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Title of the Thesis
Developmental competence of sheep oocytes and zygotes selected using the brilliant cresyl blue (BCB)

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Developmental Competence of Sheep Oocytes and Zygotes Selected Using the Brilliant Cresyl Blue (BCB) test

Abstract

The aims of the present study was to evaluate the relationship between follicle size and oocyte growth and the usefulness of the brilliant cresyl blue (BCB) test before in vitro maturation in the selection of high competent sheep oocytes and zygotes for in vitro embryo production (IVEP). Sheep oocytes were exposed to BCB stain diluted in Dulbecco's phosphate-buffered saline modified with 0.4% BSA (mDPBS) for 90 min; those with or without blue coloration of the cytoplasm were designated as BCB+ and BCB-, respectively. This study was conducted in four separate experiments: in the experiment 1, the percentages of selected oocytes by BCB test and oocyte diameter was evaluated after classification of oocytes by BCB test (13, 26 or 39 μM BCB in mDPPS). In the experiment 2, the relationships between follicle size (small: <3 mm, medium: 3-5 and large: >5 mm), oocyte diameter and intraovarian growth phase of oocyte were analyzed. In the experiment 3, the utility of varying concentrations of BCB test to select the oocytes most competent for IVP of embryos were evaluated. Therefore, oocytes were placed into three groups: control (immediately cultured); holding-control (kept in mDPBS for 90 min before cultured); and treatment-incubation (incubated with BCB). In the experiment 4, the utility of BCB test to select the zygotes most competent for IVP of embryos was evaluated. The zygotes were exposed to BCB for 10 min at 38.5 ˚C and categorized into 3 categories according to whether they were still stained (category 1), moderately stained (category 2) or unstained (category 3). After BCB categorization, the zygotes were maintained undisrupted in the culture medium for 7 days. The percentage and diameter of BCB+ oocytes obtained after exposure to different BCB concentrations were similar. The oocyte diameter and percentage of fully grown (BCB+) oocytes were increased with follicular size. BCB+ oocytes yielded more blastocysts than BCB- oocytes, control and holding-control oocytes. The category 3 zygotes yielded more blastocyst rate (31.1%) than control (untested; 18.8%, $P<0.0001$), category 2 (17.1%, $P<0.0001$) and category 1 (11.6%, $P<0.000001$) zygotes. In conclusion, the BCB test would be useful way to select developmentally competent sheep oocytes and zygotes to improve IVEP.

Key words: oocyte selection, zygote categorization, brilliant crezyl blue, developmental competence

Chapter 1:

INTRODUCTION

Follicular immature oocytes recovered from ovaries of slaughtered sheep are commonly used to study the processes of in vitro maturation (IVM), in vitro fertilization (IVF) and the technique of in vitro production (IVP) of embryos. The inefficiency of IVP of embryos has been attributed to oocyte quality at the start of maturation (Manjunatha *et al*. 2007). Oocyte developmental competence is defined as the ability of an oocyte to resume meiosis, to cleave following fertilization, to develop to the blastocyst stage, to induce a pregnancy and bring offspring to term in a good health (Krisher, 2004; Sirard *et al.* 2006). This competency is acquired gradually during the process of folliculgenesis as the oocyte grows and its companion somatic cells differentiate (Eppig *et al*. 1994).

One of the main problems concerning IVF laboratories is the need for zygote selection after in vitro fertilization. The relatively low level of efficiency achieved using in vitro embryo production, manifested by the frequent failure of recovered zygotes to reach the blastocyst stage after fertilization, is almost certainly related to the quality of the zygotes at the end of the fertilization. However, a zygote scoring system, as well as other embryo development markers, may be useful in determining the number and quality of embryos to transfer.

Precise selection of oocytes and zygotes and prediction of developmental potential is probably the most pressing issue in the IVF unites. A limited of studies have demonstrated that the developmental potential of oocytes was affected by the age of the animals (Amstrong 2001) size of follicles (Tsuji *et al.* 1985; Trounson *et al.* 2001) or oocytes (Durinzi *et al.* 1995), follicle dominance (Russell 1998), and oocyte quality (Guienne 1999). It has been suggested that follicular size and oocyte diameter should be considered as selection parameters (Arlotto *et al.* 1996; Roca *et al.* 1998). Recent data have indicated a relationship between follicle size, oocyte diameter and developmental competence (Otoi *et al.* 1997; Hyttel *et al.* 1997). Mammalian immature oocytes are routinely selected for IVF on the basis of the visual assessment of morphological features such as thickness and compactness of the cumulus investment and the homogeneity the ooplasm (Alm *et al,* 2005). Similar to the oocyte, morphological criteria, namely the position of second polar body, the pronuclear morphology, and the nuclear morphology have been employed to correlate with the developmental capacity of zygotes. Morphologically best looking oocytes do not necessarily have the highest developmental competence (Blondian and Sirard, 1995) and those oocytes with better cumulus expansion during maturation may not necessarily show the highest blastocyst rate. In addition, oocytes and zygotes selection based on their morphology is more often influenced by personal judgments and lacks universal standards. Moreover, there is no standard zygote grading system used thought assisted reproduction laboratories (Wittemer *et al,* 2000). Therefore, investigating other non-invasive and non-perturbing techniques is required to predict and select competent oocytes and zygotes to increase the efficiency of in vitro embryo production programes (Wittemer *et al,* 2000).

Several authors found positive relationships between oocyte diameter and oocytes ability to develop to blastocyst stage in ewes and goats (Russell 1998; Otoi *et al.* 1997; Crozet *et al.* 1995; Fair *et al.* 1995). The brilliant crysil blue (BCB) test has been used for selection of

grown oocytes. Studies in large animals showed that oocytes stained with BCB (BCB+) were larger and more competent in maturation and development than those that were unstained (BCB-) and control oocytes (untested with BCB) (Roca *et al*. 1998; Rodriguez-Gonzalez *et al.* 2002).

BCB stain is an electron acceptor, can be used to semi-quantitate the level of glucose-6-phosphate dehydrogenize (G6PDH) activity of oocytes by modification of a visual color (Tian *et al.* 1998). The BCB test is based on the capability of the G6PDH to convert the BCB stain from blue to colorless (active G6PDH: BCB-, unactive G6PDH: BCB+) (Ericsson *et al.* 1993). During the growth phase, immature oocytes synthesized a variety proteins (Wassarman 1988), including G6PDH; and oocyte volume and diameter increases as a result of the accumulation of water, ions, carbohydrates and lipids (Fair *et al.* 1997). G6PDH is known to be a component of the pentose phosphate pathway (PPP) which provides ribose phosphate for nucleotide synthesis and formation of fatty acids. The G6PDH enzyme is active in the growing oocyte (Mangia and Epstein 1975), but has decreased activity in oocytes that have finished their growth phase (Wassarman 1988). This enzyme is synthesized within the oocytes during oogenesis (Mangia and Epstein, 1975) and embryos during embryogenesis. In addition, glucose metabolism is necessary for successful fertilization in the mouse. Both spermatozoa and oocytes metabolize glucose through the PPP, and NADPH appears required for gamete fusion. After sperm-oocyte fusion, an increase in glucose uptake by the fertilized oocytes was observed but not before the formation of the male and female pronuclei (Urner and Sakkas, 2004). When glucose metabolism was measured following IVF, both glycolysis and PPP activities were dramatically increased in fertilized oocytes and that the level of glucose metabolism was correlated with ability of the spermatozoon to determine an early (high in vitro fertility) or a delayed onset of S phase (low in vitro fertility; Comizzoli et al, 2003).

Rodrigues-gonzalez *et al.* (2002) reported that selection of oocytes using the BCB test (BCB+ oocytes) improved the nuclear maturation, normal fertilization (2PN) and embryo development up to the morulla stage. Wongsrikeao *et al.* (2006) reported that selection of oocytes using the BCB test before IVM improved the rates of nuclear maturation, monospermic fertilization of porcine oocytes, and subsequent embryonic development after IVF, as well as enhancement of embryo quality.

The present study was conducted to standardize the BCB test: 1) to select developmentally competent ovine oocytes before IVM and thereby increase the efficiency of blastocyst development after IVM/IVF; 2) to select developmentally competent sheep zygotes to enhance IVP of embryos.

Chapter 2:

LITERATURE REVIEW

2-1- Overview of oocyte growth and developmental competance

In all mammalian species, follicle and oocyte development follow a characteristic sequence of events that commences with the establishment of the ovary shortly after conception and terminates with the ovulation of a fertile, metaphase II oocyte. Throughout the lengthy preantral phase of mammalian folliculogenesis, the development of the oocyte is dependent on, and is concurrent with that of the follicular granulosa cell (GC) layers (Diaz *et al.* 2007). Effective communication between the different follicular cell types is achieved via homologous and heterologous gap junctional contacts (Picton *et al.* 2007). Indeed, the cross talk between the somatic GCs and the oocyte is vital to sustain oocyte growth because the major source of nutrients for the gamete is the somatic compartment (Harris and Picton 2007). During follicle growth, oocyte volume and diameter increases as a result of the accumulation of water, ions, carbohydrates and lipids (Fair *et al.* 1997) such that, in mice for example, there is a 150 fold increase in oocyte volume between primordial and preovulatory oocytes (Pan *et al.* 2005).

The morphological changes and developmental time frames associated with follicle and oocyte development are well characterized across the species (McNatty *et al.* 1999) and antral cavity formation commonly occurs when follicular diameters of between 180–250 mm are reached. By this stage, the majority of oocyte growth has been completed. In contrast, species specific differences have been recorded in a number of parameters including: i) the overall timeframe for completion of folliculogenesis and oogenesis; ii) the size of the ovulatory follicles and mature oocytes; and iii) differences in the nature, concentrations and effects of the putative growth factors that moderate follicle and oocyte production in vivo. These differences are highly relevant when developing systems that support the complete IVG and IVM of follicles and oocytes.

Recent research has revealed that oocyte secreted factors regulate the initiation of primordial follicle growth (see Picton 2001 for review) and moderate the known trophic actions of the gonadotrophins FSH and LH on preantral and antral follicle growth (Webb and Campbell 2007). A plethora of putative regulators of folliculogenesis in vivo have been identified, some examples of these factors are shown in Fig. 1. The tyrosine kinase receptor Kit and the two different isoforms of its ligand, kit ligand (KL), have been localized to oocytes and GCs and have been shown to promote oocyte growth and maintenance of meiotic arrest in response to FSH receptor (FSHR) levels. While low concentrations of FSH promote oocyte growth by increasing KL-2 expression and by reducing the ratio of KL-1/KL-2, high concentrations of FSH enhance follicle development but impair oocyte growth (Thomas & Vanderhyden 2007). Other regulators of follicle growth include epidermal growth factor (EGF; Qu *et al.* 2000) and its receptor; activin (Hulshof *et al.* 1997; Telfer *et al.* 2008); basic fibroblastic growth factor (bFGF; Shikone et al. 1992); members of the insulin like growth factor (IGF) family and their binding proteins (Thomas *et al.* 2007), transforming growth factor-b (TGFB) superfamily members (Knight and Glister 2006) including somatic derived anti-Mullerian hormone (AMH), oocyte derived growth differentiation factor-9 (GDF9; Dong et al. 1996); and the bone

morphogenetic proteins (BMPs) especially BMP4, BMP7 and BMP15 (Otsuka *et al.* 2001). Other factors such as retinoblastoma protein (RB1) may also be important (Bukovsky *et al.* 1995). Changes in follicle morphology and cell number, together with the stage specific follicular responsiveness to the growth factors detailed here and the development of steroidogenic capacity can be used as functional markers to confirm the normality of follicle development in vitro.

Oocyte developmental competence is progressively acquired following a coordinated series of structural and functional changes in the gamete and surrounding cumulus cells. However, it is not until the final days of follicular development that oocytes acquire the capacity to undergo meiotic progression to metaphase II. The redistribution of the cortical granules and changes in mitochondrial number, activity and distribution occur during the terminal stages of oocyte maturation in vivo (Van Blerkom *et al.* 2002). Cytoplasmic and nuclear maturation of the oocyte is further characterised by a cascade of molecular events and check points which include changes in the transcription and translation of RNA, DNA replication and repair, chromosome condensation, spindle formation and development of the mechanisms for sperm head penetration (Swain and Smith 2007). Oocyte maturation in vivo is triggered by the preovulatory surge of gonadotrophins and particularly by high levels of LH. The LH driven follicular cascade results in a shift in steroid production by GCs from predominately oestrogen to a progesterogenic environment and a decrease in intracellular cAMP in the oocyte, which is induced at least in part by the loss of junctional contacts between the cumulus cells and oocyte (Sela-Abramovich *et al.* 2006).Activation of the molecular pathways leading to the production of hyaluronic acid by the cumulus cells occurs in response to the LH surge leads to the modification and expansion of the cumulus (Schoenfelder and Einspanier 2003). The loss of junctional contacts between the oocyte and somatic cells in turn triggers the resumption of meiosis in mature oocytes as it further reduces cAMP concentrations (Vaccari *et al.* 2008), leads to deactivation of cAMP dependent protein kinase A and reduces the inhibitory influences of purines on the maintenance of meiotic arrest (Swain and Smith 2007). All the molecular and cellular events which occur during oocyte maturation in vivo must be mimicked in vitro to produce fertile gametes.

2-2- Oocyte metabolism

Oocyte maturation encompasses a variety of cellular changes necessary for normal fertilization and successful embryonic development. These changes can be roughly categorized as nuclear or cytoplasmic maturation (Eppig 1996). Nuclear maturation refers to the progression of the chromatin through meiosis from the germinal vesicle (GV) stage to arrest at the second metaphase (MII). In contrast, cytoplasmic maturation includes all of the other changes within the oocyte, such as accumulation of mRNA and protein, reorganization of the cytoskeleton and organelles, and changes in cellular metabolism. Although nuclear and cytoplasmic maturation are often discussed separately, the numerous processes involved are interrelated and critical for successful embryonic development.

Similarly to other cells, oocytes metabolize glucose via glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid cycle (mice, Downs and Utecht 1999; cats, Spindler *et al.* 2000; cattle, Rieger and Loskutoff 1994, Krisher and Bavister 1999; sheep, O'Brien *et al.* 1996; pigs, Durkin *et al.* 2001). Resumption of meiosis is associated with elevated activity of glycolysis and the PPP, as well as increased activity of hexokinase (glycolsis and PPP), phosphofructokinase (glycolysis), and glucose-6 phosphate

dehydrogenase (PPP) within the cytoplasm of the oocyte (Tsutsumi *et al.* 1992; Downs *et al.* 1996; Downs and Utecht 1999; Cetica *et al.* 2002). In mice, gonadotropininduced meiosis is dependent on the presence of glucose (Fagbohun and Downs 1992; Downs and Mastropolo 1994). Increased metabolism of glucose through one or more metabolic pathways also occurs simultaneously with the progression of meiosis to MII in oocytes from cats (Spindler *et al.* 2000) and cattle (Rieger and Loskutoff 1994; Steeves and Gardner 1999). In primate oocytes, glucose is necessary for cytoplasmic maturation, although nuclear maturation can occur in the absence of carbohydrates (Zheng *et al.* 2001). In addition, elevated glucose metabolism in mature oocytes is correlated with, and predictive of, improved embryonic development in cats and cattle (Krisher and Bavister 1999; Spindler *et al.* 2000; Spindler and Wildt 2002). Similarly, oocytes matured in vitro from cats and pigs metabolize significantly less glucose and have lower developmental potential than oocytes matured in vivo (Spindler *et al.* 2000; Durkin *et al.* 2001). These findings highlight the importance of glucose metabolism in oocyte maturation and the interactions between nuclear and cytoplasmic maturation.

Another important aspect of oocyte maturation is the accumulation of glutathione (GSX). This tripepetide (Glu-Cys-Gly) is involved in many aspects of oocyte physiology including cumulus cell expansion, sperm decondensation, male pronuclear formation and embryonic development (cattle, de Matos *et al.* 1995; Sutovsky and Schatten 1997; Furnus *et al.* 1998; sheep, de Matos *et al.* 2002; pigs, Yoshida 1993, Yoshida *et al.* 1993; hamsters, Zuelke *et al.* 2003). Both steps in the synthesis of GSX, addition of glutamate and cysteine and the addition of glycine to this dipepetide, are dependent on ATP, and therefore dependent on the metabolism of the oocyte (Stryer 1995). Once synthesized, GSX cycles between the reduced (GSH) and oxidized (GSSG) form through the actions of GSX reductase and GSX peroxidase. The peroxidase transfers electrons from GSH to oxidized molecules within the cytoplasm, minimizing the actions of various oxidative stressors and resulting in the production of GSSG (Guerin *et al*. 2001). The cellular pool of GSH is maintained by reduction of GSSG to GSH by GSX reductase, as well as further GSH synthesis. The reduction of GSSG requires NADPH, a product of the PPP. Therefore, intracellular concentrations of GSH, and the many processes affected by GSH, are tightly linked to the metabolic activity of the oocyte. Glucose metabolism and GSX accumulation are important for successful oocyte maturation and embryonic development in pigs (Abeydeera *et al.* 2000; Durkin *et al.* 2001; Brad *et al.* 2003a,b; Herrick *et al.* 2003).

Improved understanding of the physiology of mammalian ovarian follicle growth and how this is affected by long-term culture is critical to the development of technologies for preservation of fertility. Moreover, long-term culture of ovarian follicles may become an important strategy for the production of oocytes in vitro (Picton *et al.* 2003). The potential value of this procedure is widely recognized; however, the technology remains imperfect, hindered by extremely demanding methodologies and incomplete understanding of the biology of oogenesis and folliculogenesis in vivo and in vitro. Depending upon species, it may take many weeks or months for a primordial follicle to undergo growth initiation and development to support the production of a fully grown fertile oocyte. The process of folliculogenesis involves many intricate and timely developmental events, which must be replicated in the laboratory.

Avascular preantral follicles survive relatively well in vitro and culture of preantral follicles is possible in several species, including mouse (Nayudu and Osborn 1992; Boland *et al.* 1993; Cortvrindt *et al.* 1996; Rose *et al.* 1999), sheep (Newton *et al.* 1999; Picton *et al.* 2003) and human (Roy and Treacy 1993; Picton and Gosden 2000). However, after long-term culture, oocyte viability is reduced and this is especially a problem in large animals and humans.

Culture conditions are probably suboptimal in all species, resulting in low yields of oocytes potentially causing epigenetic alterations and imprinting defects as well as compromised cytoplasmic maturation and reduced developmental competence (Obata *et al.* 2002; Fernandez-Gonzalez *et al.* 2004). Culture conditions required to improve follicle and oocyte growth in vitro must be re-evaluated based, in part, on the energy and nutritional requirements of follicles and oocytes grown in vivo. Two carbohydrates stand out with obvious importance: glucose is a major energy substrate for mouse ovarian follicles (Boland *et al.* 1993, 1994a, 1994b), whereas pyruvate is essential for the mouse oocyte (Biggers *et al.* 1967; Harris 2002).

Glucose metabolism by the late preantral and antral mammalian ovarian follicle is thought to be predominantly glycolytic (Leese and Lenton 1990; Boland *et al.* 1993; 1994a, 1994b; Gull *et al.* 1999), thus reducing the demand for oxygen which would otherwise be exhausted within the outer follicle layers (Gosden and Byatt-Smith 1986). This strategy presumably conserves oxygen for use by the oocyte, whose ATP production is confined to catabolism of nutrients, such as pyruvate (Biggers *et al.* 1967), through oxidative metabolism. Oxygen is an absolute requirement for energy metabolism by the oocyte and, hence, hypoxia in antral follicles is expected to reduce oocyte developmental competence (Van Blerkom 1998). While glycolysis is an important pathway for glucose utilization by larger follicles, its contribution to carbohydrate metabolism by smaller follicles has not been reported. Preantral follicles are less likely to suffer from the limitations of diffusion because they are 0.3–0.4 mm in diameter (Gosden and Byatt-Smith 1986), and could feasibly utilize other, more efficient, pathways for energy production.

The immature oocyte remains in an arrested state from the completion of oogenesis during foetal development, and oocyte maturation is completed within antral stage follicles in post-pubertal mammals in response to the ovulatory surges in gonadotrophins (Fig. 2-1A). Alternatively, spontaneous completion of maturation will occur following mechanical release of the oocyte from ovarian antral follicles (Edwards 1965). This latter phenomenon is referred to as in vitro oocyte maturation (IVM) and is commonly used to study oocyte development in several mammalian species, and has application to assisted reproduction in humans and livestock animal production (Trounson *et al.* 2001; Gilchrist and Thompson 2007; Hashimoto 2009).

Oocyte maturation involves the resumption of meiosis from prophase I (germinal vesicle stage, GV) to the extrusion of the first polar body (metaphase II, MII; Fig. 2-1B and C); expansion of the surrounding somatic cell compartment (cumulus cells) and maturation of the cytoplasm to support fertilization and early embryonic development. As mentioned, the oocyte is surrounded by numerous layers of cumulus cells forming the cumulus oocyte complex (COC), and bi-directional paracrine and gap junctional communication between the oocyte and cumulus vestment are essential for oocyte viability (Eppig 1991; Albertini *et al.* 2001). The cumulus cells provide the oocyte with nutrients and regulatory signals to facilitate the progression of maturation, in particular nuclear maturation. Conversely, oocyte secreted factors allow for cumulus cell differentiation from mural granulosa cells and mucification of the cumulus vestment (Gilchrist *et al.* 2004).

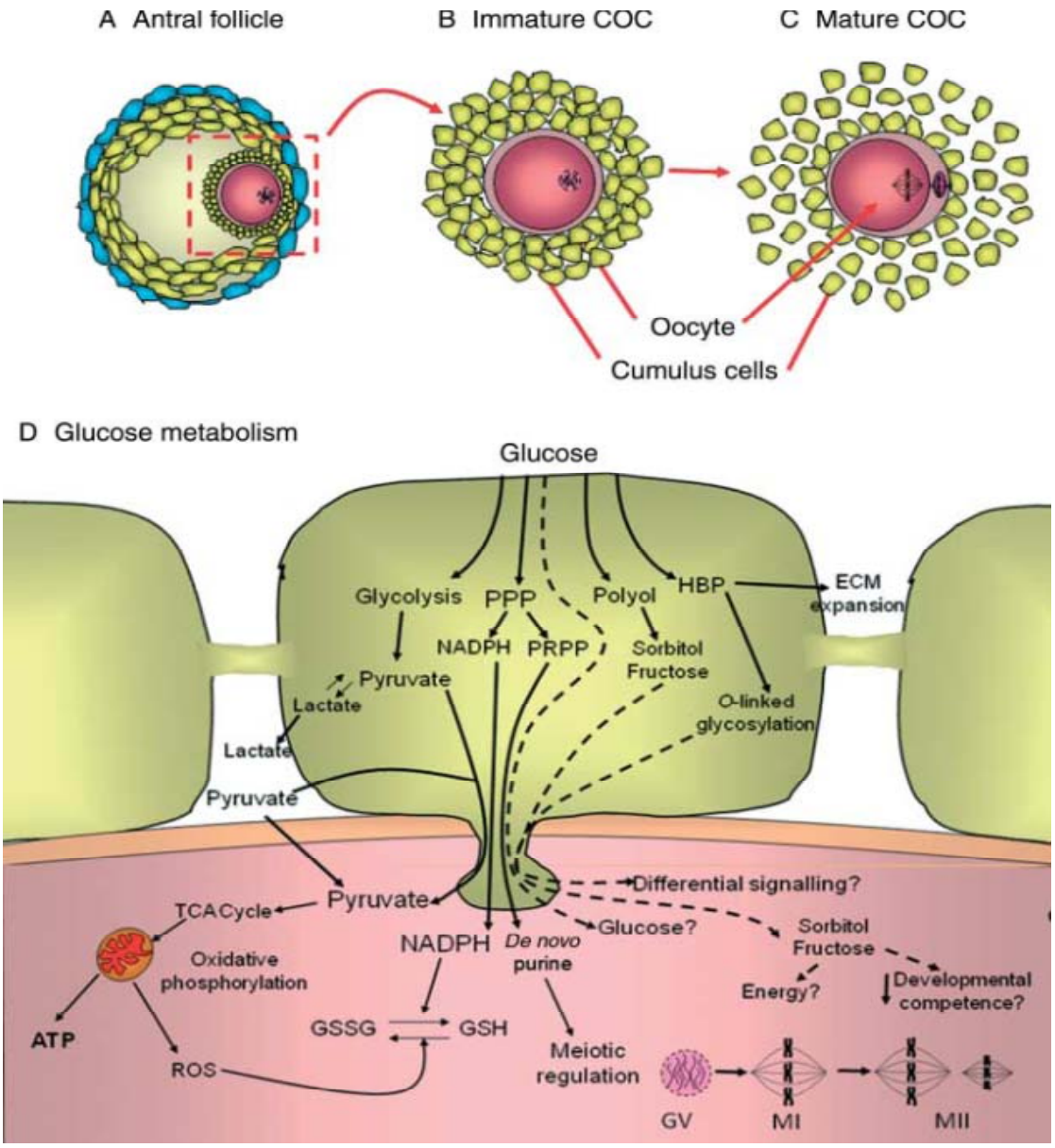

Figure 2-1 (A) Immature cumulus oocyte complexes (COCs; within red square) within antral follicles are characterized as having compact cumulus vestments and are arrested at prophase I (germinal vesicle stage, GV) of meiosis (B). Maturation occurs in response to gonadotrophin surges in vivo or release of the COC in vitro and is characterized by (C) expansion of the cumulus vestment and extrusion of the first polar body (metaphase II; MII). (D) Within the COC, glucose can be metabolized via four pathways. Glycolysis results in the production of pyruvate, which can be further metabolized via the tricarboxylic acid (TCA) cycle, followed by oxidative phosphorylation for energy production (ATP). The pentose phosphate pathway (PPP) produces NADPH for the reduction of the anti-oxidant glutathione (GSSG, oxidise glutathione; GSH, reduced glutathione). Phosphoribosyl pyrophosphate (PRPP) is also produced by PPP and is a substrate for de novo purine synthesis, important for meiotic regulation within the oocyte. Products of the polyol pathway (polyol) include fructose and sorbitol. The hexosamine biosynthetic pathway (HBP) is important for producing substrates for extracellular matrices (ECM) for cumulus expansion and O-linked glycosylation (cell signaling). MI, metaphase I; ROS, reactive oxygen species.

The rapid and dynamic nature of the final stages of oocyte maturation means that COCs require different compounds, such as fatty acids, amino acids, electrolytes, purines and pyrimidines and metabolites. With regard to metabolites, mature bovine COCs consume two fold more glucose, oxygen and pyruvate than immature COCs (Sutton *et al.* 2003a). A long-established tenet of COC energy metabolism is that the oocyte itself has a poor capacity to utilize glucose (Biggers *et al.* 1967), and that the cumulus cells metabolise the bulk of the glucose consumed by the COC to supply metabolic intermediates to the oocyte. The maturing COC uses glucose for energy production and numerous other cellular processes such as nucleic acid and purine synthesis, mucification and cellular homoeostasis (Sutton *et al.* 2003b).

The environment to which the COC is exposed during maturation (both in vivo maturation and IVM) affects oocyte developmental competence and subsequent embryonic development. Suboptimal culture conditions during IVM result in reduced blastocyst development post-fertilization (van de Sandt *et al.* 1990; Rose and Bavister 1992; Rose-Hellekant *et al.* 1998). Moreover, it is becoming increasingly evident that the consequences of poor maternal health, such as diabetes, obesity and poor diet, all result in hyper glycaemic increases in intra-follicular glucose levels, and that this is associated with poor oocyte viability in mice (Moley *et al.* 1998). Hence, the aim of this review is to examine the impact of glucose concentration and altering levels of glucose metabolism on oocyte and COC function. Considering the importance of the bi-directional communication between the oocyte and the cumulus vestment, the focus of this review will largely be on the metabolic activity of the COC as a whole unit. A general overview of glucose utilization by the COC will be discussed, followed by the roles of glucose metabolic pathways during maturation, including glycolysis, the pentose phosphate pathway (PPP), the hexosamine biosynthesis pathway (HBP) and the polyol pathway, and the consequences of maternal hyperglycaemia on oocyte development competence will be examined (fig, 2-1D).

2-3- An overview of glucose metabolism

2-3-1- COC versus the oocyte

Prior to the ovulatory signal, the oocyte is coupled to its cumulus cells through trans-zonal processes, and this intimate physical association facilitates bi-directional communication between the oocyte and the cumulus vestment via gap junctional and paracrine signaling (Gilchrist *et al.* 2004). The oocyte relies on the cumulus vestment to facilitate the transport of glucose into the oocyte or to provide the oocyte with substrates it can utilize (Biggers *et al.* 1967).

Mathematical modelling of glucose concentration gradients within the bovine COC suggests that the oocyte is exposed to glucose levels 31–82% of follicular fluid concentrations when concentrations are at physiological levels (Stokes *et al.* 2008). However, modeling shows that increases in follicular fluid glucose concentrations cause a disproportionately greater change in the concentration of glucose at the cumulus–oocyte boundary. In contrast, minimal oxygen is lost during diffusion from the follicular fluid, through the cumulus vestment to the oocyte (Clark *et al.* 2006). Both mathematical models support the notion that glucose is preferably metabolised by the cumulus vestment to provide the oocyte with metabolites.

Perturbations of oocyte competence in low glucose concentration environments are thought to be manifested by a decreased flux of glucose through the PPP and through glycolysis in mice (Downs *et al.* 1998), hence limiting substrates for nucleic acid synthesis and energy production. Conversely, high glucose levels during IVM can result in increased production of reactive oxygen species, increased O-linked glycosylation via upregulation of the HBP and decreased concentrations of reduced glutathione (GSH), an endogenous anti-oxidant (Hashimoto *et al.* 2000).

2-3-2- Oocyte-secreted factors and metabolism

Considering the importance of the cumulus vestment to oocyte developmental competence, the influence of oocyte-secreted factors on cumulus cell metabolic activity was examined. Glucose consumption by intact cattle COCs and oocytectomised complexes (OOX, surgical removal of the ooplasm, while retaining the cumulus vestment intact) was measured over a 24-h IVM period. Oocyte-secreted factors did not appear to affect the rate of glucose consumption, as cattle COCs, OOX and OOX co-cultured with denuded oocytes all showed similar rates of glucose consumption over a 24-h culture period (Sutton *et al.* 2003a). In contrast, mouse OOX display decreased expression of genes encoding glycolytic enzymes (including PFK, PFKP and lactate dehydrogenase, LDHA) and a tenfold decrease in glycolytic activity compared to intact COCs and OOX co-cultured with denuded oocytes (Sugiura *et al.* 2005).

There are several possible explanations for the differences in the two patterns of glucose metabolism reported between cattle and mouse cumulus cells. First, differences in glucose metabolism may be attributed to the addition of FSH to the cattle COCs and OOX cultures, whereas no hormones were added to mouse IVM system. Sugiura *et al.* (2005) suggest hormone stimulation during IVM negates the oocyte-mediated promotion of glycolysis that was seen within mouse cumulus cells. Furthermore, in the mouse study, COCs and OOX were cultured in the presence of milrinone, a modulator of nuclear maturation, hence arresting or delaying nuclear maturation. In comparison, the bovine study used a spontaneous maturation model. Alternatively, oocyte-mediated regulation of cumulus cell glycolysis may be a species-specific phenomenon, as is the case for FSH-stimulated cumulus expansion, which requires oocyte-secreted factors in the mouse (Buccione *et al.* 1990), but not in bovine and porcine COCs (Ralph *et al.* 1995; Nagyova *et al.* 1999).

2-4- Metabolic pathways important to oocyte and zygote development

The accessibility and ease of studying oocyte maturation using IVM systems have meant that the majority of studies describing oocyte glucose metabolism have utilized in vitro culture, rather than measurements of the metabolic activities of oocytes during in vivo maturation (Sutton *et al.* 2003b). Glucose consumed by the COC can be utilized for energy production, cellular homoeostasis, nuclear maturation, substrates for matrices production and signaling. To date, four metabolic pathways have been identified: glycolysis (energy production), PPP, HBP and the polyol pathway.

2-4-1- Pentose phosphate pathway and oocyte development

The PPP is an important glucose metabolic pathway during maturation, and although it has never been measured within the whole COC, anecdotally only a small proportion of glucose is metabolized via this pathway as the majority of glucose consumed by the COC is metabolized by the glycolytic pathway (Downs and Utecht 1999). Likewise, in the oocyte, PPP activity accounts for 3% of the small amount of glucose metabolized by mouse oocytes (Urner and Sakkas 1999). Bovine oocytes have relatively high glucose-6-phosphate dehydrogenase (G6PDH, rate-limiting enzyme of the oxidative stage) activity compared to cumulus cells (Cetica *et al.* 2002); suggestive of higher potential PPP activity in the oocyte compared to individual cumulus cells.

The pathway can be divided into oxidative and non oxidative stages, and glucose can enter the PPP at either stage. The oxidation of glucose-6-phosphate to 6-phosphogluconolactone by G6PDH results in the production of NADPH, and fructose-6-phosphate can also be utilized via the non-oxidative arm of the PPP by transketolase. Products of the PPP include NADPH, which is utilized for cytoplasmic integrity and redox state through the reduction of glutathione (GSSG to GSH). Another product of PPP is phosphoribosylpyrophosphate, a substrate for de novo and salvage purine synthesis and subsequent control of nuclear maturation.

It is well established that the addition of glucose to IVM medium results in increased rates or acceleration of nuclear maturation (Sutton-McDowall *et al.* 2005; Sato *et al.* 2007; Funahashi *et al.* 2008). However, Downs *et al.* (1996, 1998) were the first to demonstrate that the flux of glucose through PPP, rather than through glycolysis, influences the resumption of nuclear maturation in mouse COCs. Inhibition of glycolysis does not affect mouse oocyte nuclear maturation (Downs *et al.* 1996) and media containing pyruvate as the sole metabolite result in less mouse COCs completing nuclear maturation (MII) compared to media containing glucose (Downs and Hudson 2000). Furthermore, stimulation of PPP using electron acceptors such as phenazine ethosulphate and pyrroline-5-carboxylate results in a dose-dependent increase in the rate of meiotic resumption (GV breakdown) and increased glucose metabolism (Downs *et al.* 1998).

While Downs et al. explored the influence of glucose and PPP activity on the resumption of meiosis, PPP is also involved in progression of all stages of meiosis in the oocyte, including the resumption of meiosis, MI–MII transition and the resumption of meiosis post-fertilization (Sutton-McDowall *et al.* 2005; Herrick *et al.* 2006). Supplementing IVM medium with diphenyleneiodonium (inhibits NADPH oxidase) decreased PPP activity in porcine oocytes, resulting in reduced meiotic resumption and completion and decreased cleavage and blastocyst development post-fertilization (Herrick *et al.* 2006).

2-4-2- Pentose phosphate pathway and zygote development

In mammals, the maternal contribution to the development of the embryo arises from mRNA stored in the oocyte, and it acts to sustain embryo development until embryonic genome activation. An equivalent paternal contribution is as yet not evident. The spermatozoon is believed to function largely as the bearer of the haploid set of paternal genes that restore diploidy after fusion with the maternal genome, and as a carrier of the activating mechanism that allows the oocyte to proceed from the metaphase II stage. Except in rodents, the spermatozoon also supplies a centriole that becomes the microtubule organizing center for fertilization. The complexity of the fertilization process is evident in its duration, because in many mammals the first cell cycle lasts about 1 day and plays a key role in subsequent embryo development. During this period the spermatozoon must form a

pronucleus before the first DNA replication (S phase) (Howlett and Bolton, 1985; Bouniol-Baly *et al,* 1997; Grisart *et al,* 1994; Eid *et al,* 1994). Both parental nuclei are transcriptionally inactive at fertilization and the mechanism driving the very first hours that follow sperm entry into the oocyte are exclusively dependent on modifications brought about by the surrounding cytoplasm (Howlett and Bolton, 1985; Bouniol-Baly *et al,* 1997; Grisart *et al,* 1994; Eid *et al,* 1994).

Previous studies have identified paternally linked differences in the fertilization process between bulls of high and low fertility that manifest themselves during the first cell cycle. In addition, previous studies have identified beneficial paternal effect from spermatozoa recovered from bulls of high in vitro fertility is manifested during the first G1 phase after fertilization. This beneficial effect is then evidenced by an earlier onset and a longer duration of the first DNA replication in both male and female pronuclei, which more importantly, translates into higher rates of blastocyst formation 7 days later (Comizzoli *et al,* 2000). In bulls with low and high fertility capability the percentage of fertilization and overall timing of pronuclear formation is the same, however, we have found that the absence of a beneficial effect in low fertility spermatozoa was linked to a delay in both pronuclei of the onset of the first DNA synthesis, which was subsequently shorter (Comizzoli *et al,* 2000). The origin of this paternal control has not as yet been characterized.

In mice, sperm entry followed by decondensation in zonafree oocytes is associated with an increase of glucose metabolism (through glycolysis and pentose phosphate pathway [PPP]), whereas it remains at a basal level in parthenogenetically activated oocytes (Urner and Sakkas, 1999). The presence of the male pronucleus within the oocyte cytoplasm is also correlated with an increase in glucose metabolism (Pantaleon *et al,* 2001). Glucose metabolism occurs through a number of pathways; however, the PPP has been demonstrated to be activated shortly after sperm penetration into the oocyte in sea urchins (Swezey and Epel, 1986) and in mice (Urner and Sakkas, 1999). Glucose metabolized through glycolysis provides energy by generating ATP. The PPP generates NADPH, which is used in reductive reactions, and ribose 5-phosphate, which is precursor of nucleotide synthesis for subsequent DNA replication (Urner and Sakkas, 1999). Its implication in developmental processes has not, however, been evidenced. The activity of the PPP may vary according to cell types. In mice, about 0.6% and 2.5% of glucose is metabolized through the PPP in spermatozoa and oocytes, respectively (Urner and Sakkas, 1999).

Previous studies of heterospecific in vitro fertilization models have proposed that the sperm nucleus might bring a catalytic component or trigger its production to promote DNA synthesis in the female pronucleus (Naish *et al,* 1987; Comizzoli *et al,* 2001). The expression of the paternal genome is not, however, involved in the onset of the S phase (Memili and First, 1999), whereas the male chromatin remodeling seems to be a prerequisite before DNA replication in both pronuclei (Ramalho-Santos *et al,* 2000). Using the bovine model, we have therefore examined whether the differences in the paternal contributions of low- and high-fertility spermatozoa will provide insights into the interrelationship between the paternal and maternal pronuclei during the first cell cycle.

2-5- The effect of oocyte quality on embryo development and oocyte selection

2-5-1- Follicle size

Maturation of mammalian oocytes is correlated with ovarian follicular growth. This relationship was confirmed *in vitro* in many species by studying the effect of follicle size on the embryonic development capacity of the oocytes following *in vitro* maturation, fertilization and *in vitro* culture. This occurs with cattle (Pavlok *et al.* 1992; Lonergan *et al.* 1994); goats (Crozet *et al.* 1995); sheep (Cognie *et al.* 1998). Oocytes originating from large follicles of cattle from 4 to 8mm (Pavlok *et al.* 1992), or >6mm in size (Lonergan *et al.* 1994; Lequarre *et al.* 2005); goats >5mm in size (Cognie *et al.* 1996; Crozet *et al.* 1995); sheep >3–5mm (Cognie *et al.* 1998) are more competent in terms of *in vitro* embryo development than oocytes from small follicles. This occurs in cattle with follicles >1–2mm (Pavlok *et al.* 1992); 2–6mm (Lonergan *et al.* 1994; Lequarre *et al.* 2005); goats with follicles 2–3mm (Crozet *et al.* 1995; Cognie *et al.* 1996); sheep with follicles 1–2mm (Cognie, *et al.* 1998). The mammalian oocyte undergoes significant changes while enclosed within the ovarian follicle, especially after establishing dominance and as the time of ovulation approaches. These changes have been described as capacitation (Hyttel *et al.* 1997) and play a key role in the oocyte's acquisition of developmental competence. A recent study comparing the *in vitro* developmental rate of oocytes from cows in which superovulation was induced that were collected at different time points before ovulation showed that the final steps of oocyte maturation are critical for embryonic development (Humblot *et al.* 2005). The same study demonstrated that the preovulatory period is important for oocytes to acquire developmental capacity as more oocytes recovered during this period developed than oocytes recovered in earlier follicular development stages i.e. during pre-capacitation or capacitation period; (Humblot *et al.* 2005).

2-5-2- Oocyte diameter

There are many criteria for the selection of good quality oocytes in order to improve the in vitro maturation of oocytes in different farm animal species. Some criteria are, e.g. the size of the follicle (Pavlok *et al.* 1992; Lonergan *et al.* 1994; Blondin and Sirard, 1995), level of atresia (Hagemann et al., 1999; de Wit and Kruip, 2001) and progesterone concentration in the follicular fluid (Hazeleger *et al.* 1995). It has been shown that the oocyte developmental competence is also related to the morphology of the cumulus–oocyte-complexes (COCs) (Blondin and Sirard, 1995; Boni *et al.* 2002), morphology of the corona radiata cells (Laurincik *et al.* 1996) and size of the oocytes (Motlik and Fulka, 1986; Durinzi *et al.* 1995; Otoi *et al.* 1997, 2000).

Oocyte size can generally be used as an indicator of oocyte growth, as there is an intensive synthesis of RNA during this phase that causes an increase in size (Crozet *et al.* 1981; Lazzari *et al.* 1994). The association between the oocyte's diameter and its ability to resume and complete meiotic division during in vitro maturation has been described in humans (Durinzi *et al.* 1995), cattle (Otoi *et al.* 1997), buffalo (Raghu *et al.* 2002), pigs (Motlik and Fulka, 1986), and the dog (Otoi *et al.* 2000). In cattle, the oocytes derived from peripheral follicles, regardless of their size, show similar meiotic competence, whereas those collected from cortical follicles display a size-related competence (Arlotto *et al.* 1996).

In this regard, smaller oocytes tend to follow an abnormal path of meiotic maturation, resulting in disturbances in the maturation process (Lechniak *et al.* 2002). In cattle the influence of follicle size on oocyte performance during IVM and IVF (Pavlok *et al.* 1992; Lonergan *et al.* 1994) and the relation between size of the oocyte and follicular diameter has also been established (Hytell *et al.* 1997; Otoi *et al.* 1997). In pigs, both oocyte and follicular diameters are related to the degree of oocyte nuclear maturation (Lucas *et al.* 2002).

2-5-3- Morphologic characteristics of oocyte

Morphological criteria have been routinely used to select the oocytes with the best competence for embryo development. The relationship between cumulus cells and cytoplasm aspect with embryo development has been shown (Fukui and Sakuma, 1980). According to these morphological criteria, Guienne (1999) described four categories of cumulus–oocyte complex (Grades 1–4), considering Grade 1 the most competent for in vitro embryo production (IVEP) and Grade 4 as the least suitable for IVEP.

Grade 1: COCs with a homogeneous cytoplasm and a complete cumulus oophorus

Grade 2: COCs with an incomplete but compact cumulus oophorus with more than five layers and a homogeneous cytoplasm

Grade 3: COCs with heterogeneous cytoplasm and more than three cumulus cells layers or more than five layers in some parts.

Grade 4: COCs with cumulus oophorus partly or totally absent and cytoplasm strongly heterogeneous.

Although immature oocytes are routinely selected based on compaction of the cumulus corona investment and homogeneity of the ooplasm (Chauhan *et al,* 1998), this may reduce the yield of transferable embryos, as some oocytes with apparently normal morphology are in the early stages of degeneration. Buffaloes in developing countries are usually slaughtered due to subfertility, poor body condition, and advanced age; immature oocytes from buffaloes with reduced reproductive performance or at reproductive senescence were heterogeneous in quality, with low developmental competence (Raghu *et al,* 2002).

2.6- The effect of zygote quality on embryo development and zygote selection

One of the most important problems in *In Vitro* Fertilization (IVF) treatments is the selection of the best embryos for transfer, a crucial point to maintain high performance in terms of pregnancy rate, reducing at the same time the risk of multiple pregnancy. Nowadays, the quality evaluation and the selection of *in vitro* obtained embryos are made on the basis of morphological parameters involving embryo development, uniformity of blastomeres, percentage of fragmentation, cytoplasmic irregularities, and rate of cleavage, blastomeres multinucleation and other visible features (Cummins *et al*, 1986; Puissant *et al,* 1987; Shulman *et al,* 1993; Balaban *et al,* 2004; La Sala *et al,* 2005). These assessments are non-invasive for the embryo development, but on the other hand can not provide any information about embryonic chromosomal arrangement, one of the most relevant aspects

of human reproduction, both *in vivo* and *in vitro*. In fact, alterations of chromosomic copy number (aneuploidies) are common in human oocytes and embryos and seem to be mostly implicated in the first-trimester abortions, a complication affecting 50%-70% of all spontaneous conceptions (Menasha *et al,* 2002). As described, autosomal trisomies and sex chromosome monosomies, followed by polyploidy and structural rearrangements show a global range from 50% to 80% in first-trimester miscarriages (Jackson *et al,* 2002; Gabbe, 2007).

2-6-1- Morphologic characteristics of zygote

An important contribution in the evaluation of embryo quality seems to come from the pronuclear and nucleolar characteristics, proposed as an indicator of embryo development and chromosomal complement in human fertilized oocytes (Gianaroli *et al,* 2003; Scott, 2003; Payne *et al,* 2005; Chen and Kattera, 2006). Nucleoli are the sites of the synthesis of pre-RNA, and ribosomal RNA (rRNA) is necessary for the translational process whereby the embryonic genome becomes fully activated (Braude *et al,* 1988). Despite the high number of studies conducted, to date there are conflicting data about the clinical efficacy of zygote-score: in fact, recent data show that scoring system based on pronuclear morphology seems to provide a good criterion to select embryos for transfer when combined with embryo morphology evaluation on Days 2 and 3 (Alvarez *et al,* 2008) other authors concluded that late parameters (such as the cell number and embryo grade) have a better prognostic value than zygote score when selecting embryos for transfer (James *et al,* 2006; Nicoli *et al,* 2007).

Precise selection of embryos and prediction of implantation is probably the most pressing issue in assisted reproduction. Various embryo scoring system have been described to assess the developmental potential of human day 2 or 3 preimplantation embryos. In the most commonly used systems the blastomeres cleavage rate (i.e. number of blastomeres), the shape and size of the blastomeres and the amount of anucleated fragments are estimated. Several studies have shown that regardless of minor fragmentation, the optimal cleavage rate would be the most important criteria when selecting embryos for transfer (Ziebe *et al,* 1997; Sakkas *et al,* 1998). Variation in zona thickness, embryo symmetry and the presence of multinucleated blastomeres has also been shown to affect implantation rates (Ziebe *et al,* 1997; Cohen *et al,* 1989; Pelinck *et al,* 1998).

In recent years, there has been growing interest in the assessment of pronuclear morphology to select the most viable and competent embryos. In this regard in vitro fertilization (IVF) human pronucleate zygotes are scored on the basis of pronuclear alignment, size, number, equality and distribution of nucleoli, cytoplasmic heterogenity and presence or absence of cytoplasmic halos (Scott and Smith, 1998; Kattera and Chen, 2004). This scoring system correlated positively with the implantation and delivery rates in pronuclear stage transfers. Many different pronuclear scoring systems have been proposed to select high- quality embryos. Unfortunately, there is no standard zygote grading system used thought assisted reproduction laboratories. The same holds true for other systems of evaluating embryo morphology. As a result, comparisons of the systems used correlations of embryo quality with success rates between different laboratories are vague. Two main systems for assessing pronuclear morphology were developed by Scott and Smith (1998) and Tessarik *et al.* (2000) (Scott and Smith, 1998; Tesarik *et al,* 2000). A pronuclear scoring system, as well as other embryo development markers and patient status, may be useful in determining the number and quality of embryos to transfer. The early scoring