland, Proc, 53 (Abstr)

Mohammadi-Sangcheshmeh A, Held E, Rings F, Tesfaye D, Schellander K, Hoelker M (2009a): Prediction of horse oocyte quality: Morphological and cellular aspects, 36. Jahrestagung der Arbeitsgemeinschaft Embryotransfer deutschsprachiger Länder (AET-d), 28./19.6.2009, Landshut, Deutschland (Abstr), 19-20

Mohammadi-Sangcheshmeh A, Koester M, Schimming T, Tesfaye D, Montag M, Schellander K, Hoelker M (2009b): Polarized light microscopy reveals an association between zona birefringence intensity and embryonic development in bovine oocytes and zygotes, 35th Annual Conference of the International Embryo Transfer Society (IETS). Reproduction, Fertility and Development, San Diego, Ca, USA (Abstr), pp. 204-205

Held E, **Mohammadi-Sangcheshmeh A,** Rings F, Tholen E, Tesfaye D, Schellander K, Hoelker M (2009): Beschaffenheit der Zona pellucida equiner Eizellen in Relation zu deren Entwicklungspotential, 36. Jahrestagung der Arbeitsgemeinschaft Embryotransfer deutschsprachiger Länder (AET-d), 28./19.6.2009, Landshut, Deutschland (Abstr), 21-22