

# **The Rational Development of Improved In Vitro Maturation of Bovine Oocytes**

Melanie Lisa McDowall, BSc. (Hons)

Research Centre for Reproductive Health  
Department of Obstetrics and Gynaecology  
Faculty of Health Science  
The University of Adelaide

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## Abstract

In vitro embryo production has vastly improved over the past decade through the study of the in vivo environment and the metabolic requirements of embryos. In contrast, in vitro oocyte maturation (IVM) culture conditions have remained relatively unchanged and are suboptimal. The aim of this thesis was to create improved systems for bovine IVM by studying the metabolic profiles and requirements of intact cumulus oocyte complexes (COCs) during IVM and determining the ion and energy substrate composition of bovine follicular fluid (FF).

Glucose, pyruvate and oxygen consumption of bovine COCs increased 2-fold over the 24 h IVM period, with glucose being the preferred energy substrate. While initially the majority of glucose consumed by COCs is metabolised via glycolysis (L-lactate production), a considerable proportion of glucose is used as a substrate for extracellular matrix (ECM) synthesis towards the end of IVM. Glucosamine (an intermediate substrate of hyaluronic acid) supplementation of IVM media lead to decreased glucose consumption and incorporation into ECM during FSH-stimulated expansion.

Biochemical analyses of bovine FF demonstrated that the concentration of some ions and energy substrates varied with follicle size. Although follicular glucose concentrations increased with follicle size, levels were ~2-fold lower than that found in Tissue Culture Medium (TCM199), the most commonly employed medium for bovine IVM. Synthetic Follicular Fluid Medium (SFFM) was created, based on the FF data and also contained glucosamine. Two different glucose concentrations were examined, 2.3 mM glucose to represent physiological concentrations and 5.6 mM glucose, the same concentration as is in TCM199. Culturing COCs in different glucose concentrations manipulated the completion of nuclear maturation and this was dependant on concentration, gonadotrophin supplementation and the timing of media changes, demonstrating the importance of this substrate to meiotic competence.

Although glucosamine had no effect on oocyte nuclear maturation, supplementation during IVM led to a dose-dependent decrease in blastocyst rates. The detrimental effects of glucosamine manifested during early cleavage and were associated with a 0.6-fold decrease in protein synthesis levels within the oocyte compared to oocytes cultured in media with no glucosamine, suggesting a detrimental effect on developmental competence. Interestingly oocytes cultured in media containing glucosamine and EGF had significantly higher protein synthesis compared to the control group.

The biochemical profiles of COCs during IVM and FF were determined and used to create new media that allowed manipulation of oocyte nuclear maturation but compromised cytoplasmic maturation. Further research is required to optimise SFFM and to investigate the detrimental effects of glucosamine on developmental competence.

## **Declaration**

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief contains no material previously published or written by another person except where due reference has been made in the text.

I consent to this copy of my thesis, when deposited in the University of Adelaide library, being available for loan and photocopy.

November 2004

Melanie McDowall

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## Abbreviations

Akt	Protein kinase B pathway
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMP-15	Bone morphogenic protein
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
COC	Cumulus oocyte complex
DO	Denuded oocyte
ECM	Extra cellular matrix
EGF	Epidermal growth factor
FCS	Fetal calf serum
FF	Follicular fluid
FF-MAS	Follicular fluid meiosis activating sterol
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GDF-9	Growth differentiation factor 9
Glc	Glucosamine
GFAT	Glutamine:Fructose-6-phosphate transferase
GLUT	Glucose transporter
GSH	Glutathione
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HA	Hyaluronidase treatment
hCG	Human chorionic gonadotrophin
IVC	In vitro embryo culture
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro embryo production
LDH	Lactate dehydrogenase
LH	Luteinizing hormone

MI	Metaphase I
MII	Metaphase II
MPF	Maturation promoting factor
MTF	Mouse oviductal fluid
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OOX	Oocytecomised complex
PI3K	Phosphatidylinositol pathway
PPP	Pentose phosphate pathway
PRPP	Phosphoribosylpyrophosphate
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrolidone
ROS	Reactive oxygen species
SFFM	Synthetic Follicular Fluid Medium
SOF	Synthetic oviductal fluid
TCA cycle	Tricarboxylic acid cycle
TCM199	Tissue culture medium 199
T-MAS	Testicular meiosis activating sterol
TZP	Transzonal cytoplasmic processes

## Publications

**Sutton-McDowall ML**, Gilchrist RB and Thompson JG 2004 The effect of hexoses and gonadotrophin supplementation on bovine oocyte nuclear maturation during in vitro maturation in a synthetic follicular fluid medium *Reproduction, Fertility and Development* submitted.

**Sutton-McDowall ML**, Gilchrist RB and Thompson JG 2004 Cumulus-expansion and glucose utilisation by bovine cumulus-oocyte complexes during in vitro maturation: the influence of glucosamine and follicle stimulating hormone *Reproduction* **128** 313-319.

**Sutton ML**, Cetica PD, Beconi MT, Kind KL, Gilchrist RB and Thompson JG 2003 The influence of oocyte-secreted factors and culture conditions on the metabolic activity of bovine cumulus cell complexes *Reproduction* **126** 27-34.

**Sutton ML**, Gilchrist RB and Thompson JG 2003 Effects of in vivo and in vitro environments on metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity *Human Reproduction Update* **9** 35-48.

## Provisional Patents

**Sutton-McDowall ML**, Gilchrist RB and Thompson JG 2003 Media for oocyte maturation, Provisional Patent (DN-1429).

## Conference Proceedings

**Sutton-McDowall, ML**, Gilchrist, RB and Thompson, JG 2005 Glucosamine supplementation during in vitro maturation leads to perturbed developmental capacity of bovine cumulus oocyte complexes *International Embryo Transfer Society Copenhagen, Denmark accepted*.

**Sutton-McDowall, ML**, Gilchrist, RB and Thompson, JG 2004 Regulation of bovine oocyte meiotic and developmental capacity by glucose and glucosamine

*Society for Reproductive Biology, Reproduction, Fertility and Development Abstract 204.*

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# **Chapter 1**

## **Introduction: Effects of in vivo and in vitro environments on the metabolism of the cumulus-oocyte complex and influence on oocyte developmental capacity**

Melanie L. Sutton, Robert B. Gilchrist and Jeremy G. Thompson

Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, University of Adelaide, The Queen Elizabeth Hospital, Woodville Road, Woodville 5011, South Australia.

## **Abstract**

There has been an improvement in the blastocyst rates achieved following in vitro embryo production that can largely be attributed to improved embryo culture conditions based on an increased knowledge of the in vivo environment, as well as the metabolic needs of the embryo. Despite this, in vitro oocyte maturation (IVM) conditions have remained largely unchanged. Within the antral follicle, numerous events affect oocyte maturation and the acquisition of developmental competence. For example: interactions between somatic cells of the follicle, in particular cumulus cells, and the oocyte; the composition of follicular fluid; the temperature and vascularity of the follicular environment. Many of these factors change with follicle size and oocyte growth. In contrast, culture conditions for IVM are based on somatic cells that often do not reflect the follicular environment, have complex compositions or additives such as macromolecule supplements that are undefined in nature. Metabolites included in media such as glucose, pyruvate, oxygen and amino acids have been shown to have a differential influences on oocyte maturation and competence. Manipulation of these factors and application of gained knowledge of the in vivo environment may result in improved in vitro oocyte maturation and overall in vitro embryo production.

## **Introduction**

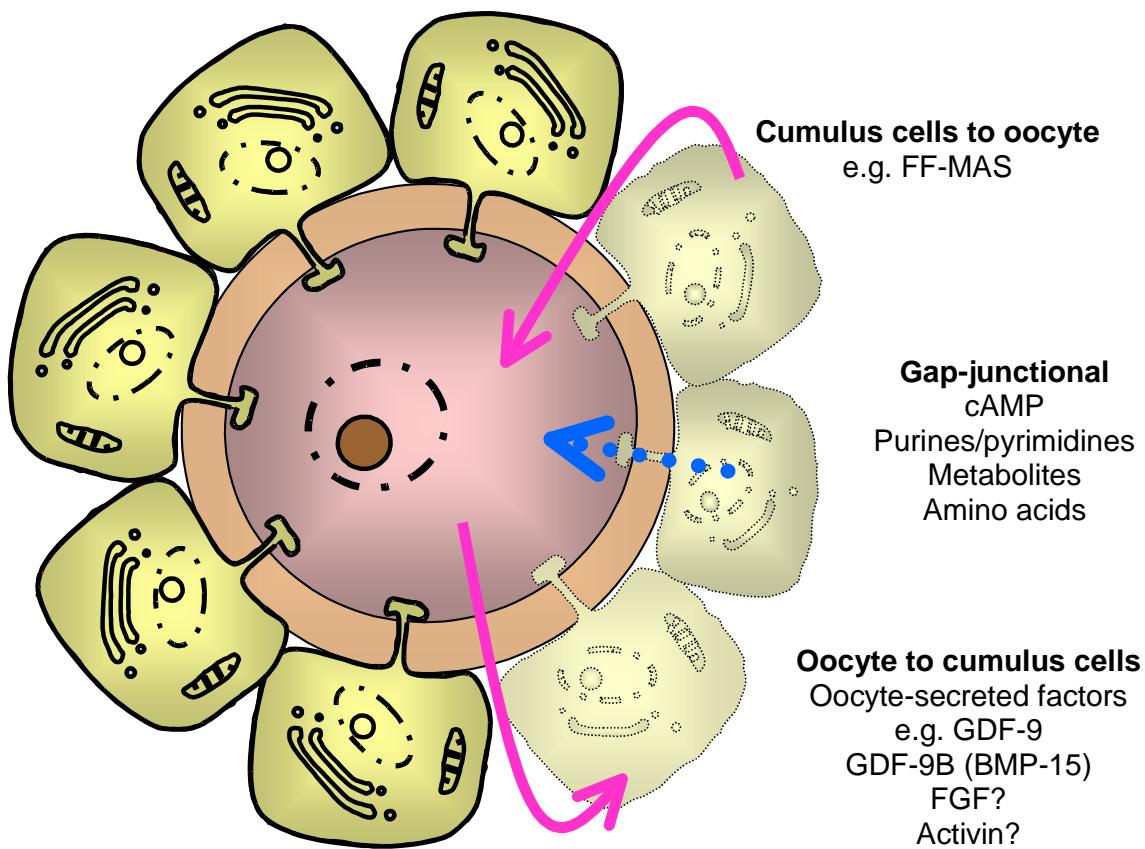
Oocyte maturation is the culmination of a prolonged period of oocyte growth and development within the growing follicle, and the short interval of meiotic maturation at ovulation. It is over the long phase of weeks to months that the oocyte, in a highly coordinated manner, gradually acquires the cellular machinery required to support early embryonic development. This capacity of the oocyte to sustain early development, called oocyte developmental competence, is intrinsically linked to the process of folliculogenesis and to the health of the developing follicle. The follicular environment also maintains oocytes in an arrested state of meiosis, at the diplotene stage of prophase I (also called the germinal vesicle; GV stage). The last phase of oocyte maturation, meiotic maturation of the immature GV oocyte, germinal vesicle breakdown (GVBD) and progression to metaphase II (MII), is induced *in vivo* by the pre-ovulatory gonadotroph surge. Alternatively, artificial release of the oocyte from the inhibitory environment of the follicle leads to spontaneous meiotic maturation *in vitro* (Pincus and Enzmann 1935). Oocyte *in vitro* maturation (IVM) is a viable phenomenon as oocytes matured, fertilized and cultured *in vitro* can generate embryos with full developmental potential after embryo transfer. Pincus (Pincus and Enzmann 1935) first described meiotic maturation following liberation of the oocyte from the follicle, but it was Edwards (Edwards 1965) who recognized the potential for *in vitro* maturation as a step in the process of embryo production. However, the ability of the oocyte to undergo meiotic maturation is a poor marker of oocyte developmental capacity (Moor and Trounson 1977). In most species examined, oocytes matured *in vitro* are compromised in their developmental capacity compared to oocytes matured *in vivo* (Bousquet *et al.* 1999; Farin *et al.* 2001; Yang *et al.* 2001; Combelles *et al.* 2002; Dieleman *et al.* 2002; Holm *et al.* 2002). Furthermore the proportion of pregnancies achieved following IVM of human oocytes from unstimulated patients is minute (Trounson *et al.* 1994; Cha *et al.* 2000). With further research, IVM has the potential to become a viable alternative to ovarian stimulation, especially for the treatment of patients with fertility disorders who are at an increased risk of developing ovarian hyperstimulation syndrome when treated with exogenous hormones such as polycystic ovarian syndrome.

Our understanding of what constitutes a developmentally competent oocyte recovered from antral follicles remains poor, although it is clear that the quality of the follicular environment from which the oocyte originates is a major determining factor. Despite this, little is known about how the nutrient requirements of the cumulus-oocyte complex (COC) impact on subsequent embryo development. For example, the most commonly used oocyte maturation media today are formulations designed many years ago for culture of non-ovarian somatic cells. There are no studies that directly correlate the metabolic needs of the COC with developmental outcomes. However, the pioneering work of Downs and colleagues clearly showed that availability of energy substrates can regulate meiotic resumption in oocytes from antral follicles, with small alterations in substrate concentrations either suppressing or inducing meiosis (Downs and Mastropolo 1994; Downs and Hudson 2000). In contrast, the effect of cell-cell signalling between the oocyte and granulosa cells during the earliest stages of folliculogenesis on metabolism of the oocyte is unknown and is likely to remain technically difficult to study. In this review we will examine: the composition of the antral follicular environment and how this relates to developmental outcome; the metabolism of the oocyte and the surrounding cellular vestment and relate these to developmental outcome; the current development of in vitro maturation media.

## Oocyte-follicular cell interactions

### *Oocyte-follicular cell communication pathways*

The follicular environment “programmes” oocyte developmental competence. Clearly oocyte growth and development are absolutely dependent on the nurturing capacity of the follicle, in particular of the granulosa cells. Communication between the germ cell and somatic cell compartments of the follicle occurs via paracrine and gap-junctional signalling (Figure 1). Indeed both forms of communication are essential for normal oogenesis and folliculogenesis (Dong *et al.* 1996; Simon *et al.* 1997). Traditionally research has focused on just one direction of this communication axis, that is, on granulosa cell support of the developing oocyte but recent studies demonstrate the importance of a bi-directional communication axis (Albertini *et al.* 2001). It is now becoming clear that oocyte paracrine signals are



**Figure 1.** Oocyte-cumulus cell communication. Both paracrine (bold arrow) and gap-junctional (dashed arrow) communication between the oocyte and cumulus cells are required for normal oocyte and follicle development. Both communications pathways are bi-directional. Factors transmitted via these pathways include follicular fluid meiosis-activating sterol (FF-MAS), cAMP, purines and pyrimidines, metabolites, amino acids, growth differentiation factor-9 (GDF-9) and GDF-9B or bone morphogenic protein (BMP-15), fibroblast growth factor (FGF) and activin.

pivotal regulators of granulosa cell and ovarian function (Eppig 2001). Two key oocyte molecules identified so far are growth differentiation factor 9 (GDF-9) and GDF-9B (also called bone morphogenic protein 15; BMP-15). These oocyte growth factors are critical for progression of the very earliest stages of folliculogenesis (Dong *et al.* 1996; Galloway *et al.* 2000), and then in late follicular development these oocyte-secreted factors play an important role in the differentiation of different granulosa cell lineages (Eppig *et al.* 1997; Li *et al.* 2000) and in the regulation of key granulosa cell functions (Elvin *et al.* 1999; Joyce *et al.* 2000; Otsuka *et al.* 2001).

The highly specialised cumulus cells have distinctive trans-zonal cytoplasmic processes (TZPs), which penetrate through the zona pellucida and abut the oolemma. Gap junctions at the ends of these TZPs (and between cumulus cells) allow the transfer of small molecular weight molecules between oocyte and cumulus cell, and also between cumulus cells (Eppig 1991). Gap junctional communication in the follicle is essential for development and fertility. Both folliculogenesis and oogenesis fail in mice homozygous null for either connexin-37 (the protein building block of oocyte-cumulus cell gap junctions; Simon *et al.* 1997), or connexin-43 (the protein associated with gap junctions between granulosa cells; Ackert *et al.* 2001). Glucose metabolites, amino acids and nucleotides are all able to pass between oocyte and cumulus cells. In addition gap junctions participate in oocyte meiotic regulation by allowing the passage of small regulatory molecules such as cAMP and purines (Dekel and Beers 1980; Salustri and Siracusa 1983; Eppig and Downs 1984; Racowsky 1985; Racowsky and Satterlie 1985). Such intimate metabolic contact between oocyte and cumulus cells is thought to play a key role in disseminating local and endocrine signals to the oocyte via the cumulus cells. Hence an understanding of the nutritional, metabolic or hormonal factors conferring oocyte developmental competence, by necessity, must entail an examination of the COC as a whole (as opposed to isolated oocytes). However, the majority of studies investigating energy substrates for maturing oocytes involve the addition of substrates to intact COCs and determining either developmental outcome or the metabolism of the denuded oocyte. Clearly the metabolic profile of denuded oocytes (DOs) differs significantly to that of COCs (Colonna and Mangia 1983; Zuelke and Brackett 1993; Khurana and Niemann 2000a).

### *Importance of cumulus cells to oocyte IVM*

Apart from the importance of granulosa cells and cumulus cells to the oocyte throughout follicle growth, the cumulus cells also play a critical role during spontaneous meiotic maturation in vitro. At around the time of meiotic resumption, cumulus cell-TZPs begin to withdraw from the oocyte and there is almost complete loss of gap-junctional communication by the time oocytes reach metaphase I (MI). Considerable extracellular production of hyaluronic acid by cumulus cells causes dispersion of cumulus cells or cumulus expansion [Eppig, 1981 #296; Salustri, 1989 #282; Chen, 1990 #280]. However, during this phase cumulus cells presumably continue to communicate with the oocyte, as removal of the cumulus cells prior to IVF results in compromised fertilization and embryo development compared to removing them post-IVF, regardless of co-culture with cumulus cells (Zhang *et al.* 1995; Fatehi *et al.* 2002).

One of the most commonly used selection criteria for IVM is the morphology of the COC, in particular the cumulus vestment. Factors such as increased cell layers and degree of compaction are related to improved developmental outcome compared to oocytes surrounded by compromised vestments and DOs (Shioya *et al.* 1988; Madison *et al.* 1992; Lonergan *et al.* 1994; Goud *et al.* 1998), as well as there being a positive relationship between increased cumulus cell number in co-culture and developmental competence (Hashimoto *et al.* 1998).

### **Follicular fluid composition**

The follicular antrum is formed early in folliculogenesis. Follicular fluid (FF) bathes the COC and contains a variety of proteins, cytokine/growth factors and other peptide hormones, steroids, energy metabolites and other undefined factors. Granulosa cells are separated by 20 nm diameter channels, potentially allowing molecules up to  $M_r$  500000 in size to enter the antrum (Gosden *et al.* 1988). The porous nature of the follicular epithelium results in FF composition being comparable to ‘filtered’ venous plasma (Table 1).

**Table 1.**The composition of sheep, pig, human and cow follicular fluid (FF) from pooled, small or large follicles.

	Sheep		Pig		Human		Cow			
	Pooled	Pooled	PCOS	Post LH	Pooled		Unstimulated (pre LH)		Stimulated (post LH)	
Na <sup>+</sup> (mM)	149 <sup>b</sup>	128 <sup>h</sup>	133.5 <sup>b</sup>		132 <sup>b</sup>	177.7 <sup>k</sup>	109.2 <sup>k</sup>	102.7 <sup>k</sup>	88.1 <sup>k</sup>	
					139 <sup>b</sup>					
K <sup>+</sup> (mM)	4.7 <sup>b</sup>	15.9 <sup>h</sup>	4.9 <sup>b</sup>		9.2 <sup>b</sup>	10.2 <sup>k</sup>	7.4 <sup>k</sup>	11.4 <sup>k</sup>	5.6 <sup>k</sup>	
					8.05 <sup>b</sup>					
Cl <sup>-</sup> (mM)	107 <sup>b</sup>	97.3 <sup>b</sup>	124.5 <sup>b</sup>		149.5 <sup>b</sup>					
Ca <sup>2+</sup> (mM)	2.29 <sup>b</sup>	2.34 <sup>h</sup>	0.94 <sup>b</sup>		3.1 <sup>b</sup>	1.9 <sup>k</sup>	2.1 <sup>k</sup>	2.2 <sup>k</sup>	1.8 <sup>k</sup>	
					2.3 <sup>b</sup>					
Mg <sup>2+</sup> (mM)	0.89 <sup>b</sup>	0.75 <sup>b</sup>	0.76 <sup>b</sup>			0.90 <sup>k</sup>	0.89 <sup>k</sup>	1.3 <sup>k</sup>	0.73 <sup>k</sup>	
Protein (g/100ml)	6.84 <sup>h</sup>		7.28 <sup>c</sup>		7.08 <sup>a</sup>	247 <sup>q</sup>	33 <sup>q</sup>			
					6.94 <sup>f</sup>					
Albumin (mg/ml)			48.2 <sup>c</sup>			43.4 <sup>k</sup>	36 <sup>k</sup>	54.1 <sup>k</sup>	47.4 <sup>k</sup>	
Total aa (μg/ml)			236 <sup>c</sup>							
Glucose (mM)				3.44 <sup>d</sup>						
				3.39 <sup>e</sup>						
Lactate (mM)				6.27 <sup>d</sup>						
				3.17 <sup>e</sup>						
pO <sub>2</sub> (mmHg)				59.8 <sup>g</sup>						
				100.5 <sup>s</sup>						
pCO <sub>2</sub> (mmHg)				46.9 <sup>g</sup>						
				34.8 <sup>s</sup>						
pH				7.33 <sup>g</sup>						
				7.35 <sup>s</sup>						
NH <sub>4</sub> <sup>+</sup> (μM)						134 <sup>q</sup>				

Superscripts indicate reference. <sup>a</sup>(Desjardins *et al.*, 1966), <sup>b</sup>(Gosden *et al.*, 1988), <sup>c</sup>(Velazquez *et al.*, 1977), <sup>d</sup>(Leese and Lenton, 1990), <sup>e</sup>(Gull *et al.*, 1999), <sup>f</sup>(Andersen *et al.*, 1976), <sup>g</sup>(Fischer *et al.*, 1992), <sup>h</sup>(Schuetz and Anisowicz, 1974), <sup>i</sup>(Mendoza *et al.*, 2002), <sup>j</sup>(Takagi *et al.*, 1998), <sup>k</sup>(Wise, 1987), <sup>l</sup>(Wise *et al.*, 1994), <sup>m</sup>(Eppig *et al.*, 1985), <sup>n</sup>(Homa and Brown, 1992), <sup>o</sup>(Malamitsi-Puchner *et al.*, 2001), <sup>p</sup>(Van Blerkom *et al.*, 1997), <sup>q</sup>(Hammon *et al.*, 2000), <sup>r</sup>(Hazeleger *et al.*, 1995), <sup>s</sup>(Huey *et al.*, 1999). PCOS = polycystic ovary syndrome.

### *Protein content*

Mean protein concentration is significantly lower in bovine FF compared to blood serum, regardless of follicle size (Desjardins *et al.* 1966), and this is largely accounted for by the partial exclusion of most proteins MW >250000 (i.e.  $\alpha_1$ -lipoprotein,  $\alpha_2$ -macroglobulin and IgM; Andersen *et al.* 1976). There is a positive relationship between increasing follicle size and the concentration of proteins with high molecular weight, indicative of increased permeability of serum proteins with follicular growth. In general the concentration of globulins in human FF are not significantly different to that in plasma, while albumin is 35% higher in FF compared with plasma (Velazquez *et al.* 1977). The total concentrations of amino acids in FF are also higher than in blood plasma, with the exception of cysteine (0.19 mM in plasma vs. 0.062 mM in FF; Velazquez *et al.* 1977), possibly due to its oxidation to cystine or use by the COC. The concentration of cysteine in a commonly used medium for IVM (Tissue culture medium 199, TCM199) is 0.6  $\mu$ M, which is 10-fold lower than physiological levels.

### *Electrolytes*

The concentrations of electrolytes such as chloride, calcium and magnesium in FF from large follicles (mostly preovulatory) are highly comparable to serum and plasma levels (Gosden *et al.* 1988). Potassium levels may be elevated (1.5- to 3-fold) in FF in some species (possibly indicating active transport systems; Schuetz and Anisowicz 1974; Gosden *et al.* 1988).

### *Energy substrates*

The concentration of energy metabolites in human FF has been studied with samples obtained from preovulatory follicles of hyperstimulated patients undergoing assisted reproduction treatments. Leese and Lenton (Leese and Lenton 1990) reported that follicular lactate levels were 3 to 4-fold higher than serum levels (6.12 mM vs. 1.5-2 mM) and exist in a 2:1 ratio with glucose. This contradicts later studies showing that glucose and lactate levels in human FF were 3.39 mM and 3.17 mM respectively

(Gull *et al.* 1999). Differences may have arisen from the methods used for analysis of the FF and serum and the storage of samples. Glucose-6-phosphate dehydrogenase activity and lactate dehydrogenase-1 (LDH-1) synthesis increase significantly with oocyte growth, plateauing in medium sized follicles (Mangia *et al.* 1976). A positive correlation between glucose utilisation and lactate production exists and it is postulated that as the follicle grows, energy requirements increase with decreasing O<sub>2</sub> availability (due to thickening of the avascular epithelium), leading to an increase in glycolysis and increased lactate production (Boland *et al.* 1993; Gull *et al.* 1999). This is supported by a 2-fold decrease in FF O<sub>2</sub> tension (59.8 mmHg in FF vs. 102 mmHg in maternal blood) and higher CO<sub>2</sub> tension (46.9 mmHg in FF vs. 38.3 mmHg in blood), resulting in a lower pH of FF compared to blood (7.33 vs. 7.41 respectively) (Fischer *et al.* 1992). All of these events are associated with increasing follicular growth leading to ovulation.

Follicular vascularity and dissolved O<sub>2</sub> content in FF are positively related to oocyte developmental outcome in humans. Measurements of follicular vascularity prior to oocyte collection demonstrated that oocytes derived from follicles with >50% blood flow on their circumference had significantly higher rates of clinical pregnancies following in vitro fertilization (IVF) and embryo transfer, compared to oocytes with poor vascularity (Chui *et al.* 1997; Coulam *et al.* 1999). Furthermore, only embryos resulting from oocytes collected from follicles with a high degree of vascularity (blood flow identified on 76-100% of the follicular circumference) resulted in successful pregnancies following embryo transfer. Poor vascularity and low dissolved O<sub>2</sub> content are associated with developmental defects such as aneuploidy, abnormal spindle organization and cytoplasmic structure (Van Blerkom *et al.* 1997). Oocytes from follicles with higher dissolved O<sub>2</sub> in FF are more competent than oocytes from lower oxygenated follicles (as measured by development to 6 to 8-cell stage; Van Blerkom *et al.* 1997; Huey *et al.* 1999). These studies suggest hypoxic conditions have adverse effects on subsequent oocyte quality.

### *Lipids*

In general, fatty acid concentration of follicular fluid decreases with follicle size (Yao *et al.* 1980). In particular, linoleic acid is negatively correlated to follicle size,

and addition to culture medium inhibits GVBD in bovine oocytes, possibly by indirectly stimulating cAMP levels by affecting adenylate cyclase activity (Homa and Brown 1992). In general there appears little information on the role of lipids during oocyte growth and maturation. There is, however, an important exception to this and that is with regard to a group of sterols, the meiosis activating sterols that are intermediates in the cholesterol biosynthetic pathway. Follicular fluid meiosis activating sterol (FF-MAS) and testicular-MAS (T-MAS, first purified from testicular tissue) are present in follicular fluid of preovulatory follicles in micromolar concentrations (Byskov *et al.* 2002). Their potential roles in the regulation of oocyte maturation are discussed later.

#### *Temperature and pH*

Temperature gradients exist within the ovarian environment with pre-ovulatory follicles approximately 1.5-2°C cooler than the ovarian stroma in pigs (Hunter *et al.* 1997; Hunter *et al.* 2000), humans (Grinsted *et al.* 1985) and cows (Grøndahl *et al.* 1996). How such temperature gradients are established and maintained is difficult to explain and may yet reflect inadequate technologies to make such measurements. However, no differences in temperature were observed between the stromal tissue and small antral follicles (Grøndahl *et al.* 1996; Hunter *et al.* 1997). Hunter (Hunter *et al.* 1997) argues that the variations in temperature are established due to the follicle becoming largely avascular compared to the surrounding tissue, as well as an increase in endothermic activity associated with ovulatory processes. Decreased temperatures may decrease the viscosity of porcine FF, which would facilitate the oocyte's entry into the oviducts. However application of temperature gradients to IVM did not alter the developmental rates of bovine oocytes (Shi *et al.* 1998), indicating that although the temperature used for IVM is based on visceral temperature (and are higher than that within the ovary; Grøndahl *et al.* 1996; Hunter *et al.* 2000; Hunter *et al.* 1997), this seems to be adequate for IVM. The adverse effects of short-term heat shock during IVM are seen when temperatures are increased by approximately 4°C and for >30 mins culture periods (Ju *et al.* 1999).

## **IVM media**

### *Commercially available cell culture media*

The maturation of oocytes in vitro is typically undertaken in commercially available complex medium, originally intended for the culture of non-ovarian somatic cells. Several commercially supplied media are commonly used for the base of IVM systems, such as TCM199, Waymouth MB 752/1, Ham's F-12, Minimum Essential Medium (MEM), and Dulbecco's modification of Eagle's medium (DMEM). The composition of the most commonly used IVM media are given in Table 2.

A range of different IVM base media is commonly used since oocytes from different species vary in their response to different media. Bovine oocytes matured in TCM199, SFRE (serum free medium based on TCM199) and MEM have superior blastocyst development rates (12-19%) compared to oocyte matured in Waymouth MB 752/1, Ham's F-12 (3% and 1%, respectively; Rose and Bavister 1992) or Menezo's B2 (Hasler 2000). This is contrary to murine oocytes, where the highest cleavage rates were observed with IVM systems that used Waymouth MB 752/1 and MEM + non-essential amino acids (NEA), Ham's F-12 and  $\alpha$ MEM (van de Sandt *et al.* 1990). For porcine IVM, the composition of Waymouth MB 752/1, more favourably supports male pronucleus formation than TCM199 or TLP-PVA media (Yoshida *et al.* 1992). This may be related to high cysteine and cystine levels in Waymouth MB 752/1 leading to increased cytoplasmic integrity through elevated axoplasmic glutathione (GSH) levels (Yoshida *et al.* 1993).

Given the apparent need to test the different IVM base media in different species, the choice of base medium for human IVM is particularly difficult. Clearly it is not possible to conduct an experiment large enough using human oocytes to thoroughly test different IVM media. IVM of human oocytes is typically conducted using either TCM199 (Trounson *et al.* 1994; Cha and Chian 1998; Mikkelsen *et al.* 1999) or Ham's F10 (Cha *et al.* 1991). Waymouth MB 752/1 has been used for IVM of marmoset monkey oocytes (Gilchrist *et al.* 1995; Gilchrist *et al.* 1997) and modified Connaught Medical Research Laboratories medium (CMRL-1066) is the most commonly used rhesus oocyte IVM medium (Schramm and Bavister 1994; Schramm *et al.* 1994; Schramm and Bavister 1996).

**Table 2.** The composition of commercially supplied media commonly used for in vitro oocyte maturation.

Compound (mM)	TCM199 MB 752/1	Waymouth F-12	Ham's F-12	MEM	DMEM	mBM3	HECM
<b>CaCl<sub>2</sub></b>	1.802	0.82	0.23	1.36	1.36	1.9	1.9
<b>MgSO<sub>4</sub></b>	0.788	3.96	0.58	0.79	0.79		
<b>KCl</b>	5.367	2.01	3	5.37	5.37	3	3
<b>NaCl</b>	116.359	102.67	130.05	116.36	109.51	113.8	113.8
<b>NaHCO<sub>3</sub></b>		26.66	14	26.19	44.04	25	25
<b>Na<sub>2</sub>HPO<sub>4</sub></b>	1.017	2.5	1.18	1.17	1.04		
<b>DL-alanine</b>	0.561		0.1				
<b>L-arginine</b>	0.332	0.36	1	0.6	0.4		
<b>DL-aspartic acid</b>	0.451	0.45	0.1			0.01	0.01
<b>Asparagine</b>						0.01	0.01
<b>L-cysteine</b>	6.980x10 <sup>-4</sup>	0.51	0.22			0.01	0.01
<b>L-cystine</b>	0.083	0.06		0.1	0.2		
<b>DL-glutamic acid</b>	0.908	1.02	0.1			0.01	0.01
<b>L-glutamine</b>	0.684	2.4	1	2	4	0.2	0.2
<b>Glycine</b>	0.666	0.67	0.1		0.4	0.01	0.01
<b>L-histidine</b>	0.104	0.78	0.17	0.2	0.2	0.01	0.01
<b>Hydroxy-L-Proline</b>	0.0763						
<b>DL-isoleucine</b>	0.305	0.19	0.03	0.4	0.8		
<b>DL-leucine</b>	0.915	0.38	0.1	0.4	0.8		
<b>L-lysine</b>	0.479	1.64	0.25	0.5	1	0.01	0.01
<b>DL-methionine</b>	0.201	0.34	0.03	0.1	0.2		
<b>DL-phenylalanine</b>	0.303	0.3	0.03	0.2	0.4		
<b>L-Proline</b>	0.348	0.43	0.3			0.01	0.01
<b>DL-serine</b>	0.476		0.1		0.4	0.01	0.01
<b>Taurine</b>						0.5	0.5
<b>DL-threonine</b>	0.504	0.63	0.1	0.4	0.8		
<b>DL-tryptophan</b>	0.0979	0.20	0.01	0.05	0.08		
<b>L-tyrosine</b>	0.256	0.26		0.23	0.46		
<b>DL-valine</b>	0.427	0.56	0.1	0.4	0.8		
<b>Glucose</b>	5.55	27.75	10	5.55	24.97	2	
<b>DL-lactate</b>						4.5	4.5
<b>Pyruvate</b>					1	0.25	
<b>Glutathione</b>	1.62x10 <sup>-4</sup>	0.16					
<b>Hypoxanthine</b>	0.0022	0.18	0.04				

TCM = Tissue culture medium; MEM = Minimum essential medium; DMEM = Dulbecco's modification of Eagle's medium; mBM-3 = Basic salt medium 3; HECM = Hamster embryo culture medium.

The use of simple inorganic salt-based media is useful in determining which of the multitude of factors in complex media are important for successful oocyte maturation. In serum-free systems, mBM-3 supplemented with glucose and a mixture of 11 amino acids (in particular glutamine; Rose-Hellekant *et al.* 1998), or supplemented with NEA alone, or NEA + essential amino acids (EA; Avery *et al.* 1998) during IVM, lead to improved blastocyst development compared to that achieved with TCM199. Embryo development has also been achieved from human oocytes matured in simple balanced salt solutions, such as human tubal fluid (HTF; Jaroudi *et al.* 1997; Hwu *et al.* 1998) and human oocyte maturation medium (HOM; Trounson *et al.* 1998; Trounson *et al.* 2001). As IVM media trials are exceptionally difficult using human oocytes, such experiments are more feasible using non-human primate oocytes. With appropriate amino acid additives, a simple protein-free medium such as hamster embryo culture medium-10 (HECM-10) is equally effective as the complex medium, CMRL-1066 during IVM, at supporting development of rhesus oocytes through to the blastocyst stage (Zheng *et al.* 2001a).

The formulation of IVM media specifically based on the composition of follicular fluids has not been attempted. Substantial improvements in embryo culture media have been made over the past decade by basing media formulations on the major cation and anion concentrations and metabolic substrates of reproductive tract fluids, e.g. SOF; synthetic oviduct fluid (Tervit *et al.* 1972), HTF (Quinn *et al.* 1985) and G1/G2; human tubal and uterine fluids (Gardner *et al.* 1996), MTF; mouse oviductal fluid (Gardner and Leese 1990) and PL<sub>3</sub>; based on bovine blood and oviductal fluid (Park and Lin 1993). IVM efficiency may be improved with the design of an IVM medium along similar principals.

#### *Macromolecule supplementation*

There is a long running debate as to whether protein and macromolecule supplements should be added to IVM media and subsequent IVF and IVC media. Numerous protein supplements are used (Fukui and Ono 1989; Wiemer *et al.* 1991) such as fetal calf serum (FCS), oestrous cow serum, oestrous gilt serum, anoestrous cow serum, steer serum, newborn calf serum, bovine serum albumin (BSA) and for human IVM, autologous patient serum and human serum albumin. FCS and BSA are the most

commonly used protein supplements in IVM, with bovine oocytes matured in the presence of FCS having higher frequencies of oocyte nuclear maturation, cleavage and blastocyst formation compared to supplementation with or without other macromolecules (Fukui and Ono 1989; Wiemer *et al.* 1991; Ocaña-Quero *et al.* 1999; Hasler, 2000). Fetal serum contains numerous factors thought to be beneficial to oocyte maturation and embryo development such as growth factors, lipids, albumin, hormones, steroids, cholesterol, peptides and many other undefined factors. The highly undefined nature of protein supplements makes them undesirable for many research aspects, due to the risk of batch variation and contaminating compounds of undefined nature. Although high grade BSA has some degree of variability, it is less variable than serum itself. BSA has also been shown to contain steroids, especially estradiol, at levels high enough to allow for adequate cytoplasmic and nuclear maturation that supplementation with estradiol alone is unnecessary (Mingoti *et al.* 2002).

Polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) are commonly used non-biological alternatives to protein supplements to aid in the handling of oocytes and embryos. Although oocytes matured in media supplemented with PVA or PVP have lower rates of polyspermic fertilization, development to the blastocyst stage is compromised compared to oocytes matured in the presence of proteins (Eckert and Niemann 1995; Fukui *et al.* 2000). Despite this, supplementation of PVA based IVM media with hormones (luteinizing hormone, LH; follicle stimulating hormone, FSH; and estradiol), growth factors (epidermal growth factor) and other beneficial factors ( $\beta$ -mercaptoethanol, hypotaurine) can increase blastocyst development to rates comparable to oocytes matured in the presence of proteins (Avery *et al.* 1998; Abeydeera *et al.* 2000; Mizushima and Fukui 2001). This indicates that inorganic macromolecules together with defined protein additives can potentially replace serum/BSA supplements in IVM medium.

#### *Follicular fluid as a medium*

When FF is used as a substitute for serum in IVM media, embryo development is not influenced by the size of the follicle which the fluid originated from, nor is there any differences between bovine oocytes matured in the presence of FF or serum

(Lonergan *et al.* 1994; Carolan *et al.* 1995; Kim *et al.* 1996). Although the size of the follicle from which the FF is sourced has little influence on embryo development, fluid obtained from non-atretic follicles supported oocyte developmental competence to a greater extent than FF from atretic follicles (Cogniéa *et al.* 1995). In contrast FF from non-atretic dominant follicles when added to IVM media with serum improved embryo development, compared to the use of pooled fluids from small or medium follicles (Sirard *et al.* 1995).

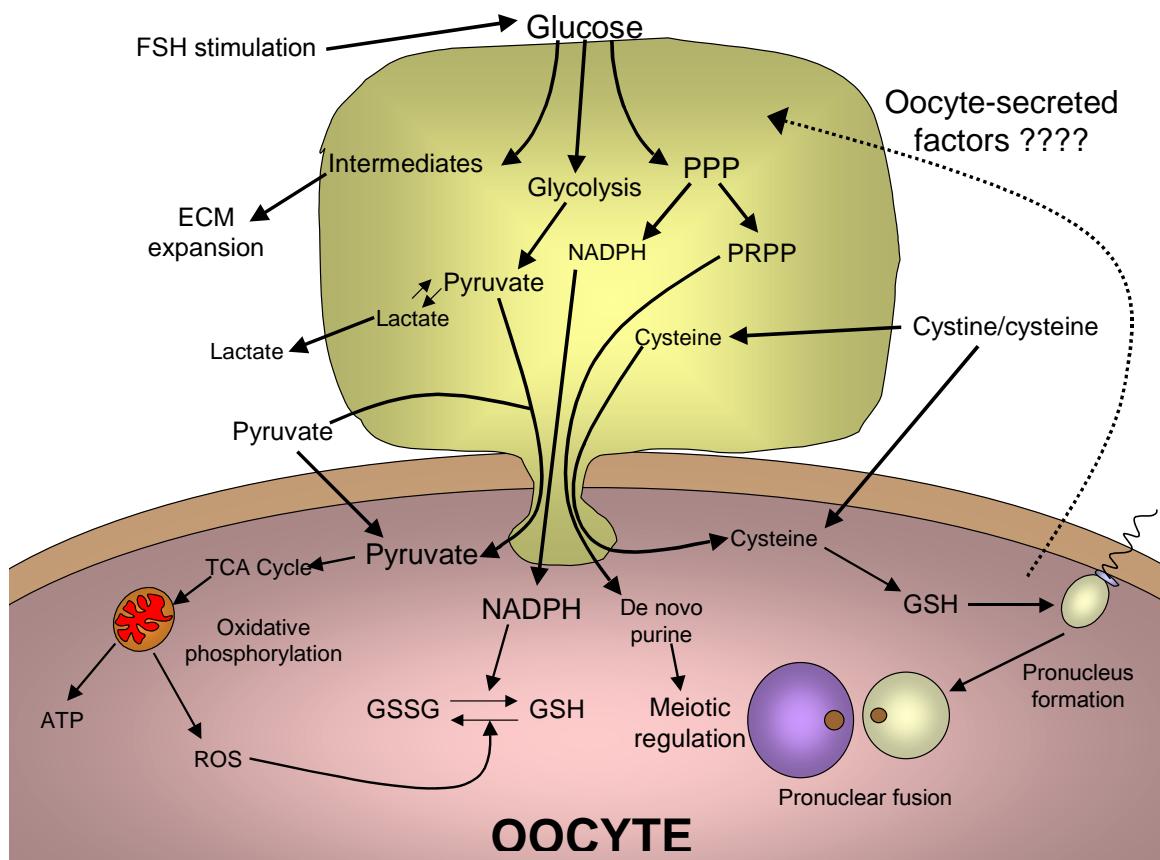
The concentration of FF used in IVM media can influence oocyte meiotic maturation, with the addition of concentrations of 60% or higher having inhibitory effects on nuclear maturation (in particular a high incidence of oocytes being blocked at GV and MI stage), resulting in higher rates of oocyte degradation (Kim *et al.* 1996). Although the use of 100% FF during maturation resulted in increased cumulus expansion (Takagi *et al.* 1998), there was a higher incidence of meiotic inhibition that was reversible when oocytes were placed in conventional media, but there was a lower blastocyst development compared to oocytes cultured in TCM199 plus serum (Ayoub and Hunter 1993; Choi *et al.* 1998).

In general, the supplementation of FF from antral follicles to IVM media as a substitute for or in addition to other organic or inorganic supplements provides no additional benefits to oocyte competence and subsequent embryo development although it has the potential of being an alternative protein source which could be collected in unison with oocyte aspiration (in particular for human IVM; Cha *et al.* 1991).

## Oocyte and cumulus-oocyte complex metabolism

As the earliest stages of embryonic development are largely dependant on events that occur during oocyte maturation, adequate supply of appropriate metabolic substrates to the COC during IVM is likely to impact on subsequent embryo developmental potential (Krisher and Bavister, 1998). Numerous energy substrates are supplied to the oocyte in IVM media with glucose, pyruvate, lactate and amino acids being important metabolites for full embryo developmental potential (Figure 2). Despite

## CUMULUS CELL



**Figure 2.** Proposed model of the metabolic interactions and activity of cumulus cells and the oocyte. Numerous energy substrates are supplied to the cumulus-oocyte complex (COC) by the surrounding fluid, including glucose, pyruvate, lactate and amino acids. Glucose can be utilised via three major pathways, glucose oxidation (the combination of glycolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation), the pentose phosphate pathway (PPP) or it can be converted to intermediates and utilised for extracellular matrix expansion (ECM). FSH stimulates glucose metabolism by cumulus cells. Glucose utilization begins with glycolysis (within cumulus cells) where glucose-6-phosphate is converted to pyruvate, which can then enter the oocyte directly or be converted to lactate. Pyruvate is further oxidised by the TCA cycle within ovum mitochondria, followed by oxidative phosphorylation in the mitochondrial intermembrane where ATP is released by electron transfer. PPP also begins with the oxidation of glucose to glucose-6-phosphate within cumulus cells with the one of the products of the pathway, phosphoribosylpyrophosphate (PRPP) being used by the oocyte for purine synthesis. Purines are involved in regulation of nuclear maturation. PPP is also involved in general cytoplasmic homeostasis since  $\text{NADP}^+$  is reduced to NADPH. Amino acids cystine and cysteine are involved in the production of glutathione (GSH), accumulation of which appears essential for early embryonic development. Although oocyte-secreted factors are known to have major effects on development and differentiation of cumulus cells, there is no data concerning their effects on metabolism of cumulus cells. GSSG = oxidised GSH; ROS = reactive oxygen species.

this, metabolic profiles and requirements of human oocytes have not been investigated.

#### *Oxygen and oxidative phosphorylation*

Oocyte nuclear maturation is absolutely dependent on oxygen availability and oxidative phosphorylation. Rat oocytes cultured under anaerobic conditions or in the presence of oxidative phosphorylation inhibitors were all arrested at the GV stage (Zeilmaker and Verhamme 1974).

Several reports demonstrate that cattle IVM under low (<10% O<sub>2</sub>) oxygen atmospheres leads to oocytes with increased polyspermic fertilization and reduced developmental competence (Pinyopummintr and Bavister 1995). This effect may be dependent on extracellular glucose concentration as low O<sub>2</sub> (5%) and high (20 mM) glucose was associated with improved developmental capacity (Hashimoto *et al.* 2000b). Furthermore, 5% O<sub>2</sub> was also associated with improved developmental competence when oocytes were released from a specific cyclin-dependent kinase-induced maintenance of meiotic arrest (Hashimoto *et al.* 2002).

#### *Glucose metabolism*

Cumulus cells play a pivotal role in glucose utilisation by the COC. Whereas mature human oocytes express only one of the facilitative glucose transporter isoforms (GLUT-1), cumulus cells express four isoforms (Dan-Goor *et al.* 1997). Indeed, in the absence of cumulus cells, immature mammalian oocytes demonstrate very low levels of glucose uptake, glycolytic activity and glucose oxidation (Zuelke and Brackett 1992; Rieger and Loskutoff 1994; Saito *et al.* 1994). Several authors have suggested that the fate of glucose within the COC is primarily the production of pyruvate/lactate, these being the oocyte's preferred substrates (Biggers *et al.* 1967; Downs and Utecht 1999; Eppig *et al.* 2000; Khurana and Niemann 2000a; Khurana and Niemann 2000b; Cetica *et al.* 2002), but there is little data comparing the relative changes in substrate uptake during the course of oocyte maturation. Following maturation, glucose utilisation by denuded mature oocytes is mostly through glycolysis, with pentose phosphate pathway (PPP) activity being less than 3% of

glycolytic activity (Urner and Sakkas 1999). Gamete fusion leads to a significant increase in activity of both pathways, with sperm derived factors triggering the response since parthenogenetic activation did not alter metabolism (Urner and Sakkas 1999) and the degree of polyspermy correlates with the level of glucose oxidation (Pantaleon *et al.* 2001).

Paradoxically, despite the relatively low level of glucose utilisation, both glycolysis and the PPP activity within oocytes are correlated with meiotic progression and developmental capacity. Glycolytic activity contributes to ATP production and provides pyruvate and reducing equivalents for further oxidation within the cell, as well as supplying other metabolic intermediates, especially for glycosylation. The PPP begins with the oxidation of glucose to glucose-6-phosphate and is required for the formation of ribose-sugars for DNA and RNA synthesis. The relative activity of glucose-6-phosphate dehydrogenase is high in bovine oocytes compared to cumulus cells suggesting a preference for the PPP within oocytes (Cetica *et al.* 2002). One of the products of the pathway, phosphoribosylpyrophosphate (PRPP) is used by the oocyte for purine synthesis. PPP is also linked to other cellular enzymatic processes, through the reduction of NADP<sup>+</sup> to NADPH, a key regulator substrate in essential metabolic programs such as GSH reduction (discussed below).

Blocking glycolytic activity within oocytes does not influence meiotic maturation of mouse oocytes (Downs *et al.* 1996). Furthermore, the use of PPP stimulators such as methylene blue, phenazine ethosulfate and pyrroline-5-carboxylate lead to a dose-dependent increase in GVBD and increased glucose consumption (Downs *et al.* 1998; Downs and Utecht 1999). Such data indicate that involvement of glucose in oocyte meiotic regulation is via PPP rather than glycolysis. PPP appears involved in FSH-induced meiotic maturation in murine oocytes and FSH stimulates the utilisation of glucose by the COC (Downs *et al.* 1996).

An adequate supply of glucose during IVM is a fundamental requirement of oocyte metabolism. The addition of glucose to maturation media improves the resumption of meiosis, embryo cleavage, morulae and blastocyst rates (bovine, Krisher and Bavister 1998; Rose-Hellekant *et al.* 1998 and rhesus monkey, Zheng *et al.* 2001b). However, high concentrations of glucose during IVM have adverse effects on

subsequent embryo development, thought to be due to an increased generation of reactive oxygen species (ROS) and decreased intracellular GSH pools (Hashimoto *et al.* 2000a). Oocytes derived from adult and pre-pubertal cattle differ in their metabolic profiles, in particular glycolytic activity (Gandolfi *et al.* 1998; Steeves and Gardner 1999). Pre-pubertal oocytes have an increase in glycolysis between 12 h and 24 h of culture, compared to increased glycolytic activity seen between 0 h and 12 h in adults. This delay in metabolism also corresponded with delayed resumption of meiosis. Pre-pubertal oocytes were also significantly smaller compared to adult derived oocytes, suggesting that the acquisition of developmental competence is reflected in glucose metabolism (Steeves and Gardner 1999). Comparisons between the metabolism of in vitro matured and in vivo matured oocytes has also revealed that IVM oocytes produce significantly higher levels of lactate, which could possibly reflect a stress response (Khurana and Niemann 2000b). In vivo matured porcine and feline oocytes also utilise glucose via glycolysis and PPP more efficiently than in vitro matured oocytes (Spindler *et al.* 2000; Durkin *et al.* 2001). Furthermore, oocyte maturation conditions that are associated with higher glycolytic pathway activity also promote improved oocyte developmental competence (Krisher and Bavister 1999; Spindler *et al.* 2000). This indicates that the capacity of the oocyte to utilise glucose is positively correlated with subsequent embryo developmental potential, and may be utilised as a predictive marker of oocyte quality (Krisher and Bavister 1999; Spindler *et al.* 2000).

### *Carboxylic acids*

Glucose as the sole metabolite results in compromised development, compared with the presence of both glucose and pyruvate (Downs and Hudson 2000). Supplementation of media with lactate leads to similar rates of bovine oocytes undergoing meiotic maturation to those that occur when pyruvate is added, although exogenous addition of NAD<sup>+</sup> is required for lactate utilisation to occur (Cetica *et al.* 1999). This result is somewhat surprising, as NAD<sup>+</sup> is not considered cell permeable. This suggests a leakage of lactate dehydrogenase from the COC into the surrounding matrix (which may occur if cumulus cells undergo apoptosis during maturation). Nevertheless, overall LDH activity is significantly higher in COC compared to DOs, but the LDH-1 isoform activity is greater in DOs than cumulus cells, hence lactate

can be utilised within the oocyte under appropriate REDOX conditions (Cetica *et al.* 1999). Pyruvate consumption by murine COCs was directly correlated to oocyte maturation; hence increasing requirement for pyruvate occurs as the oocyte matures (Downs *et al.* 2002).

### *Purines and pyrimidines*

Reversible inhibition of oocyte nuclear maturation during IVM may allow for improved oocyte cytoplasmic maturation and enhanced embryo developmental competence (Lonergan *et al.* 1997; Guixue *et al.* 2001). The inhibitory effects of purines, in particular hypoxanthine, on meiotic progression, are well documented in the mouse and are thought to play a role in inhibiting the hydrolysis of cAMP (high intra-oocyte levels of cAMP are associated with meiotic arrest (Magnusson and Hillensjo 1977; Salustri and Siracusa 1983; Eppig and Downs 1984; Mattioli *et al.* 1994; Thomas *et al.* 2002). Hypoxanthine, adenosine and guanosine are present in FF (Kadam and Koide 1990) and have a synergistic effect on arresting spontaneous meiosis in rodent oocytes (Downs *et al.* 1985; Eppig *et al.* 1985; Tornell *et al.* 1990). As mentioned previously, Downs (Downs and Mastropolo 1994; Downs *et al.* 1998; Downs and Utecht 1999; Downs and Hudson 2000) has demonstrated an interaction between purines and glucose metabolism via PPP within the murine oocyte. A product of this pathway, PRPP, is thought to be a substrate for the salvage and de novo purine synthesis pathways; in particular PRPP concentrations are positively related to the rate of the salvage pathway. However, the mechanisms as to how combinations of glucose and purines inhibit meiotic resumption are complex and are still not clearly understood. In addition, purines are generally either ineffective or have a transient effect on meiotic maturation of non-rodent models (Sirard and First 1988; Miyano *et al.* 1995; Xia *et al.* 2000).

### *Amino acids*

Amino acids may be utilised by oocytes as an energy source with cumulus cells playing an important part in amino acid flux into the oocyte (Colonna and Mangia 1983). This is particularly evident when chemically defined media supplemented with NEA and EA result in enhanced embryo developmental rates, higher cell

numbers in blastocysts and increased oocyte maternal mRNA levels compared to defined media without amino acids and macromolecule supplementation (Watson *et al.* 2000).

LH stimulates cumulus cells to convert glutamine to  $\alpha$ -ketoglutarate, which is then oxidised through the TCA cycle to generate ATP (Rose-Hellekant and Bavister 1995; Rose-Hellekant *et al.* 1998). When glutamine is added as the sole energy substrate during murine IVM, COCs initiate GVBD but fail to complete meiotic maturation (Downs and Hudson 2000). This meiotic block may be due to a reduction or absence of adequate PPP activity.

GSH is a thiol tripeptide, comprising cysteine, proline and glutamine and is an important reducing agent and scavenger that protects cells against ROS. GSH synthesis is highly dependent on levels of cysteine, a highly unstable amino acid that is readily oxidised to cystine. De Matos *et al* (de Matos *et al.* 1997) demonstrated that cumulus cells have the ability to reduce cystine to cysteine when cystine is added in high concentrations (Geshi *et al.* 2000).

The presence of exogenous cysteine during IVM is highly desirable since GSH pools accumulated during this period are necessary for sperm decondensation and male pronucleus formation. Furthermore GSH synthesis during IVM has greater effects on subsequent embryo development compared to synthesis during IVF and IVC (Miyamura *et al.* 1995; Sutovsky and Schatten 1997; Ali *et al.* 2001). The addition to IVM media of low molecular weight thiol compounds, such as cysteamine and  $\beta$ -mercaptoethanol results in elevated oocyte GSH levels by reducing oxidised cystine to constituent cysteine in medium (de Matos *et al.* 1995; de Matos *et al.* 1996). The addition of these compounds during IVM (in the presence of cysteine or cystine) enhances the developmental potential of oocytes (de Matos *et al.* 1995; de Matos *et al.* 1996). Furthermore, addition of cysteamine to IVC media leads to increased development of 6- to 8-cell bovine embryos to blastocysts, compared to embryos cultured in TCM199 alone (Takahashi *et al.* 1993). Hence an important component of a comprehensive IVM system is the provision of adequate levels of GSH precursors such as glutamine and cysteamine.

### *Lipids and fatty acids*

Little work has been conducted determining the role during oocyte maturation of both intracellular and extracellular lipids. Intracellular triglycerides are the most abundant lipid within mammalian oocytes, constituting over 50% of all lipid (Ferguson and Leese 1999; Kim *et al.* 2001). Intriguingly, the triglyceride content sharply decreases over the course of oocyte maturation in vitro, despite culturing in the presence of serum (Ferguson and Leese 1999; Kim *et al.* 2001). In pig, cattle and human oocytes, palmitic, stearic, oleic and linoleic acids are the most abundant intracellular fatty acids (Homa *et al.* 1986; Matorras *et al.* 1998; Kim *et al.* 2001), with stearic acid more abundant and oleic acid less abundant in less competent oocytes (Matorras *et al.* 1998; Kim *et al.* 2001). The role of these lipids in oocyte maturation is unclear, although they may have a potential role as reserve fuels (Leese 2002) or as meiotic regulators (discussed below).

### *Effects of gonadotrophins on cumulus cell metabolic activity*

The gonadotrophins FSH and LH are the most common hormone additives in an IVM system. Although FSH and LH are by no means necessary for spontaneous oocyte maturation, it is generally believed these hormones improve oocyte cytoplasmic maturation by significantly altering a range of cumulus cell activities. It is unclear, however, if this beneficial effect of gonadotrophins is mediated by changes in cumulus cell metabolic activity.

FSH is the key molecule in the murine “induced oocyte maturation” model. In mouse COCs, FSH but not LH, overrides the inhibitory effects of meiotic inhibitors such as hypoxanthine, cAMP (Downs *et al.* 1988) and phosphodiesterase inhibitors (Ryan *et al.* 2002), perhaps by the active production of a meiosis inducing substance such as FF-MAS (Byskov *et al.* 1995; Byskov *et al.* 1997). Furthermore the addition of FSH to IVM media leads to a 50% increase in the resumption of meiosis in bovine COCs compared to unstimulated complexes or hCG stimulation (van Tol *et al.* 1996). Whether sterols such as FF-MAS play any role in the regulation of oocyte maturation in non-rodents remains to be elucidated.

Glucose supplementation in IVM media is essential for both spontaneous and ligand-induced maturation (Downs and Hudson 2000). Furthermore the stimulatory effect of FSH on the resumption of meiosis requires the presence of glucose, as media containing pyruvate as the sole metabolite results in reduced rates of MII COCs (Downs and Hudson 2000). Glucose utilisation via the PPP is the most likely candidate through which FSH and glucose supplementation influences both types of meiotic maturation since inhibition of FSH stimulated glycolysis in murine COCs had little effect on the resumption of meiosis (Downs *et al.* 1996) compared to the use of PPP inhibitors that block GVBD, regardless of the presence or absence of FSH (Downs *et al.* 1998). Also, conditions that increase the resumption of meiosis also increase the flux of glucose through PPP (Downs and Utecht 1999). Although FSH increases the activities of both PPP and glycolysis in murine COCs (Downs and Utecht 1999), LH significantly increases the glycolytic pathway in bovine COCs and decreases PPP activity compared to unstimulated complexes (Zuelke and Brackett 1992).

## Conclusion

In vitro maturation of oocytes (and subsequent in vitro embryo production) is increasingly moving from a research tool to a commercially viable technique in domestic animal biotechnologies. In complete contrast, attempts to adopt this technology to human assisted reproductive technology practice has met with poor success, with just a handful of clinics around the world that offer human oocyte IVM as a routine clinical procedure, reporting less than satisfactory results. There is, however, a significant clinical demand for IVM, especially in developing countries, due to the potential to greatly reduce dependency on gonadotrophin stimulation, reducing the costs and associated side effects of hyperstimulation. Development of appropriate systems for human oocyte maturation in vitro requires a greater understanding of factors involved in the acquisition of oocyte developmental competence during folliculogenesis and during the maturation period (Hardy *et al.* 2000; Trounson *et al.* 2001).

There does appear to be an association between oocyte developmental competence and metabolism. In particular, a close association between intracellular GSH levels

and subsequent embryo developmental potential has been demonstrated in several species. Less well characterised is the association between glucose metabolism within the oocyte during maturation and subsequent development. Although increased oocyte developmental competence is associated with increased levels of glycolysis and PPP activity, it is yet to be revealed whether this is causative or merely associative. However, the activity of the PPP is linked with GSH turnover, and may therefore be causative.

Apart from the pioneering work of Downs and colleagues, there is very little work conducted on the metabolism of the COC as a whole. As it is clear that oogenesis and folliculogenesis are dependent on bi-directional oocyte-granulosa cell communication, it seems probable that understanding the metabolic processes within the whole complex, rather than the oocyte in isolation, will yield more insight into the role of metabolism in promoting developmental competence in the oocyte.

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# **Chapter 2**

## **Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes**

Melanie L. Sutton<sup>1</sup>, Pablo D. Cetica<sup>2</sup>, Marta T. Beconi<sup>2</sup>, Karen L. Kind<sup>1</sup>, Robert B. Gilchrist<sup>1</sup> and Jeremy G. Thompson<sup>1</sup>

<sup>1</sup> Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, University of Adelaide, The Queen Elizabeth Hospital, Woodville Road, Woodville 5011, South Australia.

<sup>2</sup> Area of Biochemistry, School of Veterinary Sciences, University of Buenos Aires, Chorroarin 280 (C1427CWO), Buenos Aires, Argentina.

## **Abstract**

Intercellular-communication between the cumulus cell complex and the oocyte is essential for numerous processes during oocyte maturation. With this in mind, the interaction between oocyte-secreted factors and bovine cumulus cell complex metabolism during in vitro maturation (IVM) was determined. Cumulus oocyte complexes (COCs) were aspirated from abattoir-derived ovaries and divided into 3 treatment groups; intact COCs, oocytectomised complexes (OOX), in which the ooplasm was micro-surgically removed, and OOX co-cultured with denuded oocytes (OOX + DO). Complexes were cultured individually in IVM media. After 0-4, 10-14 and 20-24 h oxygen, glucose, pyruvate and L-lactate utilisation by complexes was measured. There were no significant differences in metabolic measures between any of the treatment groups ( $P > 0.05$ ), indicating that cumulus complex metabolism is not affected by the presence of the oocyte. When metabolic measures for complexes were analysed relative to time in culture, there was an approximate 2-fold increase in the consumption of oxygen, glucose and pyruvate over the 24 h period ( $P < 0.05$ ), although L-lactate production remained constant. The relationship between total glucose uptake and L-lactate production indicated that at the start of culture the majority of glucose consumed was being utilised via glycolysis, but by the cessation of the maturation period, significant levels of glucose were being utilised elsewhere, possibly for the formation of cumulus extra-cellular matrix. These results indicate that COC metabolism does not reflect oocyte biochemical activity. Nevertheless, the metabolic requirements of the COC increase throughout maturation.

## **Introduction**

The environment in which oocytes are cultured during in vitro maturation (IVM) plays an important role in subsequent embryo development. The types and concentrations of energy substrates added to IVM medium alter the metabolic profiles and maturation of oocytes. Glucose, in particular, is an important energy substrate and its addition to medium in appropriate levels leads to improved maturation and blastocyst development (Rose-Hellekant *et al.* 1998; Lim *et al.* 1999; Khurana and Niemann 2000; Zheng *et al.* 2001). Glucose has several fates during IVM, with glycolysis leading to the production of ATP and substrates such as pyruvate for further energy production. Alternatively, its metabolism via the pentose phosphate pathway not only supplies substrates that contribute to ooplasmic integrity, but is also linked to the regulation of meiotic maturation within the oocyte (Downs *et al.* 1998; Downs and Utecht 1999). Studies focusing on the metabolism of oocytes have demonstrated that factors during maturation are associated with improved embryo development. For example, oocytes collected from adult donors or in vivo matured, are accompanied by increased levels of glucose utilization through glycolysis and the pentose phosphate pathway, when compared to oocytes from pre-pubertal donors or matured in vitro (Gandolfi *et al.* 1998; Steeves and Gardner 1999a; Durkin *et al.* 2001). Furthermore, conditions during IVM that promote higher glycolytic activity also improve developmental capacity (Krisher and Bavister 1999). Despite cumulus cells playing an important role in the utilisation of energy substrates by the oocyte, there is minimal data regarding the metabolic profiles of the cumulus-oocyte complex (COC), with most studies focusing on the profiles of denuded oocytes. In the absence of somatic cells, little glucose is utilised by the oocyte (Rieger and Loskutoff 1994; Saito *et al.* 1994). Hence the oocyte is reliant on cumulus cells to first metabolise glucose to intermediates such as pyruvate, the oocyte's preferred substrate (Biggers *et al.* 1967). Therefore, an increased understanding of the metabolic activity of the intact COC may allow the development of improved in vitro maturation conditions and hence improved developmental outcomes.

It is well known that granulosa cells play a critical role in the growth and development of the oocyte. Inter-cellular communication between cumulus cells and the oocyte occurs via paracrine factors and through gap-junctions (Albertini *et al.* 2001). Cumulus cells facilitate the transfer of nutrients and factors essential for oocyte development such as metabolites, amino acids, signal transduction molecules and other factors (Colonna and Mangia 1983; Larsen and Wert 1988; Buccione *et al.* 1990a; Eppig 1991). Cumulus cells also play an important role in the regulation of oocyte cytoplasmic and nuclear maturation. For example, the transmission of regulatory molecules such as cAMP and purines via gap junctions allow for the maintenance of meiotic arrest within the oocyte (Eppig and Downs 1984). Indeed there is a positive relationship between the developmental potential of the oocyte and cumulus cell morphology, such as cumulus cell compaction (Shioya *et al.* 1988; Madison *et al.* 1992; Lonergan *et al.* 1994; Goud *et al.* 1998; Khurana and Niemann 2000), cell number (Hashimoto *et al.* 1998) and the degree of expansion.

More recently the importance of the two-way communication axis between oocytes and granulosa cells has been revealed. Oocytes secrete paracrine growth factors that regulate a broad range of granulosa cell functions by modulating fundamental control elements. For example, oocytes regulate expression of LH receptors, kit ligand, inhibin subunits and expression of extra-cellular matrix molecules (Canipari *et al.* 1995; Eppig *et al.* 1997; Lanuza *et al.* 1998; Joyce *et al.* 1999). As such, oocytes not only promote growth of granulosa cells (Vanderhyden *et al.* 1992; Gilchrist *et al.* 2001) and of the follicle, but also regulate differentiation processes such as steroidogenesis and physical remodelling of the follicle (Vanderhyden 1993; Eppig *et al.* 2002). The primary recipients of oocyte paracrine signalling are cumulus cells. Cumulus cells have a distinct phenotype from mural granulosa cells (which line the wall of the follicle), and indeed, maintenance of the cumulus cell phenotype is dependent on oocyte secretions (Eppig *et al.* 1997; Li *et al.* 2000). Oocyte paracrine signalling is therefore essential for normal cumulus cell function, as well as for maintaining a functional morphogenic gradient across the ovarian follicle.

Since the oocyte regulates such a broad range of cumulus cell functions, we hypothesised that oocyte paracrine secreted factors would modulate cumulus cell metabolism. As the capacity of the oocyte to utilise glucose is positively correlated to

developmental potential, it may be possible to use the metabolic parameters of the cumulus complex as a predictive marker of oocyte quality. The aim of this study was to determine whether oocyte-secreted factors influence cumulus-oocyte complex metabolism.

## **Material and methods**

Unless specified, all chemicals and reagents were purchased from Sigma, St Louis, MO, USA.

### *Oocyte collection and culture*

Bovine ovaries were collected from a local abattoir and transported to the laboratory in warm (30–35°C) saline. Follicles, 3–8 mm in size and of non-atretic appearance (Yang and Rajamahendran, 2000), were aspirated using an 18-gauge needle attached to a 10 ml syringe and collected in aspiration media (HEPES-buffered Tissue Culture Medium, TCM199, ICN Biochemicals, CA, USA, supplemented with 0.5 mM sodium pyruvate and 50 µg/ml heparin). Complexes from all aspirated oocytes with smooth, ungranulated ooplasms and intact, compact cumulus vestments greater than five cell layers were selected and washed twice in HEPES-buffered TCM199. COCs were randomly divided into four treatment groups ( $n = 36$  per treatment), intact COCs, oocytectomised complexes (OOX), OOX co-cultured with denuded oocytes (OOX + DO) and DO. Six replicate experiments were performed. OOX were produced by micro-surgical removal of the oocyte, leaving the cumulus cell complex intact (Buccione *et al.* 1990b), and DO were generated by removing cumulus cells from COCs by vortexing. COC, OOX, OOX + 5 DO and 5 DO were then washed in maturation media and cultured individually in pre-equilibrated 10 µl drops of maturation media (bicarbonate-buffered TCM199 supplemented with 0.5 mM sodium pyruvate, 4 mg/ml BSA, ICPbio Ltd, Auckland, New Zealand; 0.1 IU/ml hCG, Pregnyl, Organon, Netherlands and 0.1 IU/ml FSH, Puregon, Organon) overlaid with mineral oil and incubated at 39°C with 6% CO<sub>2</sub> in humidified air. Culturing OOX together with 5 DO in a 10 µl drop gives a concentration of 0.5 oocyte/µl which is within the typical range used to examine oocyte effects on follicular cells (Gilchrist *et al.* 2001).

### *Oxygen consumption assay*

The oxygen consumption of COC, OOX, OOX+DO and DO was assayed from 0-4 h, 10-14 h or 20-24 h of culture. The protocol for the oxygen assay has been described by (Houghton *et al.* 1996) and is based on the fluorescent properties of pyrene; an oil soluble compound that is excited at 340 nm and its fluorescence dissipates in a linear fashion in the presence of increasing oxygen concentrations. Oxygen assay chambers were constructed using 5 µl polymerase chain reaction (PCR) micropipettes (Drummond Scientific Company, Broomall, PA, USA) and a stainless steel plunger. One µl of 1 mM pyrene, dissolved in mineral oil, was drawn into the micropipettes, followed by 2 µl of HEPES-buffered maturation media containing a single COC, OOX, OOX + 1 DO or 1 DO. An airtight seal was made at the open end of the chamber and the plunger was fixed using sealing wax. Negative control (0% O<sub>2</sub>) chambers were constructed in a similar manner but with 1 mM oxyrase (Oxyrase Inc, Mansfield, OH, USA) in 60 mM glucose (equilibrated overnight to remove O<sub>2</sub>) replacing the media and positive controls (20% O<sub>2</sub>) were constructed with media alone. The fluorescence emission of pyrene at the pyrene-media and the pyrene-plunger interfaces were measured using a fluorophotometric-inverted microscope (Leica, Wetzlar, Germany). Half hourly measurements were taken over a 4 h period. In between measurements, the chambers were maintained at 39°C. Oxygen consumption by each complex was determined using a computer program that describes the movement of oxygen from the pyrene-oil into the media as oxygen is consumed by the complex (Houghton *et al.* 1996). At the completion of the assay, the chambers were dismantled and the complexes and spent media were stored in separate 96-well plates (Falcon) overlaid with mineral oil at -80°C.

### *DNA quantification*

The DNA content of individual complexes or oocytes collected at the completion of the oxygen assay was quantified using PicoGreen dye (Molecular Probes, Eugene, OR, USA), a fluorescent nucleic acid dye that has a high affinity for double stranded DNA compared to single stranded DNA and other nucleic acids. Extraction and preparation of the samples and quantification of the DNA were performed entirely in

96-well plates. Total DNA was extracted from complexes by adding 50 µl of lysis buffer (50 mM Tris-HCl, 1 mM EDTA (pH 7.6), supplemented with 500 µg/ml proteinase K) and incubating for 3.5 h at 50°C and then 10 mins at 80°C. Lysates were then treated with 50 µl of 10 µg/ml DNase-free RNase (Roche Diagnostics, Basel, Switzerland) and incubated for 30 mins at 40°C. Excess liquid was evaporated from the wells and 100 µl of Tris-EDTA buffer was added to each well. PicoGreen was prepared according to the manufacturer's instructions and added to each sample. Fluorescence was measured using a FLUOstar Galaxy microplate reader and software (BMG Labtechnologies Pty Ltd, Offenburg, Germany) with excitation set at 485 nm and emission at 520 nm. Standard curves (lambda DNA) were used to determine the DNA content of the samples. The interassay CV was <3%. RNase treatment does not affect DNA concentration or contribute to background fluorescence.

#### *Metabolism assays*

Glucose, L-lactate and pyruvate consumption and/or production during the oxygen assay were determined by measuring the concentrations of each of the substrates in the spent media using microfluorometric assays (Leese and Barton 1984; Gardner and Leese 1986). All of the metabolic measurements were expressed per ng of DNA to account for the variable number of cumulus cells within each complex.

#### *Statistical analyses*

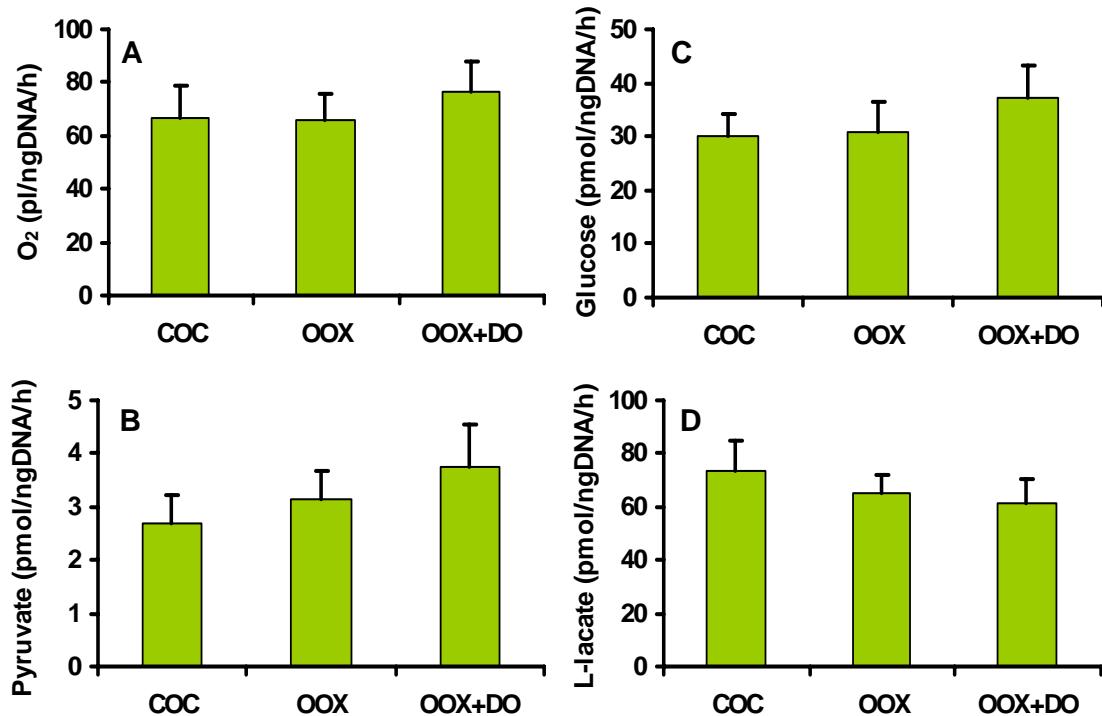
Comparisons of the means of metabolic parameters were performed using two-way analysis of variance (ANOVA) for the three treatments (COC, OOX and OOX + DO) and the three time points during maturation. As there were no interactions between time of maturation and treatment group, main effects of time of maturation and treatment are presented. The relationship between glucose uptake and L-lactate production was tested by linear regression analyses. All of the statistical analyses were performed using SigmaStat version 2.0 computer software (SPSS Inc, Chicago, IL, USA).

## Results

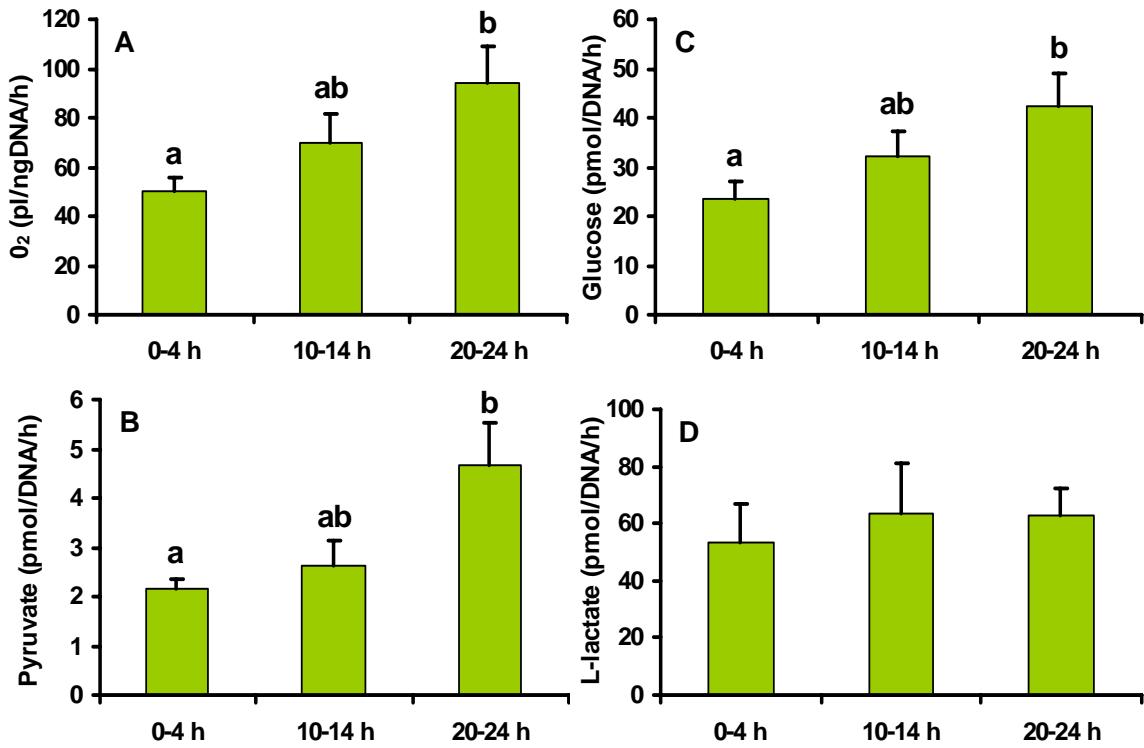
To investigate the relationship between oocyte secreted factors and the metabolic activity of bovine cumulus cell complexes throughout IVM, the utilisation of oxygen, pyruvate, glucose and L-lactate by intact COCs, oocytectomised cumulus cell complexes (OOX) and OOX co-cultured with denuded oocytes (OOX + DO) was compared from 0-4 h, 10-14 h or 20-24 h of culture. The metabolism of DO was also measured but was undetectable for all of the metabolites measured (data not shown). There were no differences in the consumption of oxygen, pyruvate or glucose or L-lactate production between intact COCs, OOX or OOX + DO (Figure 1). This demonstrates that oocyte secreted factors do not alter cumulus cell metabolic activity.

At 20-24 h, the consumption of oxygen (Figure 2A;  $94.0 \pm 14.9$  pl/ng DNA/h), pyruvate (Figure 2B;  $4.69 \pm 0.85$  pmol/ng DNA/h) and glucose (Figure 2C;  $42.4 \pm 6.38$  pmol/ng DNA/h) was significantly greater than consumption at 0-4 h (oxygen:  $50.2 \pm 5.64$  pl/ng DNA/h,  $P = 0.026$ ; pyruvate:  $2.15 \pm 0.22$  pmol/ng DNA/h,  $P < 0.05$ ; glucose:  $23.5 \pm 3.60$  pmol/ng DNA/h,  $P < 0.05$ ) for oxygen, pyruvate and glucose, respectively). When present at the concentrations utilised during this study (5.56 mM and 0.23 mM for glucose and pyruvate, respectively) glucose was the preferred substrate throughout the entire culture period, with several folds more glucose being taken up compared to pyruvate. L-lactate was the only metabolite measured in which a net production into the medium was observed and levels of production remained constant over the entire 24 h culture period (Figure 2D,  $P > 0.05$ ).

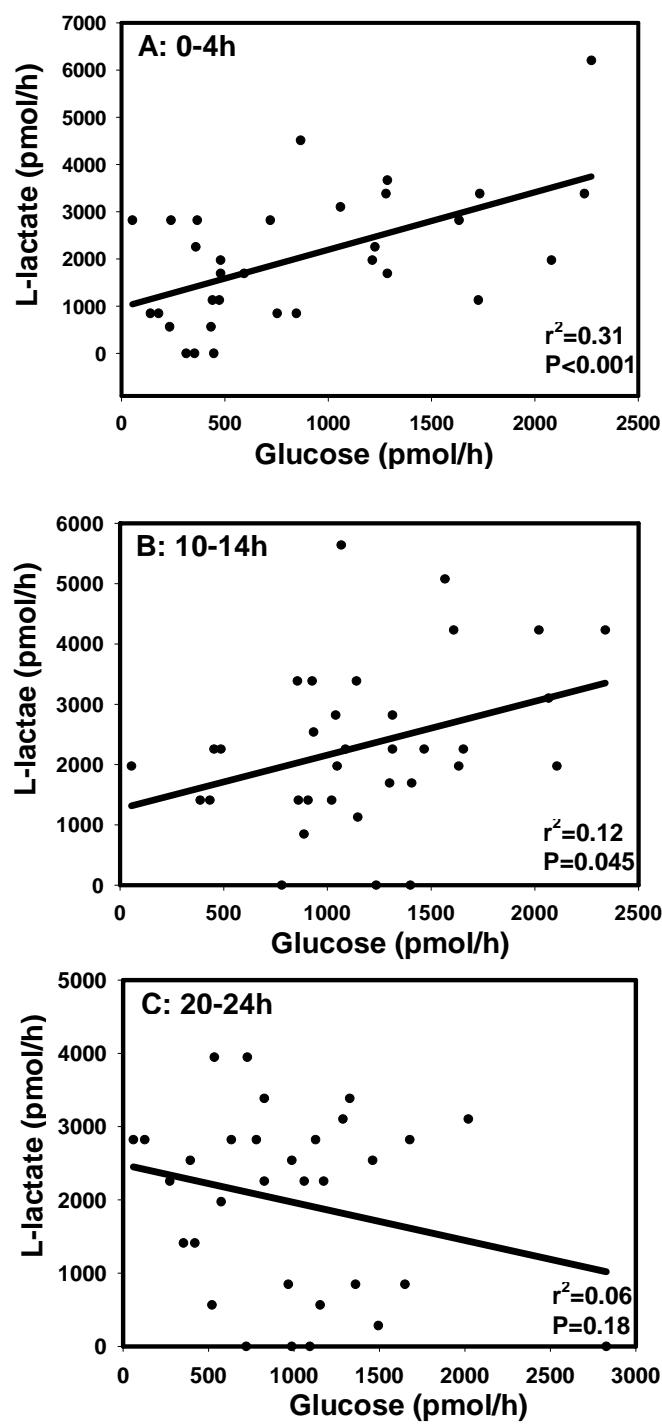
L-lactate produced by COCs could have been metabolised from either glucose or pyruvate. However, as considerable more glucose was consumed by COCs compared to pyruvate, it was realistic to assume that the majority of L-lactate produced was derived from glucose. For every molecule of glucose consumed, 2 molecules of L-lactate are produced via the glycolytic pathway. The relationship between total glucose uptake and L-lactate production was examined at each time point to estimate the proportion of glucose being utilised for lactate production. At the beginning of



**Figure 1.** The influence of oocyte-secreted factors on the metabolism of bovine cumulus cell complexes was determined by measuring the consumption of A) oxygen, B) pyruvate and C) glucose and D) the production of L-lactate by intact cumulus oocyte complexes (COC), oocytectomised complexes (OOX) and OOX co-cultured with denuded oocytes (OOX + DO). Data is expressed per ng of DNA and each bar represents the pooled means  $\pm$  SEM. There were no significant differences between any of the treatments for all the metabolites measured, regardless of culture time ( $P > 0.05$ ).



**Figure 2.** The utilisation of metabolites by bovine cumulus cell complexes was measured at three points during IVM. The consumption of A) oxygen, B) pyruvate and C) glucose and D) the production of L-lactate were measured. Data is expressed per ng of DNA and each bar represents means  $\pm$  SEM. <sup>ab</sup> Different superscripts indicate significant differences ( $P < 0.05$ ).



**Figure 3.** The relationship between total glucose consumption and L-lactate production by bovine cumulus cell complexes via the glycolytic pathway was examined using linear regression analyses after A) 0-4 h, B) 10-14 h or C) 20-24 h of culture. Points represent individual complexes.

the IVM period, the relationship between glucose and L-lactate was highly significant (Figure 3A;  $P < 0.001$ ,  $r^2 = 0.31$ ), indicating that a large proportion of the consumed glucose was converted to L-lactate at linear rates. At 10-14 h, this relationship still existed (Figure 3B;  $P = 0.045$ ,  $r^2 = 0.12$ ), albeit not as pronounced as the relationship at the start of IVM. At 20-24 h, there was no longer a relationship between the rate of glucose consumption and L-lactate production (Figure 3C;  $P = 0.178$ ,  $r^2 = 0.06$ ). This, coupled with the constant L-lactate production over time, indicates that although a substantial proportion of total glucose utilisation occurred via glycolysis, increasingly less glucose could be accounted for by L-lactate production, suggesting an alternative fate.

In concurrent studies, the IVM system utilised here resulted in blastocyst production rates  $>40\%$  (data not shown) demonstrating that oocytes derived from this system behave typically of bovine in vitro matured oocytes in general.

## Discussion

To date, apart from morphological features, there are no non-invasive methods that can be applied prior to in vitro fertilization and in vitro embryo culture to predict the developmental potential of an oocyte. The aim of this study was to determine whether oocyte-secreted factors (the activity of which may indicate oocyte “health”) influence the metabolism of oxygen, pyruvate, glucose and L-lactate by bovine cumulus cell complexes, as well as to create a metabolic profile of COC metabolism throughout IVM. This study has demonstrated that the measured metabolic parameters are not affected by the presence of the oocyte. Cumulus complexes that were in physical contact with the oocyte, as well as complexes co-cultured with oocytes had metabolic activities indistinguishable to complexes in which the oocyte had been removed (OOX). Furthermore, this was the case at each of the time points measured throughout the IVM period. Oocyte-secreted factors operate in a strictly concentration dependent manner (Buccione *et al.* 1990b; Lanuza *et al.* 1998; Gilchrist *et al.* 2001), and it is most unlikely that the lack of cumulus cell response to co-culture with oocytes was due to insufficient oocyte factor(s), as previously we have shown that OOX complexes co-cultured with DO at a density of 0.5 oocytes/ $\mu\text{l}$  is sufficient to elicit a response in cumulus cells (Li *et al.* 2000; Gilchrist *et al.* 2001).

Furthermore, the lack of difference between treatment groups is unlikely to be due to the insensitivity of the metabolic assays, since these techniques have been utilised to demonstrate significant differences in the metabolic activity of embryos (Leese and Barton 1984; Houghton *et al.* 1996; Thompson *et al.* 1996).

It is perhaps surprising that oocytes do not seem to regulate cumulus cell metabolism, given that in general, the oocyte plays a role in the regulation of most cumulus cell functions that have been examined to date (Eppig 2001). There is however precedents for cumulus cell activities that are independent of oocyte regulation. Most notably, FSH stimulated expansion of porcine and bovine COC occurs independently of the oocyte (Prochazka *et al.* 1991; Singh *et al.* 1993; Ralph *et al.* 1995; Nagyova *et al.* 1999). However, this is a species-specific phenomenon as rodent COC mucification is absolutely dependent on the presence of the oocyte (Buccione *et al.* 1990b; Salustri *et al.* 1990). In addition, it has been demonstrated that pig oocytes secrete factors that enable the expansion of both rat and mice cumulus cells (Vanderhyden 1993; Nagyova *et al.* 2000).

The observation that cumulus cell metabolic activity is independent of the oocyte is in contrast to previous studies that demonstrated that LH increased glycolytic activity in intact bovine COC but not in OOX (Zuelke and Brackett 1992). However, numerous differences exist between the two studies. The source of LH differed; our IVM media contained hCG whereas Zuelke et al (Zuelke and Brackett 1992) utilised bovine LH purified from pituitary extracts. Also we measured total glucose uptake throughout IVM, compared to the activities of specific pathways at the end of IVM (Zuelke and Brackett 1992). We demonstrated that by 24 h of culture, a large proportion of glucose consumed was not being utilised via glycolysis. It is possible that only the specific glycolytic pathway that was examined in the previous study is affected by oocyte-secreted factors.

The fact that cumulus cell metabolic activities were independent of oocyte-secreted factors, suggests that the non-invasive measurements of substrate consumption or production by COCs used in this study cannot be used as a diagnostic for the biochemical activity (i.e. health) of an oocyte. This suggests that acquisition of developmental competence of bovine oocytes may also not be determined by such

factors. This is also the case in feline oocytes, where increased glucose metabolism is positively correlated with developmental potential; whereas the metabolism of cumulus cells removed prior to fertilization was not indicative of the developmental potential of the oocyte they originated from (Spindler *et al.* 2000). It is plausible that features of the *in vivo* environment such as follicle size, classification (i.e. subordinate or dominant), health or oestrous stage of the donor at collection have a greater influence on developmental potential, as these factors have previously been associated with improved embryo development (Blondin and Sirard 1995; Hagemann 1999; Hagemann *et al.* 1999).

The metabolic activity of the COC as a whole has not been widely characterised to date, with most studies focusing on the utilisation of substrates by the oocyte itself. Since the presence of the cumulus cell vestment is essential to oocyte maturation, we investigated the metabolism of the intact COC throughout IVM as opposed to the denuded oocyte alone. Although the oocyte does not have an effect on the metabolism of the complex, the increased requirement for glucose during the maturation period is in agreement with the reported metabolic profile of the denuded oocyte (Rieger and Loskutoff 1994; Steeves and Gardner 1999b). Pyruvate consumption by COCs is also positively related to nuclear maturation (Downs *et al.* 2002); hence increased culture time leads to higher requirements for pyruvate by the oocyte. Glycolysis was the predominant pathway for glucose utilisation, since L-lactate production accounted for the majority of the glucose consumed. The level of oxygen uptake (which is indicative of oxidative phosphorylation) can be accounted for by the uptake of pyruvate (and subsequent utilisation through the tricarboxylic acid cycle).

As glucose was the preferred energy substrate and COCs consumed only a small amount of pyruvate, it was assumed that the majority of L-lactate production was via the glycolytic metabolic pathway. The constant rate of L-lactate production throughout IVM suggests that the proportion of consumed glucose that was being utilised for ATP production via glycolysis for the COC did not change over the 24 h of culture. This is in agreement with the observation that phosphofructokinase activity (the rate limiting enzyme of glycolysis) remains constant throughout oocyte maturation (Cetica *et al.* 2002). There are numerous pathways through which glucose

can be utilised, and it is possible that during different stages of oocyte maturation, there are differing requirements for the substrates that were measured. Previous studies have demonstrated that the pentose phosphate pathway is active throughout maturation and there is a positive relationship between increased glucose flux through this pathway and the induction of meiosis (Downs *et al.* 1998). It is possible that towards the end of IVM the flux of glucose through the pentose phosphate pathway increases to allow for increased production of substrates involved in nuclear maturation. An additional route of glucose utilisation is the conversion of glucose to extra-cellular matrix components. A large component of cumulus expansion is the increased synthesis of extra-cellular matrix, which is not only an energy expensive process but also requires the synthesis of matrix components. Glucose metabolised by cumulus cells can be utilised via the glycolytic pathway for energy production, but may also be used for the synthesis of glucosamine, a substrate for hyaluronic acid (Chen *et al.* 1993), a major component of cumulus matrix. These pathways are most likely linked, since the addition of FSH has previously been associated with increased glucose consumption (Downs and Utecht 1999) and mucification (Salustri *et al.* 1989). Mucification occurs in the latter part of maturation and is important in vivo for post-ovulatory events (Chen *et al.* 1993). Therefore it is more likely that the increased glucose uptake towards the end of IVM observed in this study, is attributed to an increased extra-cellular matrix formation, particularly since pentose phosphate pathway activity has been shown to be low compared to glycolytic activity (Urner and Sakkas 1999). Investigations are currently in progress to further analyse the role of glucose in cumulus matrix formation in the COC.

In conclusion, we have demonstrated that the metabolism of oxygen, pyruvate, glucose and L-lactate by bovine cumulus cell complexes is not affected by oocyte-secreted factors, hence non-invasive measurements of the utilisation of these substrates by COCs is unlikely to represent biochemical activities of the oocyte associated with developmental competence. This study has also demonstrated that the metabolic requirements of the COC increased through IVM. It is possible that further investigation of the metabolism of the COC as a unit may aid in the optimisation of culture conditions during IVM.

## Acknowledgements

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# **Chapter 3**

## **Cumulus-expansion and glucose utilisation by bovine cumulus-oocyte complexes during in vitro maturation: the influence of glucosamine and follicle stimulating hormone**

Melanie L. Sutton-McDowall, Robert B. Gilchrist and Jeremy G. Thompson

Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, University of Adelaide, The Queen Elizabeth Hospital, Woodville Road, Woodville 5011, South Australia.

## **Abstract**

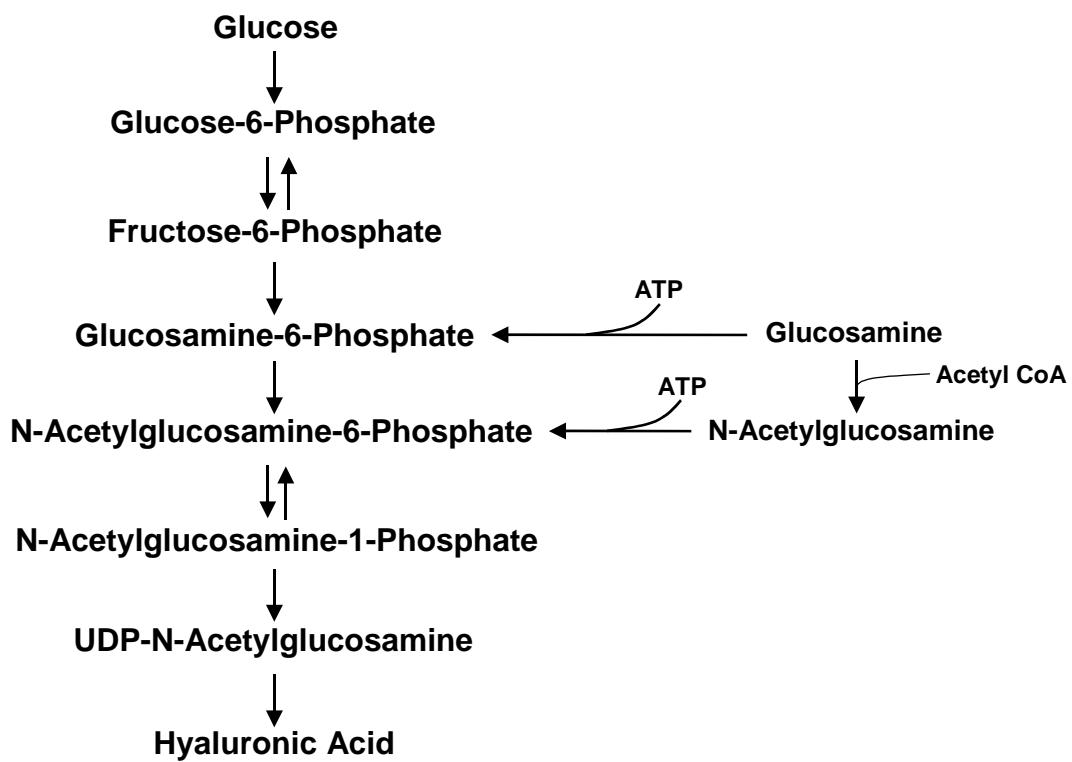
Glucose is an important metabolite and its presence during in vitro oocyte maturation (IVM) can have profound effects on the oocyte's developmental capacity. We have demonstrated that glucose uptake increases over a 24 h IVM period, with most accounted for as L-lactate production. However, as maturation proceeds, L-lactate production remained constant, suggesting an alternative role for glucose metabolism. We hypothesised that in latter stages of oocyte maturation, glucose not accounted for by L-lactate production is utilized for FSH-stimulated extracellular matrix (ECM) synthesis. To examine precursor utilization for synthesis of ECM, bovine cumulus-oocyte complexes (COCs) were matured in ± FSH and/or glucosamine (an alternative substrate of matrix components). Measurements included COC diameters, glucose consumption and L-lactate production in spent media and [ $^{14}\text{C}$ ] glucose incorporation into ECM. FSH significantly stimulated both diameter and glucose consumption during 20-24 h maturation compared to unstimulated complexes, although co-incubation with glucosamine and FSH decreased total glucose consumption 1.7-fold compared to FSH alone ( $P < 0.05$ ). Furthermore there was a linear relationship between glucose and L-lactate metabolism in the presence of glucosamine, suggesting that the majority of glucose was being utilized for L-lactate production via glycolysis. In the presence of glucosamine, 2-fold less [ $^{14}\text{C}$ ] glucose was incorporated into matrix compared to COCs cultured without glucosamine. These results support the hypothesis that there is a link between glucose and glucosamine uptake in FSH-stimulated ECM synthesis. Furthermore glucose has multiple fates within the COC during maturation and levels of utilization are dependent on the composition of the maturation environment.

## **Introduction**

Within the follicle there are several different somatic cell phenotypes that surround the oocyte. It is now widely recognised that bi-directional communication between the oocyte and follicular somatic cells is fundamentally important for folliculogenesis and oocyte growth and maturation (Eppig 2001). The morphology of the cumulus vestment surrounding an oocyte is commonly used as selection criteria prior to in vitro maturation (IVM; Shioya *et al.* 1988; Madison *et al.* 1992; Lonergan *et al.* 1994; Goud *et al.* 1998) and the degree of expansion can be used as a morphological indicator of oocyte quality following maturation. It is contentious as to whether cumulus expansion is directly related to developmental capacity of the oocyte (Ali and Sirard 2002; Luciano *et al.* 2004), although culture conditions that promote improved IVM generally also promote cumulus expansion, such as FSH and macromolecule supplementation (Eyestone and de Boer 1993; Choi *et al.* 2001). Furthermore, the presence of the cumulus vestment increases fertilization rates compared to denuded oocytes (Tanghe *et al.* 2003).

The synthesis of extracellular matrix (ECM) by cumulus cells in the latter stages of oocyte maturation leads to mucification and cumulus expansion (Buccione *et al.* 1990). Injection of mice with radio-labelled substrates of ECM (namely [<sup>3</sup>H] glucosamine) post-hCG administration leads to rapid incorporation into the cumulus vestment compared to lower incorporation rates in cumulus oocyte complexes (COCs) arrested at meiosis I (Fowler 1988). The major constituent and structural bone back of the ECM is hyaluronic acid, which can be synthesised from numerous compounds, including glucosamine and glucose (Figure 1; [Salustri, 1989 #282; Chen, 1990 #280].

Cumulus cells play a very important role in the utilization of energy substrates during IVM (reviewed by Sutton *et al.* 2003). In particular, the oocyte itself appears to lack the capacity for significant glucose uptake and utilization (Biggers *et al.* 1967; Rieger and Loskutoff 1994; Saito *et al.* 1994; Dan-Goor *et al.* 1997). Thus a major fate of glucose consumed by cumulus cells is its conversion to pyruvate or L-lactate



**Figure 1.** Hyaluronic acid synthesis from glucose and glucosamine during FSH-stimulated cumulus expansion.

via glycolysis, substrates that the oocyte can readily oxidise for energy in the form of ATP (Downs and Utecht 1999; Khurana and Niemann 2000a, 2000b; Cetica *et al.* 2002). The metabolism of glucose also plays an important role in numerous other events throughout maturation. For example, metabolism of glucose through the pentose phosphate pathway plays a role in the regulation of meiotic progression through the production of substrates for purine nucleotide synthesis (Downs *et al.* 1998; Downs and Hudson 2000). Additional products such as NADPH are involved in cytoplasmic integrity and pronuclear formation proceeding fertilization (Urner and Sakkas 1999). Indeed the presence and concentration of energy substrates, especially glucose significantly affects oocyte developmental capacity (Krisher and Bavister 1998).

FSH is an important stimulator of ECM synthesis (Salustri *et al.* 1989) as well as glucose consumption (Downs *et al.* 1996) by COCs, therefore it is plausible that glucose could be converted to glucosamine and utilized for ECM synthesis and cumulus expansion. We have previously demonstrated that as IVM of bovine COCs proceeds, glucose consumption increases but L-lactate production remained constant, suggesting an alternative fate for a proportion of the glucose consumed (Sutton *et al.* 2003). We hypothesise that during the course of IVM, glucose is increasingly utilized for matrix formation during FSH-stimulated mucification.

The aim of this study is to ascertain whether an increasing proportion of glucose during IVM is converted to matrix components by supplementing culture media with glucosamine. A further objective is to further characterise the influence of FSH on glucose utilization during bovine IVM.

## Methods and materials

Unless specified, all chemicals and reagent were purchased from Sigma, St Louis, MO, USA.

### *Oocyte collection*

Bovine ovaries were collected from a local abattoir and transported to the laboratory in warm (30-35°C) saline. Follicles of non-atretic appearance (Yang and Rajamahendran 2000) and between 3-8 mm in diameter were aspirated using an 18-gauge needle and 10 ml syringe containing ~1 ml of Handling media (HEPES buffered tissue culture medium 199; TCM199, ICN Biochemicals, CA, USA, supplemented with 0.5 mM sodium pyruvate and 4 mg/ml fatty acid free BSA; ICPbio Ltd, Auckland, NZ). Intact COCs with compact cumulus vestments greater than three cell layers and ungranulated ooplasts were collected, washed twice in Handling media and once in the corresponding maturation media.

### *Experiment 1: The influence of FSH and glucosamine on glucose utilization*

To determine the effect of glucosamine on glucose uptake, COCs were randomly divided into four treatment groups; 1) Control medium (bicarbonate-buffered TCM199 supplemented with 0.5 mM sodium pyruvate and 4 mg/ml fatty acid free BSA), 2) + FSH (control medium supplemented with 0.1 IU/ml FSH; Puregon, Organon, Oss, Netherlands), 3) + Glc (control medium plus 5 mM glucosamine) and 4) + FSH + Glc (control medium plus 0.1 IU/ml FSH and 5 mM glucosamine). COCs were cultured individually in 10 µl drops of corresponding media, overlaid with mineral oil and incubated at 39°C with 6% CO<sub>2</sub> in humidified air. The concentration of glucosamine used has previously been shown to be an adequate concentration for cumulus expansion to occur in mouse, rat and sheep COCs [Chen, 1990 #280].

At 0 h or after 20 h of culture, COCs were transferred into fresh media and cultured individually in 2 µl droplets for a further 4 h. At the completion of culture, the horizontal and vertical diameters were measured using a dissecting microscope and ocular graticle. COCs and spent media were stored individually in 96-well plates for DNA quantification and metabolite measurements, respectively. Three experimental replicates were performed and a total of 15 COCs were assayed in each replicate for each treatment and time point.

*Experiment 2: [ $U\text{-}^{14}\text{C}$ ] Glucose incorporation into extracellular matrix (ECM) during cumulus expansion*

To determine whether exogenous glucose is converted to matrix components, COCs were cultured individually in 10  $\mu\text{l}$  pre-equilibrated drops of 1) Control medium (-Glc; bicarbonate buffered TCM199 supplemented with 0.5 mM sodium pyruvate, 4 mg/ml fatty acid free BSA, 0.1 IU/ml FSH, 0.1 IU/ml hCG) or 2) + Glc (control medium supplemented with 5 mM glucosamine). Drops were overlaid with mineral oil and cultured at 39°C with 6%  $\text{CO}_2$  in humidified air. After 20 h of culture, COCs were washed and transferred into - Glc or + Glc media containing 250 Bq/ $\mu\text{l}$  [ $U\text{-}^{14}\text{C}$ ] glucose (specific activity = 111 MBq/mmol; Amersham Bioscience, Buckinghamshire, England). COCs were cultured individually in 10  $\mu\text{l}$  drops overlaid with mineral oil for a further 4 h, after which COCs were washed and cultured in 10  $\mu\text{l}$  drops of Handling medium for 1 h. COCs were washed in Handling medium and within both groups, half of the COCs were transferred into 20  $\mu\text{l}$  of Handling medium and the remaining half into 20  $\mu\text{l}$  of Handling medium supplemented with 50 IU/ml hyaluronidase. COCs were vortexed and centrifuged to sediment the cells. Aliquots (15  $\mu\text{l}$ ) of the supernatant were added to Eppendorf tubes containing 1 ml of scintillant (Opti Phase Supermix cocktail, EG&G Wallac, Turku, Finland) and  $^{14}\text{C}$  radioactivity was assessed using a Microbeta Trilux counter (EG&G Wallac). Groups not exposed to hyaluronidase served as controls, whereas hyaluronidase treatment enabled the release of soluble [ $U\text{-}^{14}\text{C}$ ] glucose that was incorporated into the matrix, hence the proportion incorporated during culture could be determined. A total of 20 COCs were cultured per replicate and five COCs pooled per group. Three replicate experiments were performed.

*Experiment 3: Glucose depletion from IVM medium*

To assess the impact of IVM on glucose concentrations, a model for glucose depletion under standard culture conditions (i.e. control medium: TCM199 supplemented with 4 mg/ml fatty acid free BSA, 0.5 mM sodium pyruvate, 0.1 IU/ml FSH and 0.1 IU/ml hCG) was created using Microsoft Excel version 7.0 software (Microsoft Corporation, Redmond, WA, USA), based on 1) a rate of glucose consumption determined previously (Sutton *et al.* 2003); 2) initial glucose

concentrations and 3) media density. The validity of the model was tested by culturing groups of 10 COCs in either 50 µl (5 µl per COC) or 100 µl (10 µl per COC) of pre-equilibrated control medium or control medium supplemented with 5 mM glucosamine (+ Glc). Culture drops were overlaid with mineral oil and were cultured at 39°C in 6% CO<sub>2</sub> in humidified air. At 0 h, 18 h and 24 h; 2 µl aliquots of media from the culture droplets were collected and stored in individual wells of a 96-well plate overlaid with mineral oil at -80°C. Glucose and L-lactate concentrations were measured as described below, to determine treatment effects on depletion of glucose and production of L-lactate. The experiment was replicated three times with each treatment being duplicated within each replicate.

#### *Glucose and L-lactate assays*

The total uptake of glucose and the production of L-lactate in the spent media were determined using microfluorometric assays that are based on measuring the production of NADH or NADPH at 340 nm using a fluorophotometric-inverted microscope (Leese and Barton 1984). The fluorescence intensity of the spent media was then converted to substrate concentrations using standard curves that were conducted simultaneously.

#### *DNA assay*

The DNA content of individual COCs was quantified using PicoGreen dye (Molecular Probes, Eugene, OR, USA) to allow metabolic measurements to be expressed per ng DNA, hence accounting for variable cell numbers between complexes, as previously described (Sutton *et al.* 2003).

#### *Statistical analyses*

Mean diameters, glucose consumption, L-lactate production and [U-<sup>14</sup>C] glucose counts were examined for treatment and time effects using two-way analysis of variance (ANOVA), followed by all pair-wise multiple comparison procedures (Tukey test). The relationship between glucose consumption and L-lactate production for all treatments was tested using linear regression analysis. All

statistical tests were performed using SigmaStat version 2.0 computer software (SPSS Inc, Chicago, IL, USA).

## Results

### *Experiment 1. The influence of FSH and glucosamine on glucose utilization*

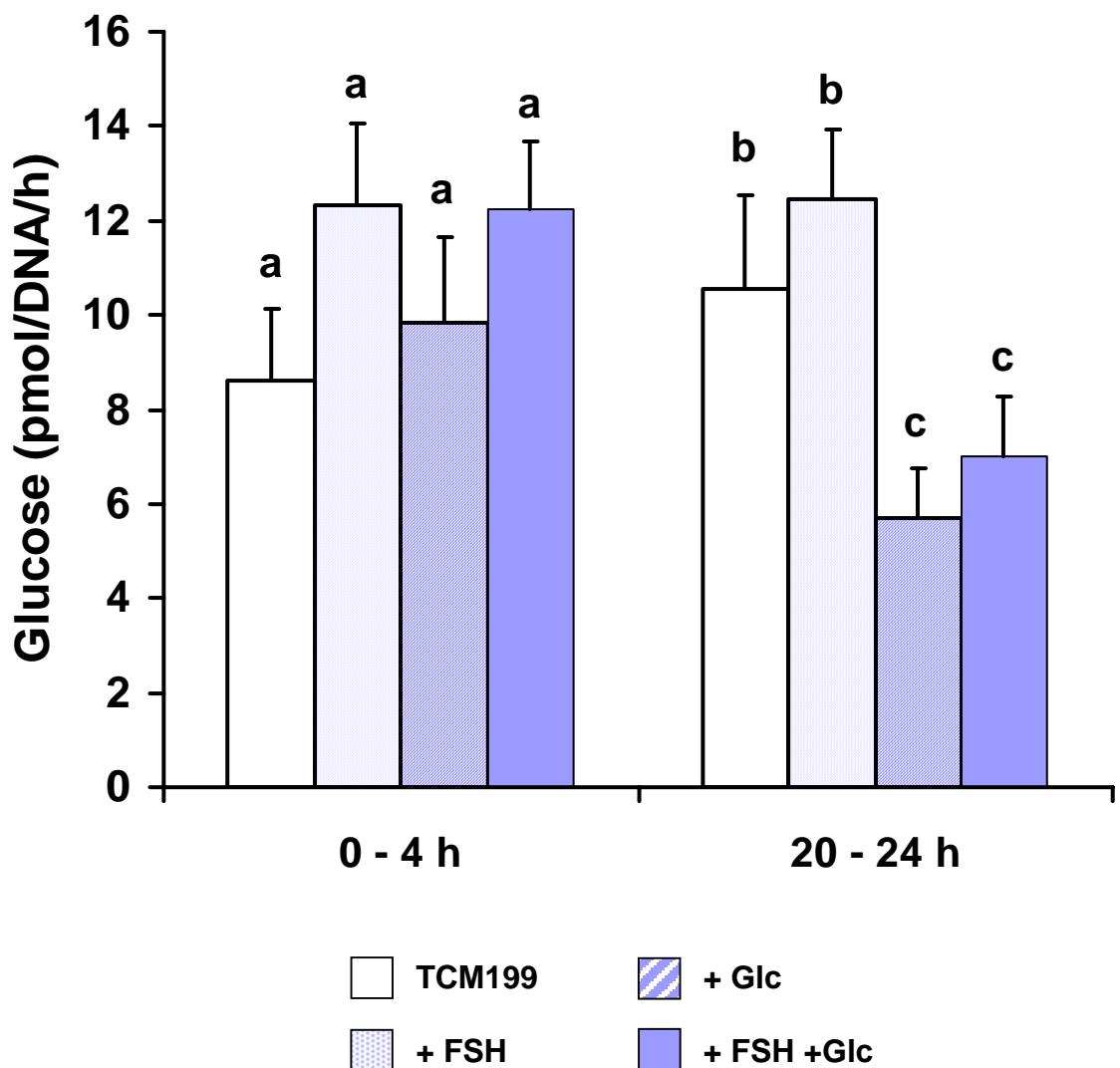
COCs were cultured with FSH, a stimulator of cumulus expansion and/or glucosamine, a substrate for hyaluronic acid, and their influence on glucose metabolism was determined. At time 0-4 h of culture, the diameters of the COCs were not affected by any of the treatments and were constant across all the groups (Table 1). Following 24 h of culture, the stimulatory effect of FSH was evident and there was a significant increase in COC diameter (+ FSH =  $525.7 \pm 55.7 \mu\text{m}$  and + FSH + Glc =  $463.7 \pm 26.2 \mu\text{m}$ ) compared to the control and COCs cultured with glucosamine alone (control =  $341 \pm 11.6 \mu\text{m}$  and + Glc =  $315.3 \pm 12.3 \mu\text{m}$ ;  $P < 0.05$ ), where there was minimal cumulus expansion.

**Table 1.** The diameters of cumulus oocyte complexes (COCs) at 4 h and 24 h cultured in Tissue Culture Medium 199 (TCM199, Control)  $\pm$  FSH and/or  $\pm$  glucosamine ( $\pm$  Glc).

Time (h)	Culture				Diameter ( $\mu\text{m}$ )
	Control	+ FSH	+ Glc	+ FSH + Glc	
0-4 h	$316.9 \pm 13.8^{\text{a}}$	$310.9 \pm 12.3^{\text{a}}$	$295.4 \pm 6.12^{\text{a}}$	$314.2 \pm 12.3^{\text{a}}$	
20-24 h	$341.0 \pm 11.6^{\text{b}}$	$525.7 \pm 55.7^{\text{c}}$	$315.3 \pm 12.3^{\text{b}}$	$463.7 \pm 26.2^{\text{c}}$	

Values are means  $\pm$  SEM. <sup>a</sup> and <sup>bc</sup> Means with different superscripts within the same time are significantly different ( $P < 0.05$ ).

There were no significant differences between treatment groups in glucose consumption by COCs during 0-4 h culture (Figure 2;  $P > 0.05$ ). Between 20-24 h culture, the presence of glucosamine in the medium significantly decreased the consumption of glucose by COCs regardless of the presence or absence of FSH



**Figure 2.** Glucose uptake by cumulus oocyte complexes (COCs) from 0-4 h or 20-24 h of culture in Tissue Culture Medium 199 (TCM199), +FSH, + glucosamine (+ Glc) or + FSH + Glc. Data bars represent means  $\pm$  SEM. <sup>a</sup> and <sup>bc</sup> Means within a time point with different superscripts are significantly different ( $P < 0.05$ ).

(main effects: - Glc =  $11.5 \pm 1.2$  pmol/ngDNA/h vs. + Glc =  $6.38 \pm 0.8$  pmol/ngDNA/h,  $P < 0.05$ ).

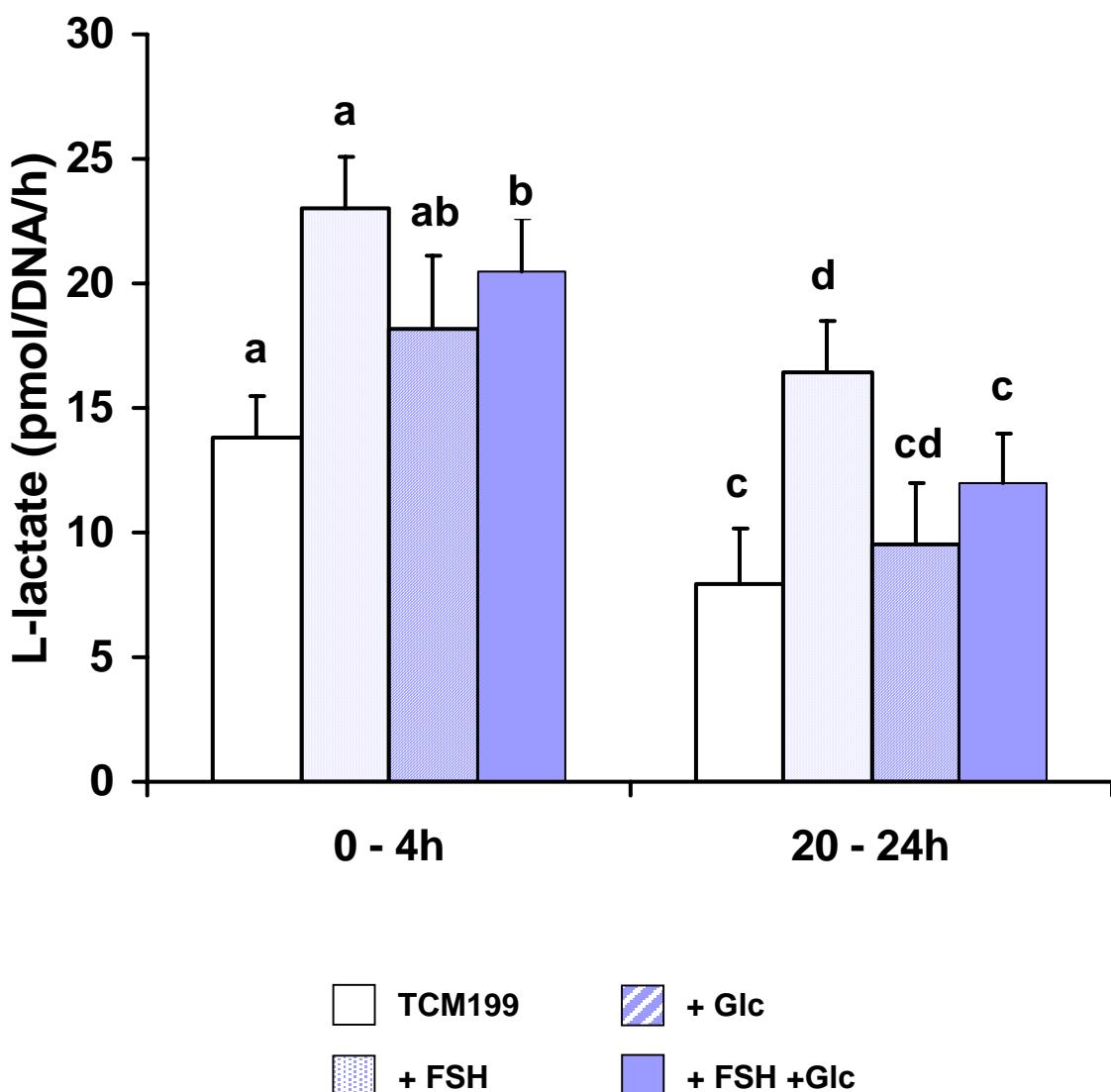
Significantly more L-lactate was produced by COCs in the presence of FSH compared to unstimulated complexes at both time points (Figure 3). Over the 20-24 h culture period there was a significant interaction between glucosamine and FSH (Figure 3;  $P < 0.05$ ). In the absence of glucosamine, FSH caused a significant increase in L-lactate production relative to the control whereas in the presence of glucosamine, no differences in L-lactate production were observed in the presence or absence of FSH.

*Experiment 2: [ $U-^{14}C$ ] Glucose incorporation into extracellular matrix (ECM) during cumulus expansion*

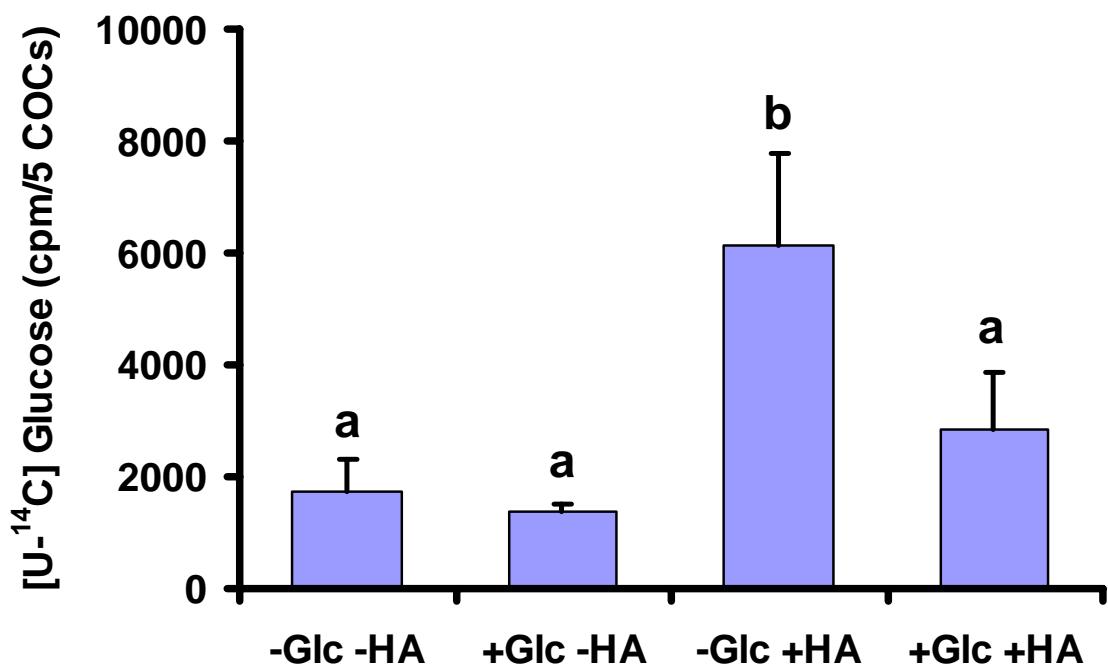
Individual COCs were cultured with [ $U-^{14}C$ ] glucose in the presence or absence of glucosamine to determine whether glucose is converted to ECM and if exogenous glucosamine effects glucose consumption for ECM synthesis. Half of the treatment groups underwent hyaluronidase digestion to release any soluble [ $U-^{14}C$ ] glucose that was incorporated into the ECM. Both of the control groups that were not treated with hyaluronidase displayed similarly low levels of  $U-^{14}C$  carbon (most likely as glucose or glycolytic intermediates) within the COC supernatant, regardless of presence or absence of glucosamine (Figure 4). In contrast, significantly more  $U-^{14}C$  carbon was liberated from the ECM upon hyaluronidase digestion when COCs were cultured in media without glucosamine (- Glc + HA) compared to COCs cultured with glucosamine and treated with hyaluronidase (Figure 4; - Glc + HA =  $6154 \pm 1623$  cpm/5 COCs vs. + Glc + HA =  $2828 \pm 1021$  cpm/5 COCs,  $P < 0.05$ ).

*Experiment 3: Glucose depletion from IVM medium*

A glucose depletion model was generated based on glucose consumption rates determined in previous experiments. We determined the validity of the model by culturing COCs in groups, utilising two densities of COCs per  $\mu$ l of maturation media. COCs were also cultured in the presence or absence of glucosamine to



**Figure 3.** L-lactate production by cumulus oocyte complexes (COCs) from 0-4 h or 20-24 h of culture in Tissue Culture Medium 199 (TCM199), + FSH, + glucosamine (+ Glc) or + FSH + Glc. Data bars represent means  $\pm$  SEM. <sup>ab</sup> and <sup>cd</sup> Means within a time point with different superscripts are significantly different ( $P < 0.05$ ).



**Figure 4.** [ $\text{U}^{-14}\text{C}$ ] glucose incorporation into extra cellular matrix (ECM) by cumulus oocyte complexes (COCs). COCs were cultured in media  $\pm$  glucosamine ( $\pm$  Glc) and underwent post maturation treatment  $\pm$  hyaluronidase ( $\pm$  HA) to determine whether glucosamine supplementation influences glucose incorporation into ECM. Data bars represent mean  $\pm$  SEM. <sup>ab</sup> Different superscripts are significantly different ( $P < 0.05$ ).

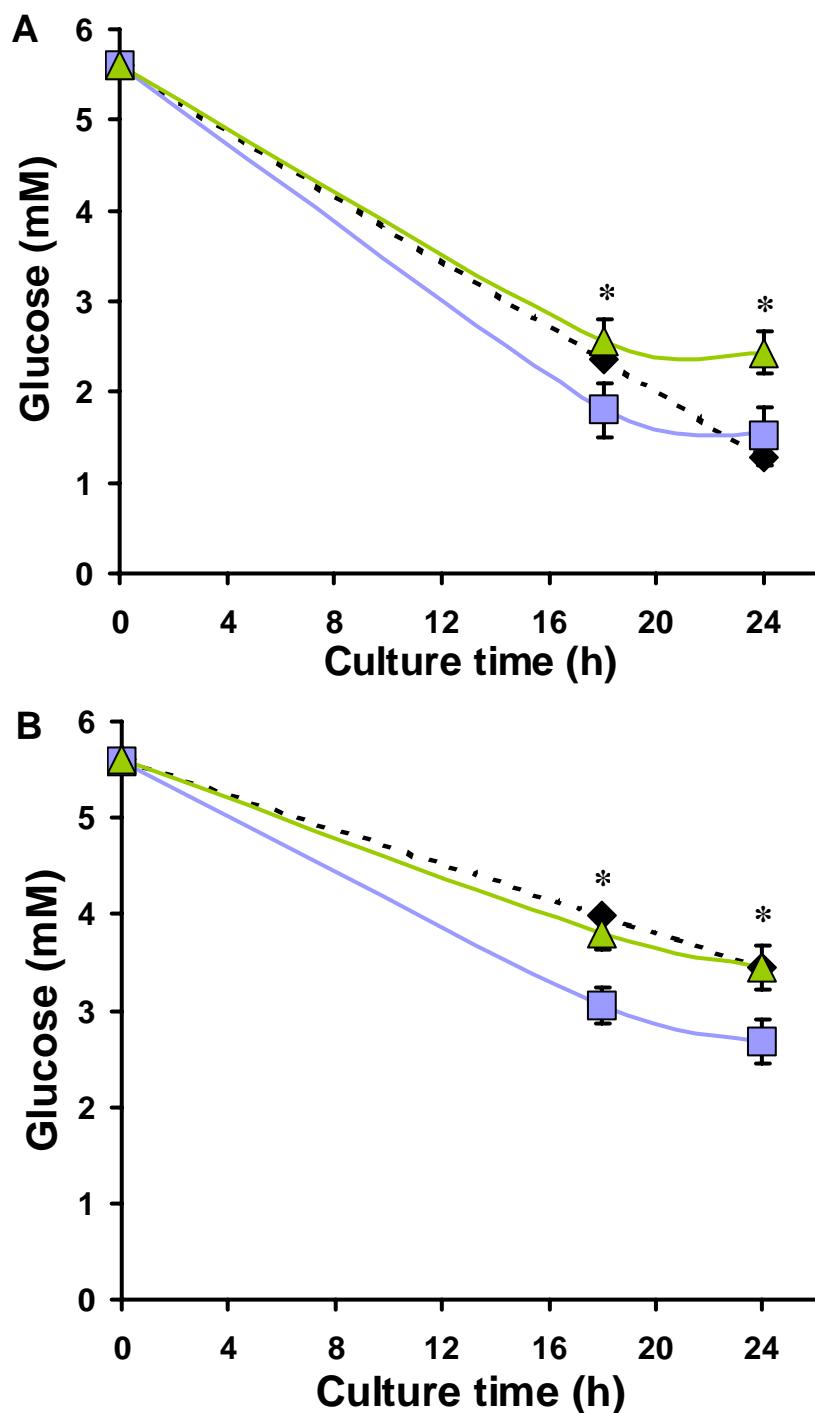
determine whether glucosamine supplementation attenuated glucose loss from the maturation media.

When COCs were cultured at a density of 5 µl/COC, the concentration of glucose remaining in media after 24 h in control media was similar to the concentration predicted by the glucose depletion model (Figure 5A). Unlike the model where a linear rate of depletion was predicted, between 18 h and 24 h the concentration of glucose in both the control and the media supplemented with glucosamine remained unchanged. After 18 h and 24 h of culture, the presence of glucosamine led to a greater than 1.4-fold decrease in glucose uptake by COCs compared to in control medium (18 h: control =  $1.80 \pm 0.29$  mM vs. + Glc =  $2.56 \pm 0.23$  mM and 24h: control =  $1.51 \pm 0.33$  mM vs. + Glc =  $2.43 \pm 0.23$  mM;  $P < 0.05$ ). Similar results were observed when COCs were cultured in 10 µl of medium per complex after 18 h (Figure 5B; control =  $3.05 \pm 0.19$  mM vs. + Glc =  $3.79 \pm 0.17$  mM;  $P < 0.05$ ) and 24 h (control =  $2.68 \pm 0.24$  mM vs. + Glc =  $3.45 \pm 0.24$  mM;  $P = 0.05$ ). However, under these conditions, glucose uptake followed the predicted rate of glucose depletion in medium containing glucosamine rather than the control groups (Figure 5B). Furthermore there was significantly less glucose in the 5 µl/COC culture system after 24 h of maturation compared to the 10 µl/COC ( $P < 0.001$ ), regardless of the presence or absence of glucosamine. In regards to L-lactate production, there were no significant differences between the control and the + glucosamine groups, regardless of culture time and the volume in which the COCs were cultured (Table 2,  $P > 0.05$ ).

**Table 2.** L-lactate production by 10 cumulus oocyte complexes (COCs) cultured at either 5 µl/COC or 10 µl/COC in Tissue Culture Medium 199 (control) or control + glucosamine (+ Glc).

		L-lactate production (mM)			
Culture Time (h)		5 µl/COC		10 µl/COC	
		Control	+ Glc	Control	+ Glc
18 h		$2.8 \pm 0.4$	$2.5 \pm 0.4$	$3.3 \pm 0.5$	$2.2 \pm 0.3$
24 h		$2.9 \pm 0.4$	$2.5 \pm 0.3$	$3.7 \pm 0.5$	$2.4 \pm 0.4$

Values are means  $\pm$  SEM.



**Figure 5.** Glucose depletion by 10 cumulus oocyte complexes (COCs) cultured in either A) 5 µl/COC or B) 10 µl/COC and ± glucosamine for 24 h. ♦ Predicted, ■ Tissue Culture Medium 199 (TCM199, Control) or ▲ + glucosamine (+ Glc). Data points represent means ± SEM. \* Significantly different to control ( $P < 0.05$ ).

## **Discussion**

Although the roles of glucose and glucosamine in ECM synthesis and cumulus expansion are well known, especially in rodents, this is the first study to directly relate total glucose uptake to its role in cumulus-derived matrix synthesis via the glucosamine-biosynthesis pathway. This study has demonstrated that the addition of glucosamine to culture medium leads to a decrease in total glucose uptake by bovine COCs during the final stages of IVM, coinciding with FSH-stimulated cumulus expansion. This was further validated by an experiment in which COCs cultured in media supplemented with glucosamine had reduced U-<sup>14</sup>C carbon derived from glucose incorporated into their ECM compared to COCs cultured without glucosamine. Hence, this data suggests that bovine COCs preferentially utilize glucosamine over glucose for ECM synthesis during FSH-stimulated mucification.

The results of this study demonstrate that supplementation of IVM media with glucosamine results in decreased glucose uptake by COCs cultured in both single and group culture systems. An investigation into the pattern of glucose depletion during maturation demonstrated that the density in which COCs are cultured alters the pattern of glucose metabolism. At high COC density (i.e. 5 µl/COC), there appears to be a concentration dependent plateau in glucose consumption and L-lactate production compared to when lower densities (i.e. 10 µl/COC) are used. This is significant as it suggests that glucose uptake and/or metabolism is inhibited at a time of significant demand. The addition of glucosamine only partly alleviates this situation, but maturing COCs at lower density combined with glucosamine was the most effective at maintaining a linear depletion of glucose from the media, while reducing L-lactate accumulation, both of which would be associated with normal cellular function.

It is of interest to note that under maximal depletion conditions (i.e. high density and no glucosamine), glucose concentrations did not fall under ~ 2.2 mM. We and others (Johnson *et al.* 2001; Berg *et al.* 2003; Sutton-McDowall *et al.* 2004) have recently reported that bovine follicular fluid glucose levels are approximately 2.3 mM. We can only speculate why COCs matured under such conditions appear to inhibit

glucose uptake. Perhaps atmospheric pO<sub>2</sub> conditions present during IVM down-regulate glucose transporters, which are known to be regulated by oxygen concentration (Elvin *et al.* 1999). The exact time during IVM at which this decreased rate of glucose consumption occurs is not known since measurements of glucose concentrations were only performed in the latter period of the cultures. An interesting observation is that when the media density of 5 µl/COC was used, a considerable proportion of glucose was depleted from the media by the end of culture, despite glucose initially being present in relatively high concentrations compared to intrafollicular concentrations (5.6 mM vs. ~2.3 mM).

A diverse range of media have been used for bovine IVM, with glucose concentrations ranging from 1.5 mM in synthetic oviductal fluid (SOF; Ali and Sirard 2002) to 28 mM glucose in Waymouth MB 752/1 (Rose and Bavister 1992). COCs cultured in substantially high glucose concentrations (10-28 mM glucose) have decreased ooplasmic glutathione levels (an important reducing agent), increased intracellular reactive oxygen species (Hashimoto *et al.* 2000) and compromised blastocyst development compared COCs cultured in low to moderate glucose levels, such as SOF (1.5 mM or 5.6 mM glucose; Hashimoto, *et al.* 2000), TCM199 and minimal essential medium (5.6 mM glucose; Rose and Bavister 1992). Conversely insufficient glucose during IVM leads to impaired the completion of nuclear maturation (Hashimoto, *et al.* 2000), cumulus expansion and blastocyst development (Rose-Hellekant *et al.* 1998), most likely due to insufficient substrates for hyaluronic acid synthesis. Although it is evident that glucose concentrations play a crucial role in oocyte maturation, it is yet to be elucidated as to the effects of culturing COCs in more physiological glucose concentrations.

Supplementation of media with glucosamine reduces the requirement for glucose for matrix components. Glucosamine supplementation during IVM may be a mechanism through which glucose levels can be reduced to more physiological concentrations while providing the cumulus vestment with adequate substrates for mucification. Decreasing glucose concentrations has been shown to regulate the rate of meiotic resumption of murine COCs (Downs, *et al.* 1998; Downs and Hudson 2000).

In conclusion we have demonstrated that a proportion of glucose uptake is linked to FSH-stimulated cumulus expansion during IVM. Glucosamine can assist in preventing significant glucose depletion in IVM media during maturation by providing an alternative and preferential substrate for matrix production and cumulus expansion.

## Acknowledgements

We would like to thank Rachel Collett and Chris Kraft for technical assistance and ovary collections and Fred Amato and Svjetlana Kireta for advance and assistance with the [ $^{14}\text{C}$ ] glucose experiment. M Sutton-McDowall is supported by the Australia Research Council (SPIRT, C00107702) and Cook Australia Pty Ltd.

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# **Chapter 4**

## **The ionic and energy substrate composition of bovine follicular fluid in relation to follicle size and the influence of glucose on oocyte meiotic progression**

Melanie L. Sutton-McDowall, Robert B. Gilchrist and Jeremy G. Thompson

Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, University of Adelaide, The Queen Elizabeth Hospital, Woodville Road, Woodville 5011, South Australia.

## **Abstract**

The developmental potential of an oocyte is determined primarily by the quality of the follicular milieu surrounding it, affected by such factors as follicle size and stage of the oestrous cycle. This study was conducted to evaluate ion and energy substrate concentrations in bovine follicular fluid (FF) derived from different sized follicles and from luteal or follicular phases of the oestrous cycle with the aim of developing a more physiological maturation medium. Composition of FF did not vary with stage of cycle ( $P > 0.05$ ). There were no differences in  $\text{Na}^+$  and  $\text{Mg}^{2+}$  concentrations, whereas with increasing follicle size,  $\text{Cl}^-$  and  $\text{HCO}_3^-$  levels significantly increased and  $\text{K}^+$ ,  $\text{PO}_4^{2-}$  and  $\text{Ca}^{2+}$  concentrations decreased ( $P < 0.05$ ). Pyruvate and L-lactate levels were not influenced by follicle size, whereas glucose concentrations were significantly higher in FF of large compared to small follicles ( $P < 0.05$ ). Maturation of oocytes in medium containing FF levels of ions and glucose (2.3 mM) in comparison with medium containing 5.6 mM glucose or TCM199, inhibited oocyte nuclear maturation. This study demonstrates that although the ionic and energy substrate compositions of FF were not influenced by stage of oestrous cycle, the concentration of some of the parameters measured did change with follicle development. Significant amongst these is glucose, the levels of which appear to influence oocyte meiotic progression.

## **Introduction**

The ovarian follicular antrum forms early in folliculogenesis and in most species this coincides with periods of rapid oocyte development (reviewed by Picton *et al.* 1998). The antral cavity is filled with follicular fluid (FF), a liquid that bathes the cumulus oocyte complex (COC) and is the major source of nutrients and other factors important to oocyte maturation. The composition of FF largely resembles filtered plasma due to the porous nature of the follicular wall (Gosden *et al.* 1988), although differences have been reported such as the exclusion of high molecular weight proteins (Andersen *et al.* 1976). Furthermore amino acids (Velazquez *et al.* 1977), K<sup>+</sup> (Schuetz and Anisowicz 1974; Gosden, *et al.* 1988) and CO<sub>2</sub> tension (Fischer *et al.* 1992) are higher in FF compared to blood plasma levels and O<sub>2</sub> tension and pH are lower (Fischer *et al.* 1992).

Numerous follicular parameters correlate with increased oocyte developmental capacity. A positive relationship exists between bovine follicle size and oocyte development to the blastocyst stage (Pavlok *et al.* 1992; Lonergan *et al.* 1994), with oocyte from follicles >6 mm having higher blastocyst rates. Hagemann and colleagues (Hagemann 1999; Hagemann *et al.* 1999) also demonstrated that bovine oocytes derived from follicles within the growth phase have higher developmental potential compared to oocytes derived from dominant phase follicles. Furthermore measurements of follicular vascularity prior to oocyte collection are positively related to improved developmental outcomes in humans (Chui *et al.* 1997; Coulam *et al.* 1999). Conversely, oocytes derived from follicles with low levels of dissolved O<sub>2</sub> have increased incidences of developmental defects such as aneuploidy, abnormal spindle organization and cytoplasmic structure (Van Blerkom *et al.* 1997). Hence in some incidences, follicular parameters are related to oocyte developmental capacity.

The environment (both *in vivo* and *in vitro*) that COCs are exposed to during maturation has the potential to alter oocyte developmental capacity (Eppig 1977; Rose and Bavister 1992; Roche 1996; Rose-Hellekant *et al.* 1998; Albertini *et al.* 2001). Wise (Wise 1987) has previously investigated biochemical changes in FF collected from heifers on every day of a 21-day oestrous cycle, focusing on follicular

steroids (i.e. progesterone, estradiol and testosterone), proteins and albumin, biochemical enzyme activity (i.e. lactate dehydrogenase) and some ions (i.e.  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ). Differences in follicular concentrations of some of these factors were observed between different follicle sizes, health and stage of oestrous (Wise 1987). The aim of this study was to determine the follicular concentrations of ions and energy substrates for the development of a basic, defined culture media, based on the composition of FF. From our analysis of FF, we also assessed the impact of altering the concentration of a major substrate (glucose) on oocyte meiotic maturation, as this was found to clearly vary between different FF samples taken and that which is commonly used in current bovine in vitro maturation systems.

## Methods

Unless otherwise stated, reagents were purchased from Sigma (St Louis, MO).

### *Ovary and follicular fluid collection*

Bovine ovaries were collected in pairs from a local abattoir, cooled rapidly and transported on ice to minimize post-mortem metabolism. The morphology of both ovaries, in particular corpus luteum, was assessed to estimate the oestrous cycle stage of the animal based on previously described criteria (Table 1; Ireland *et al.* 1980). Ovary pairs were separated into two major categories, luteal and follicular phases, using this technique. The diameter of follicles were measured in intact ovaries using a calliper; small follicles were classified as  $\leq 4$  mm, medium 5–7 mm and large  $\geq 8$  mm. Fluids were aspirated from follicles that were non-atretic in appearance (Yang and Rajamahendran 2000) using a 27-gauge 1.0 ml insulin syringe (Terumo Medical Corporation, MD, USA) and the volume of the collected fluid was estimated. Samples were centrifuged to sediment any cellular material and the supernatant was snap frozen in liquid nitrogen. FF was stored at  $-80^\circ\text{C}$  until analysed. A total of 28 ovary pairs were collected and 10 samples per size and phase were analysed with the exception of FF from small luteal phase and large follicular phase ovaries where six samples were analysed.

**Table 1.** Ovary morphology used to ascertain oestrous cycle stage.

Phase	Morphology Characteristics
<b>Luteal</b>	New/active corpus luteum (red, pink) Dominant follicle present Subordinate follicles
<b>Follicular</b>	Regressing corpus luteum (orange, yellow, white) Dominant follicle (to become ovulatory follicle) present Possible presence of regressing dominant follicle

*Measurement of follicular fluid ions and energy substrates*

FF samples were assayed for  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{HCO}_3^-$ ,  $\text{PO}_4^{2-}$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  by an automated Dade RxL Dimension chemical analyser (Institute of Medical and Veterinary Science, The Queen Elizabeth Hospital Division, Clinical Chemistry, Woodville, South Australia). The ionic composition of Tissue Culture Medium 199 (TCM199; ICN Biochemicals, Irvine, CA, USA), synthetic oviductal fluid medium (SOF; Tervit *et al.* 1972) and day 7 bovine uterine fluid (Thompson *et al.* 2000) were also compared. Glucose, pyruvate and L-lactate concentrations were determined using microfluorometric assays (Leese and Barton 1984; Gardner and Leese 1986). Standard curves were generated simultaneously and were used to determine the concentrations of energy substrates in FF.

*The effect of glucose concentration on meiotic progression*

Bovine ovaries were collected from a local abattoir and transported to the laboratory in warm (30–35°C) saline. The contents of follicles of non-atretic appearance (Yang *et al.* 2001) and 3–8 mm in size were aspirated using an 18-gauge needle and 10 ml syringe. Cumulus oocyte complexes (COCs) surrounded by intact cumulus vestments with greater than three cell layers were collected, washed twice in HEPES-buffered TCM199 supplemented with 0.5 mM sodium pyruvate and 4 mg/ml fatty acid free BSA (ICPbio Ltd, Auckland, NZ) and once in the corresponding maturation media. Groups of 10 COCs were cultured in 100 µl of either bicarbonate-buffered TCM199

supplemented with 0.5 mM sodium pyruvate or bovine FF medium (BFFM; medium based on the composition of FF, Table 2) supplemented with 100 µM glutamax (Gibco Invitrogen Corporation, Carlsbad, CA, USA), 100 µM acetyl-L-cysteine, 100 µM 2-mercaptoethylamine, 1% v/v non-essential amino acids (100X, Gibco Invitrogen Corporation), 2% v/v essential amino acids (50X, Gibco Invitrogen Corporation) and either 2.3 mM or 5.6 mM glucose. All media were supplemented with 4 mg/ml fatty acid free BSA, 0.1 IU/ml FSH (Puregon, Organon, Oss, Netherlands) and 0.1 IU/ml hCG (Pregnyl, Organon). Culture drops were overlaid with mineral oil and incubated at 39°C in 6% CO<sub>2</sub> in humidified air. At 24 h or 30 h, COCs were removed from culture, mechanically denuded by repeat pipetting and fixed in 3:1 ethanol: acetic acid for 48 h. Denuded oocytes were mounted on microscope slides and fixed in place with a cover slip supported by petroleum jelly. Nuclear dye (1% orcein dissolved in 45% acetic acid) was applied for 20 mins, followed by de-staining with 1:1:3 glycerol: acetic acid: water. Cover slips were sealed with clear nail polish and the nuclear status was assessed at 400X using a phase contrast microscope. Oocytes were classified as being at metaphase I (MI), anaphase I (AI), telophase I (TI) or metaphase II (MII). Eight replicate experiments were performed with 10 COCs used in each treatment group and time point.

#### *Statistical analyses*

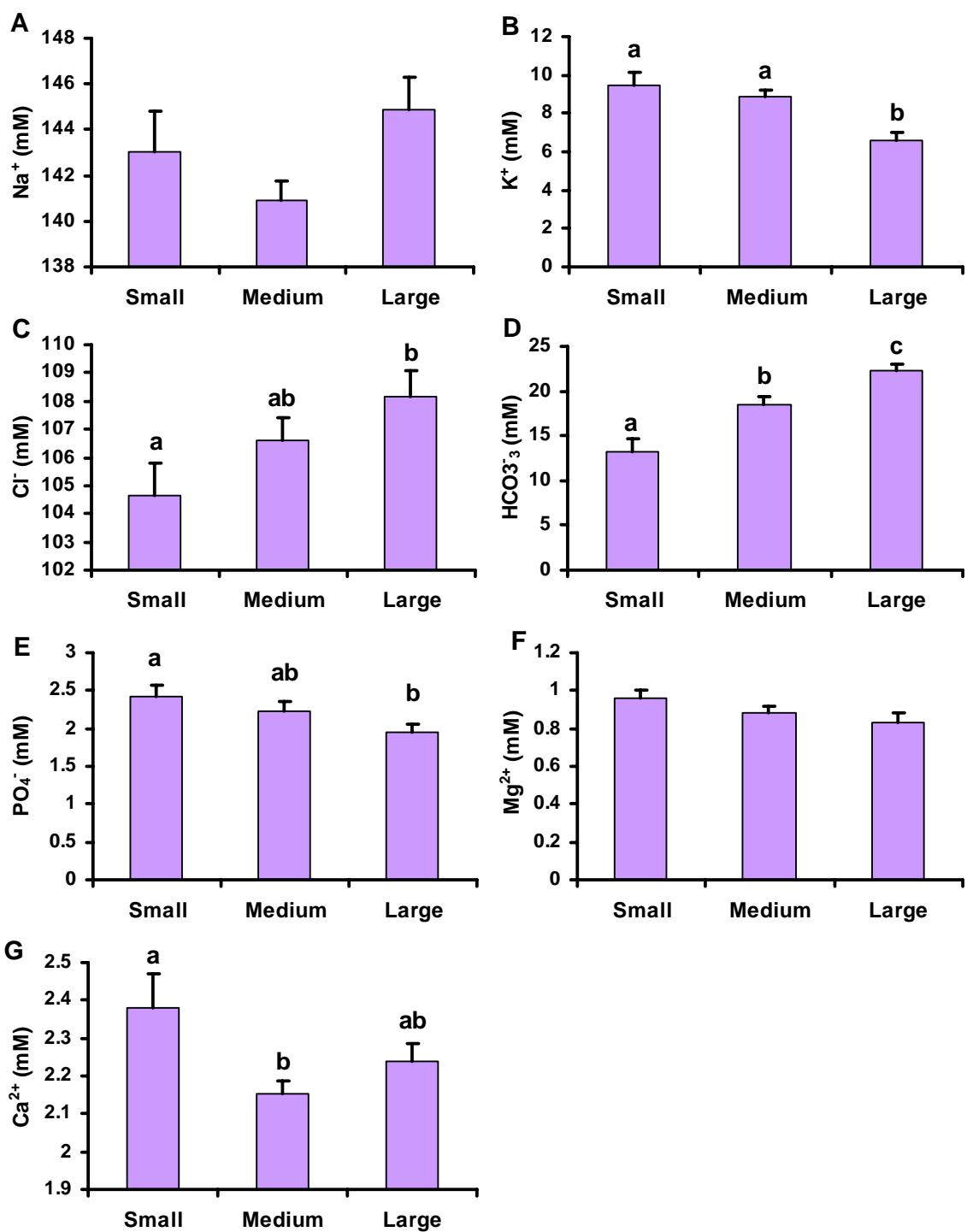
Fluids were analysed by multiple linear regression analysis with cow as the dependant variable and oestrous stage, follicle size and ion/energy substrate measurements as independent variables. The composition of fluids derived from small, medium or large follicles were compared by an analysis of variance (ANOVA), followed by Tukey test. For assessment of nuclear maturation, AI and TI were grouped with MII and the proportions of oocytes in MII were arcsine transformed and analysed using an ANOVA followed by Tukey test. All statistical analyses were performed using SigmaStat version 2.0 computer software (SPSS Inc, Chicago, IL).

## Results

### *Follicular fluid composition*

Follicular fluids were divided into two categories (i.e. originating from luteal or follicular phase ovaries) to determine whether ion and energy substrate concentrations varied between the two follicular waves. Furthermore, FF from small, medium and large follicles was compared. There were no significant differences in the concentrations of any of the ions or energy substrates measured between follicles of the same size but originating from different animals and oestrous phases ( $P > 0.05$ ); hence the FF compositions were compared according to follicle size, irrespective of the animal and cycle stage.

In regards to the ionic composition of FF, the concentrations of  $\text{Na}^+$  and  $\text{Mg}^{2+}$  were not dependent on follicle size (Figures 1A and F). Follicular concentrations of both closely resembled levels in commercially available media typically used for the in vitro maturation (IVM) of oocytes such as TCM199, although were at higher concentrations than in embryo culture medium SOF (Table 2). The concentrations of  $\text{Cl}^-$  and  $\text{HCO}_3^-$  ions significantly increased with follicle size (Figures 1C and D;  $P < 0.05$ ), samples derived from small follicles had less  $\text{Cl}^-$  than fluid from large follicles (small =  $104.7 \pm 1.2$  mM vs. large =  $108.1 \pm 1.0$  mM;  $P < 0.05$ ), whereas the concentration of  $\text{HCO}_3^-$  was significantly different for three follicle sizes, with a 1.7-fold increase in large FF compared to small FF (Figure 1D;  $P < 0.001$ ). In contrast, the concentration of  $\text{K}^+$  was lowest in large follicles (Figure 1B;  $6.6 \pm 0.4$  mM) and was significantly different from that of small and medium follicles ( $9.5 \pm 0.7$  and  $8.9 \pm 0.3$  mM respectively;  $P < 0.001$ ). Both  $\text{PO}_4^-$  and  $\text{Ca}^{2+}$  concentrations in fluid from small follicles were higher than that of either medium or large follicles ( $\text{PO}_4^-$ : small =  $2.4 \pm 0.1$  mM vs. large =  $2.0 \pm 0.1$  mM,  $P < 0.05$ ; and  $\text{Ca}^{2+}$ : small =  $2.4 \pm 0.1$  mM vs. medium =  $2.2 \pm 0.0$  mM,  $P < 0.05$ ). Whilst  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{HCO}_3^-$  and  $\text{Mg}^{2+}$  levels in the TCM199 were within the physiological range found in FF, both  $\text{PO}_4^-$  and  $\text{Ca}^{2+}$  concentrations were higher in FF compared to TCM199 and SOF (Table 2).



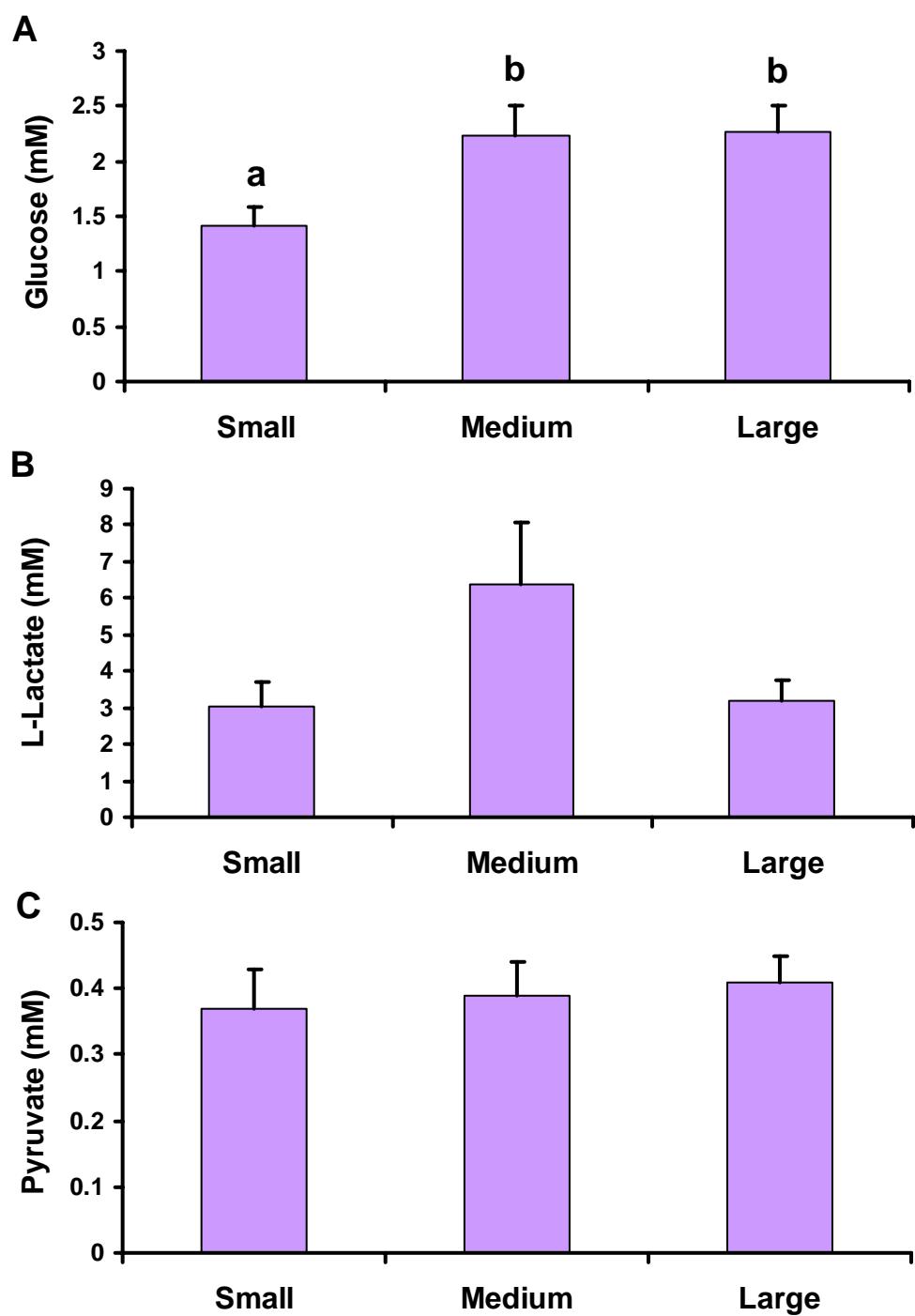
**Figure 1.** Concentration of ionic compounds in follicular fluid collected from small, medium or large follicles. A) sodium ( $\text{Na}^+$ ), B) potassium ( $\text{K}^+$ ), C) Chlorine ( $\text{Cl}^-$ ), D) bicarbonate ( $\text{HCO}_3^-$ ), E) phosphate ( $\text{PO}_4^{2-}$ ), F) magnesium ( $\text{Mg}^{2+}$ ) and G) calcium ( $\text{Ca}^{2+}$ ). Bars represent mean  $\pm$  SEM. <sup>abc</sup> Columns with different superscript letters are significantly different ( $P \leq 0.05$ ).

**Table 2.** The ionic composition of media, serum supplements and uterine fluid compared to follicular fluid (FF).

	Samples (mM)				
	FF (range)	SFFM	TCM199	SOF	BUF
<b>Na<sup>+</sup></b>	141 – 145	140.4	142	136	132
<b>K<sup>+</sup></b>	6.59 – 9.45	4	6.1	8.4	29.7
<b>Cl<sup>-</sup></b>	105 – 108	102	117	119	107
<b>HCO<sub>3</sub><sup>-</sup></b>	13.3 – 22.2	40	25	25	-
<b>PO<sub>4</sub><sup>2-</sup></b>	1.96 – 2.42	2	1.2	1.2	Undetected
<b>Mg<sup>2+</sup></b>	0.84 – 0.96	0.8	0.9	0.5	2.9
<b>Ca<sup>2+</sup></b>	2.15 – 2.38	2.3	1.8	1.7	3.5
<b>Glucose</b>	1.4 – 2.3	2.3 or 5.6	5.6	1.5	2.4
<b>Pyruvate</b>	0.37 – 0.41	0.4	-	0.33	0.2
<b>L-lactate</b>	3.0 – 6.4	4.3	-	3.3	2.2

SFFM = Synthetic Follicular Fluid Medium, TCM199 = Tissue Culture Medium 199, SOF = synthetic oviductal fluid (Tervit, *et al.* 1972) and BUF = bovine uterine fluid (day 7; Thompson, *et al.* 2000). Follicular fluid data is expressed as the range of concentrations seen between different size follicles.

Concentrations of glucose, L-lactate and pyruvate were measured in FF from different sized follicles. There was a significant increase in glucose concentration in FF from medium and large follicles compared to fluids from small follicles (Figure 2A; small =  $1.4 \pm 0.2$  mM vs. medium =  $2.2 \pm 0.3$  mM and large =  $2.3 \pm 0.2$  mM;  $P < 0.05$ ). Furthermore glucose concentrations in TCM199 were  $>2.4$ -fold higher than in FF (TCM199 = 5.6 mM). Follicle size did not significantly affect pyruvate levels (Figure 2C), and although L-lactate levels were numerically higher in FF from medium follicles compared to small and large FF, there were no significant differences (Figure 2B;  $P > 0.05$ ).



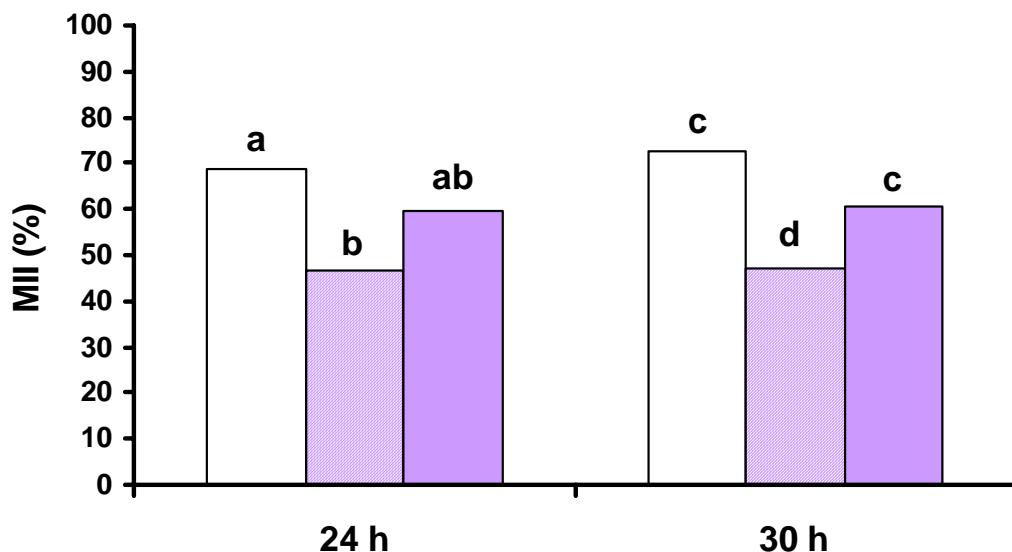
**Figure 2.** Concentration of metabolites in follicular fluid collected from small, medium or large follicles. A) Glucose, B) L-lactate and C) Pyruvate. Bars represent means  $\pm$  SEM. <sup>ab</sup> Columns with different superscripts are significantly different ( $P < 0.05$ ).

### *The effect of glucose concentration on meiotic progression*

A bovine cumulus oocyte complex (COC) IVM medium (Synthetic Follicular Fluid Medium, SFFM) was formulated, based on the concentrations of the ions and energy substrates measured in FF (Table 2). As glucose concentration in FF (2.3 mM) is lower than that commonly used in bovine IVM (TCM199, 5.6 mM), meiotic maturation capacity was compared between these levels of glucose. COCs were cultured in either SFFM (2.3 mM vs. 5.6 mM) or TCM199 to determine the influence of glucose concentration on nuclear progression. The proportion of oocytes that reached MII was significantly lower when cultured in SFFM (2.3 mM glucose) compared to TCM199 (5.6 mM glucose), regardless of culture time (Figure 3,  $P < 0.05$ ). Although there were no differences in MII proportions after 24 h when COCs were cultured in SFFM with either concentrations of glucose, by 30 h significantly more COCs reached MII when cultured in SFFM with 5.6 mM glucose compared to SFFM with 2.3 mM glucose (2.3 mM glucose = 47% vs. 5.6 mM glucose = 61%,  $P < 0.05$ ). Furthermore, COCs cultured in 5.6 mM glucose (the same concentration of glucose as TCM199) had comparable rates of nuclear maturation to TCM199 ( $P > 0.05$ ).

## **Discussion**

Over the past decade, in vitro embryo production has improved considerably and this is in large part due to the design of media based on the composition of reproductive tract fluids. Examples include synthetic oviduct fluid (SOF; Tervit, *et al.* 1972), human tubal fluid (HTF; Quinn *et al.* 1985) and G1/G2 human tubal and uterine fluids (Gardner *et al.* 1996), mouse oviductal fluid (MTF; Gardner and Leese 1990) and PL<sub>3</sub>; based on bovine blood and sheep oviductal fluids (Park and Lin 1993). Considering the most commonly used media for bovine IVM is TCM199, which was originally formulated for non-ovarian somatic cell cultures, an increased understanding of the ionic and energy substrate composition of the follicular environment may assist in the design of improved oocyte maturation media.



**Figure 3.** The influence of glucose concentrations on the progression of nuclear maturation of cumulus oocyte complexes cultured for 24 h or 30 h in either □ Tissue Culture Medium 199 (5.6 mM glucose), ■ Synthetic Follicular Fluid Medium (SFFM) with 2.3 mM glucose or ▨ SFFM with 5.6 mM glucose. Bars represent means  $\pm$  SEM. <sup>ab</sup> and <sup>cd</sup> Bars with different superscripts are significant different to SFFM ( $P < 0.05$ ).

The composition of FF from many species has previously been studied, although the main focus has been on steroids, protein, lipid and electrolyte concentrations in pooled samples (Desjardins *et al.* 1966; Schuetz and Anisowicz 1974; Wise 1987; Gosden, *et al.* 1988). The current study examined the concentration of ions and energy substrate in FF from individual follicles, rather than pooled samples, specifically with the formation of a bovine in vitro maturation medium in mind. Irrespective of follicles being derived from luteal or follicular phases, in the current study there were no differences in any of the FF components measured, although there were differences in some parameters between fluids derived from follicles of different sizes.

The concentrations of  $\text{Na}^+$  or  $\text{Mg}^{2+}$  did not vary in samples from different sized follicles, whereas increasing follicle size was associated with increased levels of  $\text{Cl}^-$  and  $\text{HCO}_3^-$  and decreased  $\text{K}^+$ ,  $\text{PO}_4^-$  and  $\text{Ca}^{2+}$ . In regards to energy substrate concentrations, follicle size was positively related to glucose concentrations but not L-lactate or pyruvate levels. The concentrations of  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  reported here are similar to previously reported levels in unstimulated bovine FF, although the  $\text{Na}^+$  levels in the current study were considerably lower than in small FF (Wise 1987). Electrolyte concentrations in FF of other animals including sheep, pig and human were highly comparable to levels measured in the current study (Schuetz and Anisowicz 1974; Gosden, *et al.* 1988). Levels of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{HCO}_3^-$  and  $\text{Mg}^{2+}$  in the most commonly employed bovine IVM medium, TCM199, are within the physiological range of bovine FF measured in this study, although  $\text{PO}_4^-$  and  $\text{Ca}^{2+}$  levels are lower in TCM199. Ions are critically involved in a multitude of intra-cellular processes and inter-cellular communication pathways; e.g.  $\text{Ca}^{2+}$  is a vital intra-cellular secondary messenger, and is also involved in cell signalling within the COC via gap junctions (Webb *et al.* 2002). The cellular implications of non-physiological electrolyte concentrations in IVM medium are unclear and are undoubtedly complex, but are presumably detrimental to oocyte developmental potential.

Samples were collected from follicles that were not atretic in appearance, i.e. follicles with clear, amber FF and clearly visible vascularization of the theca interna (Yang and Rajamahendran 2000). Since a detailed histological analysis of the

follicles was not performed, it is quite possible that some samples were collected from early atretic follicles. Further investigations would possibly determine whether follicular ionic and energy substrate concentrations vary between healthy follicles and those at different stages of atresia.

The glucose concentrations measured in the current study in bovine FF from large follicles were comparable to levels measured in FF aspirated *in vivo* or 4 h post-mortem (Berg *et al.* 2003), and to bovine blood glucose levels (Johnson *et al.* 2001). Despite ovaries being transported on ice to minimise post-mortem metabolic activity, the follicles did undergo a certain degree of stress, demonstrated by L-lactate concentrations being considerably higher compared to levels within *in vivo* aspirated fluids (Berg, *et al.* 2003). Although the levels of L-lactate found in the current study were higher than *in vivo*-aspirated fluids, they were between 1.5- to 4.7-fold lower than levels measured by Leroy *et al* (Leroy *et al.* 2002). Interestingly, the concentration of glucose in FF varies very little between studies, although a wide range of L-lactate concentrations are reported, regardless of the samples being collected *in vivo* or post-mortem. This suggests that the methods used to collect FF have little impact on glucose levels but do influence lactate production, mostly likely as a consequence of post-aspiration metabolism. This is particularly demonstrated by FF that was collected *in vivo* having significantly lower L-lactate concentrations compared to FF collected post-mortem (Johnson, *et al.* 2001; Berg, *et al.* 2003).

Physiological concentrations of glucose in FF were less than half that in commonly used bovine IVM media, TCM199, which could alter the development of *in vitro* matured oocytes, i.e. increased glucose concentrations increases resumption of meiosis in mouse oocytes (Downs *et al.* 1998). We assessed this during bovine cumulus-oocyte complex (COC) maturation *in vitro*, utilising a medium composed from FF ion and substrate concentrations. Our results in the bovine agree with Downs *et al.* (Downs and Mastropolo 1994; Downs, *et al.* 1998) that glucose concentration significantly influences meiotic progression. Downs and colleagues demonstrated that glucose concentrations influence nuclear maturation via de novo purine synthesis (Downs, *et al.* 1998; Downs and Utecht 1999). However, it is also possible that when maturation media contains low glucose concentrations, glucose levels have depleted to levels that may be detrimental to normal cellular functions

required for the completion of oocyte maturation. Furthermore, we have not measured post-LH follicular glucose concentrations in the present study so it is difficult to assess if this physiological level used truly reflects what a COC is exposed to during meiotic maturation. An analysis of post-LH follicular fluid substrate levels appears warranted.

In conclusion, we have demonstrated that, in regards to oestrous cycle stage, there were no differences in the measured ionic and energy substrate concentrations. Fluids collected from different size follicles displayed some differences in these parameters. Most noticeably glucose concentrations were significantly higher in larger follicles compared to small follicles, yet were still lower than that commonly used during bovine IVM. Reducing glucose from 5.6 mM to 2.3 mM glucose (which mimics the level observed in large antral follicles) impeded meiotic progression.

## Acknowledgements

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# **Chapter 5**

## **The effect of hexoses and gonadotrophin supplementation on bovine oocyte nuclear maturation during in vitro maturation in a synthetic follicular fluid medium**

Melanie L. Sutton-McDowall, Robert B. Gilchrist and Jeremy G. Thompson

Research Center for Reproductive Health, Department of Obstetrics and Gynaecology, The University of Adelaide, The Queen Elizabeth Hospital, Woodville Road, Woodville 5011, South Australia.

## **Abstract**

In vitro oocyte maturation (IVM) culture conditions are currently suboptimal and have remained relatively unchanged over the past few decades. In contrast, studies of the in vivo environment have led to significant improvements to in vitro embryo culture technologies. The aim of this study was to determine the influence of maturing bovine cumulus oocyte complexes (COCs) in medium based on the composition of bovine follicular fluid, in particular the effects of different glucose concentrations and glucosamine supplementation on meiotic maturation. Culturing COCs in medium containing low glucose (2.3 mM) supplemented with glucosamine resulted in comparable cumulus expansion to COCs cultured in higher glucose (Tissue Culture Medium 199, TCM199; 5.6 mM), although culturing in low glucose led to an 8 h delay in nuclear maturation. This delay in nuclear maturation was due to decreased glucose concentrations, rather than glucosamine supplementation. The use of sequential culture systems, in which COCs were transferred into media with different glucose concentrations and/or hormone supplementation demonstrated that conditions promoting faster meiotic resumption are detrimental to the completion of nuclear maturation. These results suggest that manipulation of glucose concentrations may be a mechanism to influence oocyte meiotic progression, and further investigations are required to determine the mechanisms through which glucose affects meiotic maturation and subsequent affects on oocyte developmental capacity.

## **Introduction**

In vitro oocyte maturation (IVM) has the potential to become a valuable technique to assisted reproductive technology in humans and for the dissemination of superior genetics within the livestock farming industries, as well as being a valuable research tool. Immature oocytes can be collected from naturally cycling women or domestic animals without hormone stimulation, which reduces cost and eliminates side effects associated with hyper stimulation, and from early pregnant animals or large numbers can be obtained from abattoir-derived ovaries.

Embryos that are produced in vitro (IVM, followed by in vitro fertilization and embryo culture) have perturbed developmental outcomes compared to embryos derived from in vivo matured oocytes (Leibfried-Rutledge *et al.* 1987; van de Leemput *et al.* 1996; Bousquet *et al.* 1999; Dieleman *et al.* 2002). This might be due to the heterogenous population of oocytes used for IVM (Fortune 1994) and the inability of current culture systems to accommodate oocytes with low developmental potential, such as those derived from small follicles or dominance influenced follicles (Hagemann *et al.* 1999). Minimal improvements have been made in IVM technologies over the past few decades, for example the most commonly utilised medium for bovine IVM is Tissue Culture Medium 199 (TCM199), which was formulated for non-ovarian somatic cell cultures and is complex in its chemical composition (Sutton *et al.* 2003). In contrast, in vitro embryo culture conditions have improved vastly over the past decade, which is largely attributed to the development of media, the composition of which, is based on oviductal and uterine fluids (Tervit *et al.* 1972; Quinn *et al.* 1985; Gardner and Leese 1990; Park and Lin 1993; Gardner *et al.* 1996), and an improved understanding of the biochemical requirements of embryos during in vitro culture. It is my proposal that improved IVM culture conditions could be devised using a similar approach.

Oocyte maturation consists of two components, nuclear and cytoplasmic maturation. Nuclear maturation involves the resumption of meiosis from prophase I (germinal vesicle stage, GV) to metaphase II (MII), creating a haploid oocyte. Nuclear maturation is well defined and the nuclear stage of an oocyte is easily measured with

routine microscopy, which may or may not require specific DNA dyes (depending on species and oocyte morphology). The resumption of meiosis can occur either spontaneously by releasing the COC from the follicle (Pincus and Enzmann 1935) or can be ligand induced under conditions of meiotic arrest, i.e. the administration of hormones *in vivo* or *in vitro* or FF-MAS to *in vitro* culture systems (Faerge *et al.* 2001; Byskov *et al.* 2002; Blume *et al.* 2003; Grondahl *et al.* 2003).

The second component of oocyte maturation is cytoplasmic maturation, which is loosely defined as the ability of an oocyte to undergo fertilization and support early embryonic development (de Loos *et al.* 1992; Krisher and Bavister 1998; Trounson *et al.* 2001; Combelles *et al.* 2002). In contrast to nuclear maturation, cytoplasmic maturation is undefined biochemically and the most effective measure of successful cytoplasmic maturation is fertilization and development of the embryo through to the blastocyst stage (Eppig 1996; Krisher and Bavister 1998; Avery *et al.* 2003). The synchronisation of nuclear and cytoplasmic events is believed to facilitate improved developmental capacity (Eppig 1996). There is currently considerable interest in controlling nuclear maturation using a variety of modulators including phosphodiesterase inhibitors (Thomas *et al.* 2002, 2004); protein synthesis inhibitors such as cycloheximide (Tatemoto and Terada 1998); purines such as hypoxanthine (Eppig *et al.* 1985) and other media additives (reviewed by Sirard *et al.* 1998).

Analyses of bovine follicular fluid derived from different sized follicles demonstrated that the concentrations of some ions and energy substrates differed between FF and TCM199 (Chapter 4). In particular, follicular glucose concentrations were approximately 2-fold lower than in TCM199, yet culturing COCs in a physiological concentration of glucose during IVM led to significantly less oocytes completing nuclear maturation compared to oocytes cultured in higher glucose concentrations (Chapter 4). Although Downs and colleagues (Downs *et al.* 1998; Downs and Hudson 2000) have demonstrated that glucose concentrations influence nuclear maturation of murine oocytes via *de novo* purine synthesis, it is possible that by the completion of maturation in low glucose media, glucose levels could be depleted to concentrations detrimental to normal cellular functions. The addition of glucosamine to IVM medium reduces total glucose consumption by FSH-stimulated COCs, due to the preferential use of glucosamine for extracellular matrix synthesis

over glucose (Chapter 4). Glucosamine supplementation of IVM medium may be the mechanism by which COCs can be cultured at physiological glucose concentrations, hence potentially improving oocyte developmental capacity.

I hypothesized that the creation of media based on energy substrate and ion concentrations in bovine FF may lead to improved developmental outcomes compared to currently used formulations. The aim of this study was to determine the influence of culture media components, based on physiological conditions, on the kinetics of bovine oocyte nuclear maturation. Specific aims include determining whether glucosamine supplementation supports oocyte maturation in medium containing low glucose, the influence of hormone supplementation, different glucose concentrations, and changing media coinciding with the resumption or completion of meiosis.

## **Materials and method**

Unless otherwise specified, all reagents were purchased from Sigma (St Louis, MO, USA). Handling medium was HEPES-buffered TCM199 (ICN Biochemicals, Irvine, CA, USA) supplemented with 0.5 mM sodium pyruvate and 4 mg/ml fatty acid free BSA (ICPbio Ltd, Auckland, NZ). Control medium for oocyte maturation was bicarbonate-buffered TCM199 supplemented with 0.5 mM sodium pyruvate and 4 mg/ml fatty acid free BSA (TCM199). Synthetic follicular fluid medium (SFFM) was a defined medium based on the composition of follicular fluid from antral follicles (Chapter 4) and contained non-essential and essential amino acids (ICN Biochemicals) and 4 mg/ml fatty acid free BSA. SFFM contained either 2.3 or 5.6 mM glucose and unless otherwise indicated, 5 mM glucosamine.

### *Oocyte collection*

Bovine ovaries were transported from a local abattoir in warm saline (30-35°C). Follicles of non-atretic appearance and 3-8 mm in size were aspirated using an 18-gauge needle and 10 ml syringe. Cumulus oocyte complexes (COCs) with multiple intact cell layers and smooth, ungranulated ooplasms were collected from the total

aspirates, washed twice in handling medium and once in corresponding maturation media.

*Experiment 1: TCM199 vs. SFFM containing 2.3 mM glucose*

To determine whether SFFM supports oocyte nuclear maturation, COCs were cultured in either TCM199 or SFFM + 2.3 mM glucose + 5 mM glucosamine. Both media were supplemented with 0.1 IU/ml FSH (Puregon, Organon, Oss, Netherlands) and 0.1 IU/ml hCG (Pregnyl, Organon). COCs were cultured in groups of 10 in 100 µl of pre-equilibrated media, overlaid with mineral oil; in 6% CO<sub>2</sub> in humidified air at 39°C. At 16 h, 20 h, 24 h or 28 h, diameters of individual COCs were measured in the “X” and “Y” planes using a dissecting microscope, after which oocytes were mechanically denuded and fixed for assessment of nuclear progression. Five replicate experiments were performed with 10 COCs being cultured per treatment per replicate.

*Experiment 2: Glucose concentrations and glucosamine supplementation*

To determine the influence of different glucose concentrations and glucosamine supplementation on oocyte nuclear maturation, COCs were randomly divided into five culture groups, 1) TCM199, 2) SFFM + 2.3 mM glucose - glucosamine, 3) SFFM + 2.3 mM glucose + 5 mM glucosamine, 4) SFFM + 5.6 mM glucose - glucosamine and 5) SFFM + 5.6 mM glucose + 5mM glucosamine. All media were supplemented with 0.1 IU/ml FSH and 0.1 IU/ml hCG. COCs were cultured in groups of 10 in 100 µl pre-equilibrated drops of corresponding media, overlaid with mineral oil; in 6% CO<sub>2</sub> in humidified air at 39°C. After 24 h and 30 h of culture, COCs were removed, mechanically denuded and fixed for assessment of nuclear maturation. Eight replicate experiments were performed with 10 COCs being cultured per treatment per replicate.

*Experiment 3: Sequential culture system (6 h change)*

COCs were cultured in groups of 10 in 100 µl of either 1) TCM199 + gonadotrophins (0.1 IU/ml FSH and 0.1 IU/ml hCG), 2) SFFM + 2.3 mM glucose, 3)

SFFM + 2.3 mM glucose + gonadotrophins, 4) SFFM + 5.6 mM glucose or 5) SFFM + 5.6 mM glucose + gonadotrophins. All culture drops were overlaid with mineral oil and cultured in 6% CO<sub>2</sub> in humidified air at 39°C. After 6 h of culture, COCs were washed and transferred into TCM199 + gonadotrophins or SFFM + 5.6 mM glucose + gonadotrophins. After a total culture time of 24 h or 30 h, COCs were removed, denuded and fixed for assessment of nuclear maturation (Figure 1). Six replicate experiments were performed with 10 COCs being cultured per treatment per replicate.

*Experiment 4: Sequential culture system (16 h change)*

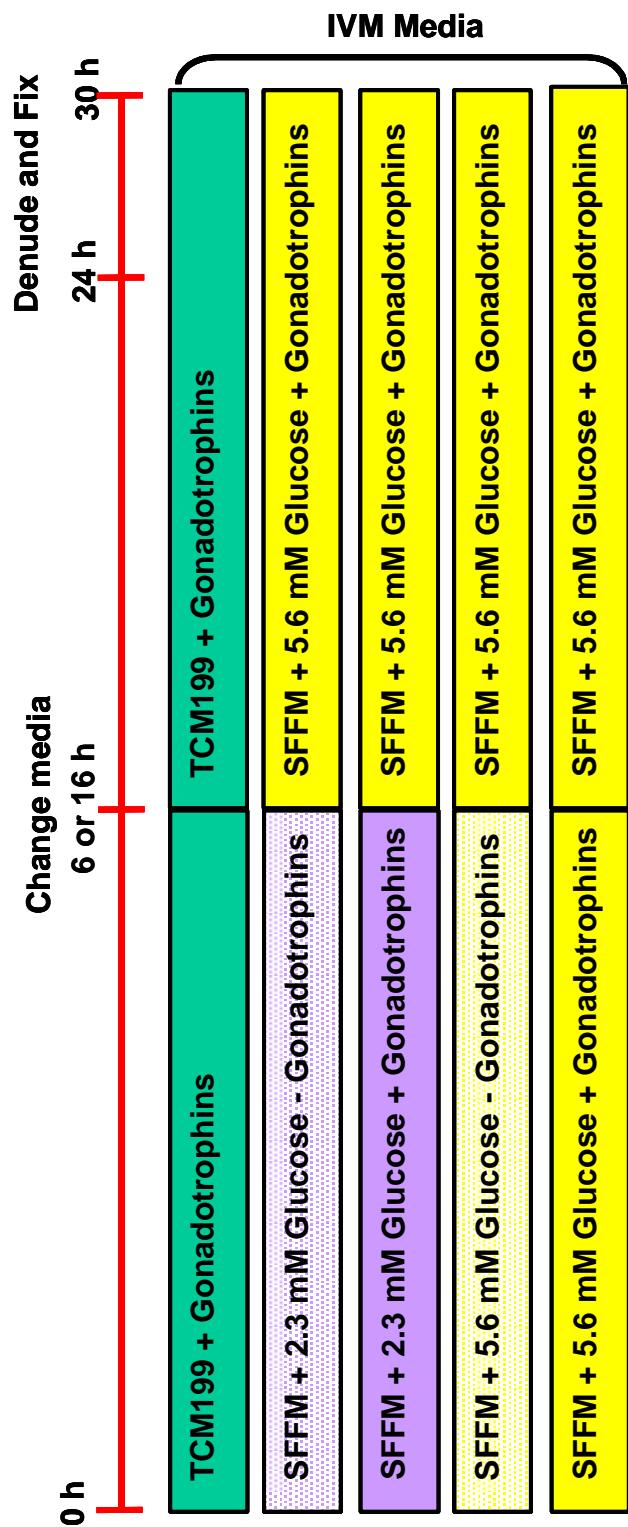
To determine the effect of duration of exposure to different glucose concentrations and hormones (FSH and hCG), Experiment 3 was repeated in its entirety except COCs were exposed to the different treatments for 16 h (rather than 6 h), and were then washed and transferred into TCM199 + gonadotrophins or SFFM + 5.6 mM glucose + gonadotrophins (Figure 1). Five replicate experiments were performed, with 10 COCs cultured per treatment and time point.

*Experiment 5: Influence of glucose and gonadotrophins on meiotic resumption*

To determine the influence of glucose concentration and gonadotropins (FSH and hCG) on meiotic resumption (germinal vesicle breakdown, GVBD), COCs were exposed to treatments and culture conditions as described for Experiment 3, but after 6 h of culture, COCs were removed, denuded and fixed for assessment of nuclear maturation. Five replicate experiments were performed with 20 COCs being cultured per treatment per replicate.

*Assessment of nuclear maturation*

At the designated time points, COCs were removed from culture and mechanically denuded in 50 IU/ml hyaluronidase. Denuded oocytes (DOs) were fixed (3:1 ethanol: acetic acid, 400 µl) for a minimum of 36 h and then DOs were mounted on microscope slides with a cover slip held with petroleum jelly. Orcein dye (1% orcein dissolved in 45% acetic acid) was drawn beneath the cover slip using capillary



**Figure 1.** Experimental design for Experiment 3 and 4. TCM199 = Tissue Culture Medium 199, Gonadotrophins = FSH + hCG, SFFM = Synthetic Follicular Fluid Medium.

action. After 20-30 mins, DOs were de-stained with 1:1:3 glycerol: acetic acid: water. Cover slips were glued with nail polish and the slides were examined at 400x with a phase contrast microscope. Nuclear maturation of DOs were classed as germinal vesicle stage (GV), diakinesis (germinal vesicle breakdown, GVBD), metaphase I (MI) or metaphase II (MII).

#### *Statistical analyses*

Diameters of COCs were compared using t-test. For assessment of nuclear maturation, the proportions of oocytes at GVBD or MII were arcsine transformed and analysed using analysis of variance (ANOVA) followed by Tukey test. All statistical analyses were performed using SigmaStat version 2.0 computer software (SPSS Inc, Chicago, IL, USA). Probabilities less than 0.05 were considered significantly different.

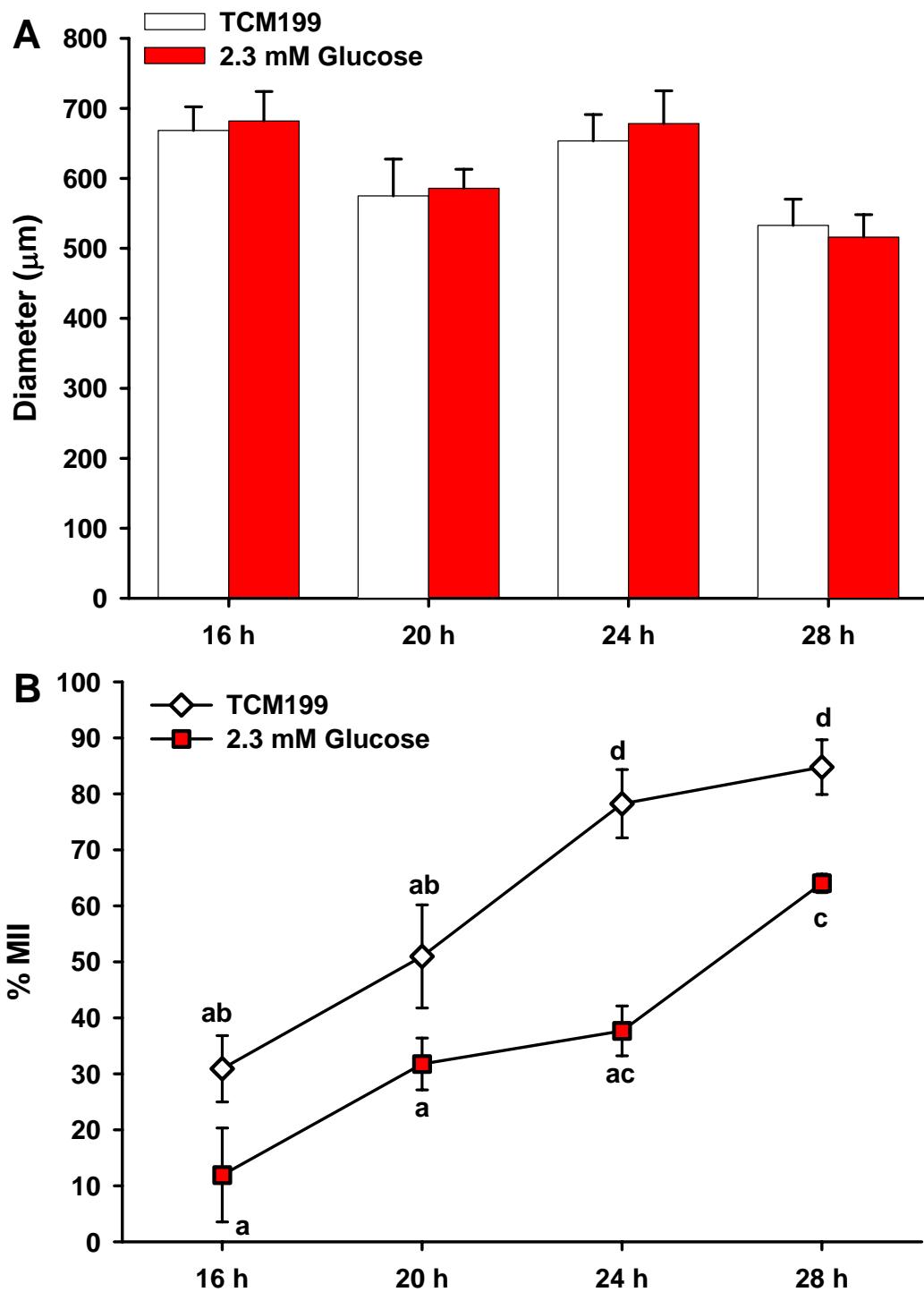
## **Results**

### *Experiment 1: TCM199 vs. SFFM containing 2.3 mM glucose*

The kinetics of COCs matured in either TCM199 (5.6 mM glucose) or SFFM containing 2.3 mM glucose were compared, to determine the influence of the new formulation, based on the composition of the in vivo environment. In particular, the combination of reduced glucose together with glucosamine was studied to ascertain whether it supported comparable cumulus expansion and to ascertain its effect on meiotic maturation.

Within each time point, the mean diameters of the COCs cultured in TCM199 and SFFM + 2.3 mM glucose were similar (Figure 2A,  $P > 0.05$ ), and the general appearance of the expanded COCs did not differ between the two media.

In regards to nuclear maturation, more COCs cultured in TCM199 completed nuclear maturation than those matured in SFFM + 2.3 mM glucose (main effect,  $P < 0.001$ ). This was particularly evident after 24 h of culture, where  $78.2 \pm 6.1\%$  of oocytes cultured in TCM199 were at MII, compared to just  $37.6 \pm 4.5\%$  of COCs cultured in



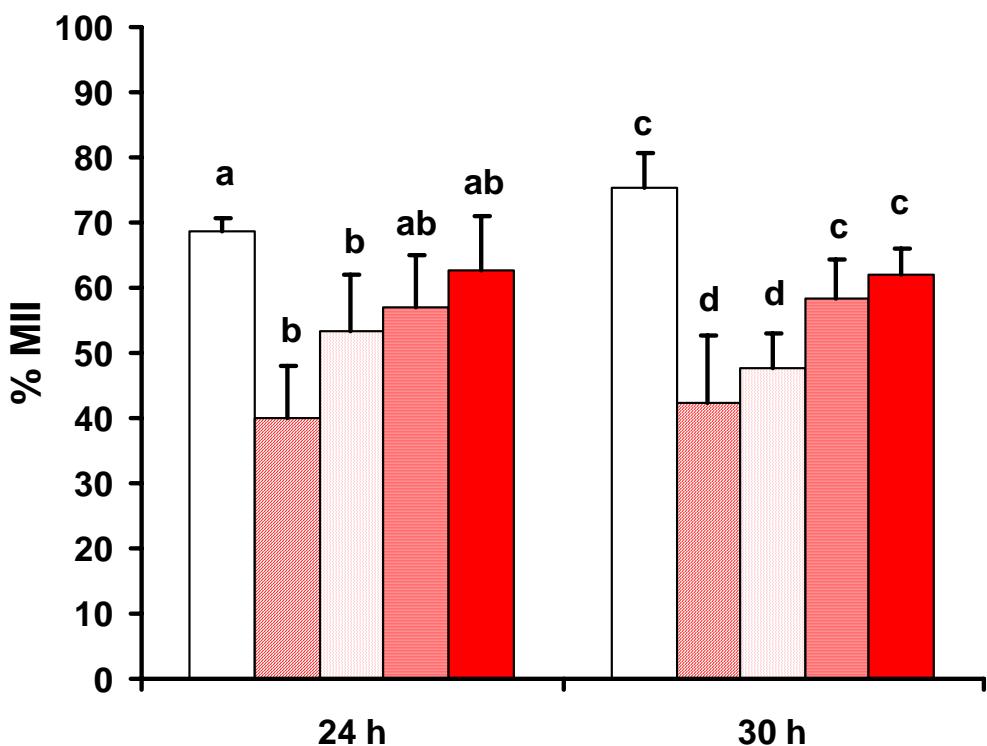
**Figure 2.** The influence of glucose and glucosamine on A) the mean values of horizontal/vertical diameters (mean  $\pm$  SEM) of cumulus oocyte complexes (COCs) and B) the ability to complete nuclear maturation (metaphase II, MII) when cultured in Tissue Culture Medium 199 (TCM199) or Synthetic Follicular Fluid Medium (SFFM) + 2.3 mM glucose + glucosamine for 16 h, 20 h, 24 h and 28 h. Media was supplemented with 0.1 IU/ml FSH and 0.1 IU/ml hCG. Data points with different letters are significantly different (<sup>ab, cd</sup> P < 0.05).

SFFM + 2.3 mM glucose (Figure 2B,  $P < 0.05$ ). There was approximately an 8 h delay in meiotic progression, since the proportion of COCs at MII after 24 h of culture in SFFM media + 2.3 mM glucose ( $37.6 \pm 4.5\%$ ) was comparable to the proportion of MII COCs after 16 h of culture in TCM199 ( $30.9 \pm 5.9\%$ ). This partial inhibition of meiotic progression continued, so that by 28 h,  $64 \pm 1.6\%$  of COCs cultured in SFFM had reached MII, compared to  $84.8 \pm 4.9\%$  of TCM199 matured oocytes.

#### *Experiment 2: Glucose concentrations and glucosamine supplementation*

The major differences between TCM199 and SFFM are the reduction in the concentration of glucose and the addition of glucosamine. SFFM with two different glucose concentrations were tested, 2.3 mM glucose representing physiological FF concentrations, and 5.6 mM glucose representing levels in TCM199. Media were either supplemented or devoid of glucosamine, to determine whether the delay in nuclear maturation in Experiment 1 was due to the decreased glucose concentration or the supplementation of media with glucosamine.

Regardless of the presence or absence of glucosamine, culturing COCs in SFFM with low glucose (2.3 mM) resulted in significantly less oocytes reaching MII after 30 h of culture compared to oocytes cultured in TCM199 (Figure 3; main effect, 2.3 mM glucose =  $45 \pm 5.7\%$  vs. TCM199 =  $75.3 \pm 5.3\%$  MII;  $P < 0.05$ ). Although there were no differences in MII proportions after 24 h when COCs were cultured in SFFM with either concentration of glucose, by 30 h, significantly more COCs had reached MII when cultured in 5.6 mM glucose compared to 2.3 mM glucose, regardless of glucosamine (main effect, 2.3 mM glucose =  $45 \pm 5.7\%$  and 5.6 mM glucose =  $60.1 \pm 3.6\%$  MII,  $P < 0.05$ ). Furthermore, COCs cultured in SFFM + 5.6 mM glucose (the same concentration of glucose as in TCM199) had comparable rates of nuclear maturation to TCM199 after 24 h and 30 h ( $P > 0.05$ ). Glucosamine supplementation had no effect on nuclear maturation after 24 h and 30 h (main effect,  $P > 0.05$ ).



**Figure 3.** The influence of glucose and glucosamine on the ability of cumulus oocyte complexes to complete nuclear maturation (metaphase II, MII). □ Tissue Culture Medium 199 (TCM199), ■ Synthetic Follicular Fluid Medium (SFFM) - glucosamine + 2.3 mM glucose, ▨ SFFM + 5 mM glucosamine + 2.3 mM glucose, ▨ SFFM - glucosamine + 5.6 mM glucose or ▨ SFFM + 5 mM glucosamine + 5.6 mM glucose. All media was supplemented with 0.1 IU/ml FSH and 0.1 IU/ml hCG. Different superscripts are significantly different (<sup>ab, cd</sup> P < 0.05).

### *Experiment 3: Sequential culture system (6 h change)*

The influences of different glucose concentrations, gonadotrophin supplementation (FSH and hCG) and a sequential culture system on nuclear maturation were determined. Initially COCs were cultured in media containing different glucose concentrations and  $\pm$  gonadotrophins. After 6 h of culture (coinciding with initiation of GVBD), COCs in all treatment groups were transferred into SFFM + 5.6 mM glucose + gonadotrophins; conditions that promote the completion of nuclear maturation.

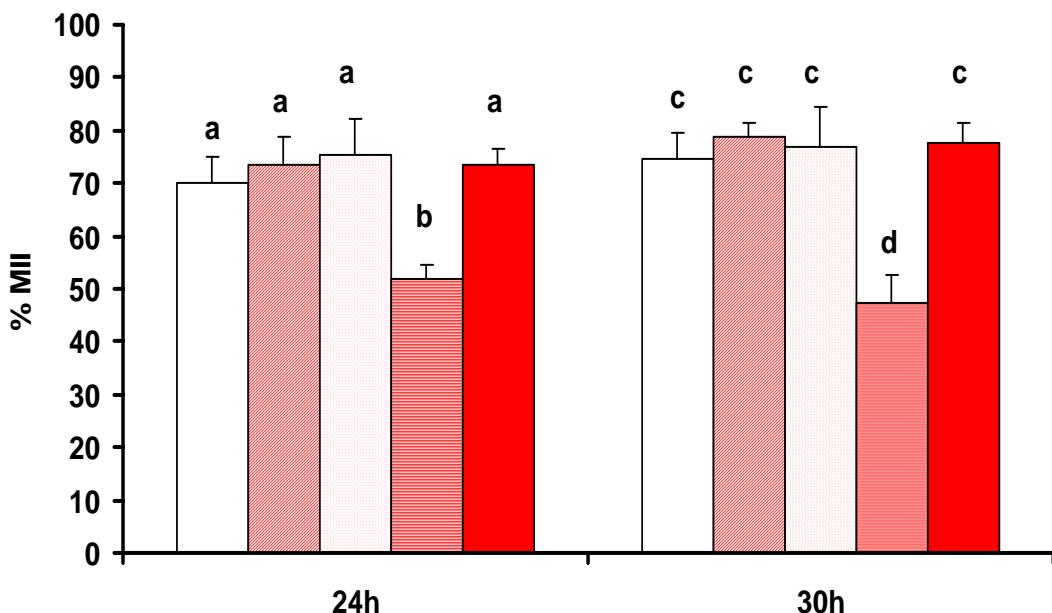
COCs cultured in SFFM + 5.6 mM glucose without gonadotrophins for the first 6 h had significantly less MII stage oocytes after 24 h compared to all other treatment groups (Figure 4,  $P < 0.05$ ). There were no significant differences between the different groups after 30 h ( $P > 0.05$ ), apart from the 1.5-fold lower fraction of MII oocytes initially cultured in SFFM + 5.6 mM glucose without gonadotrophins.

### *Experiment 4: Sequential culture system (16 h change)*

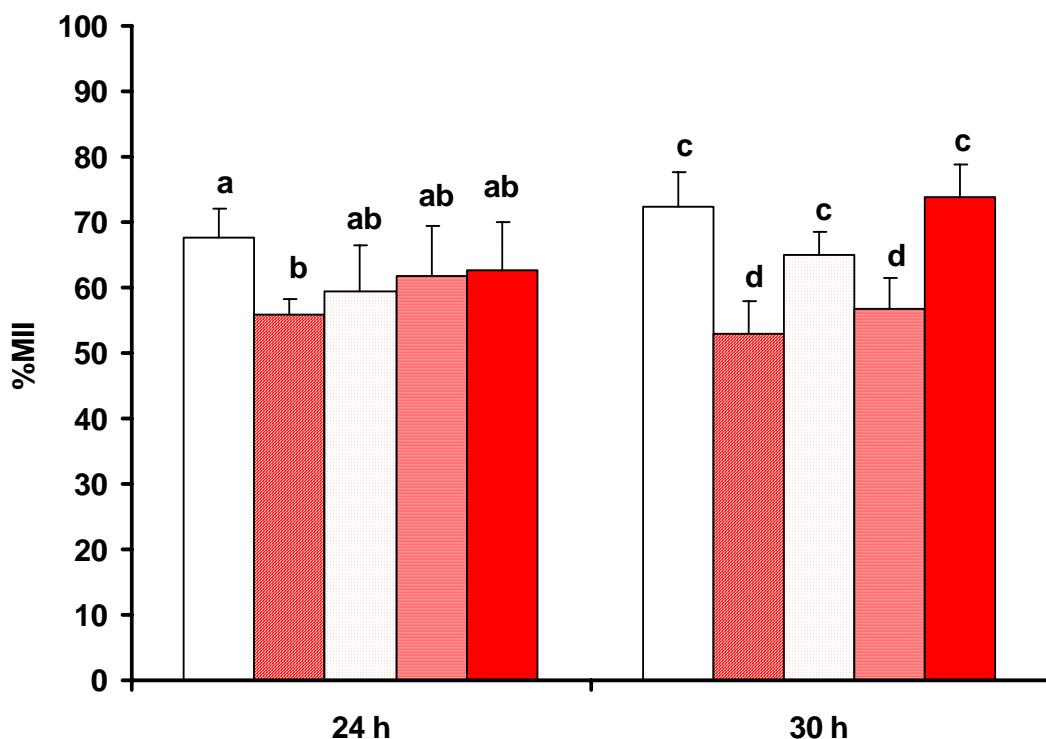
The influence of gonadotrophin supplementation and different glucose concentrations for the initial 16 h of IVM (coinciding with MI to MII progression) was determined. After 24 h of culture, COCs cultured in SFFM containing 2.3 mM glucose without gonadotrophins for the first 16 h, had significantly less oocytes at MII compared to the other treatment groups (Figure 5). By 30 h of culture, hormone supplementation led to significantly more oocytes completing nuclear maturation, regardless of glucose concentration (main effect; - gonadotrophins =  $54.1 \pm 6.2\%$  MII vs. + gonadotrophins =  $69.6 \pm 3.2\%$  MII,  $P < 0.05$ ).

### *Experiment 5: Influence of glucose and gonadotrophins on meiotic resumption*

The influences of gonadotrophins and glucose concentration on the resumption of meiosis were determined after 6 h of culture, coinciding with the initiation of GVBD. While there were no differences in the proportion of oocytes undergoing GVBD after being cultured in TCM199, SFFM + 2.3 mM glucose  $\pm$  gonadotrophins, and SFFM



**Figure 4.** The effect of gonadotrophins (FSH and hCG) for the first 6 h of IVM on meiotic progression under variable glucose concentrations. The proportion of oocytes that progressed to metaphase II (MII) after culture in □ Tissue Culture Medium 199 (TCM199), ■ Synthetic Follicular Fluid Media (SFFM + glucosamine) + 2.3 mM glucose - gonadotrophins, ▨ SFFM + 2.3 mM glucose + gonadotrophins, ▢ SFFM + 5.6 mM glucose - gonadotrophins and ▤ SFFM + 5.6 mM glucose + gonadotrophins for initial 6 h of culture. COCs were transferred into either TCM199 or SFFM + 5.6 mM glucose + gonadotrophins for the final culture period. All SFFM treatments contained 5 mM glucosamine. Different superscripts are significantly different (<sup>ab, cd</sup> P < 0.05).

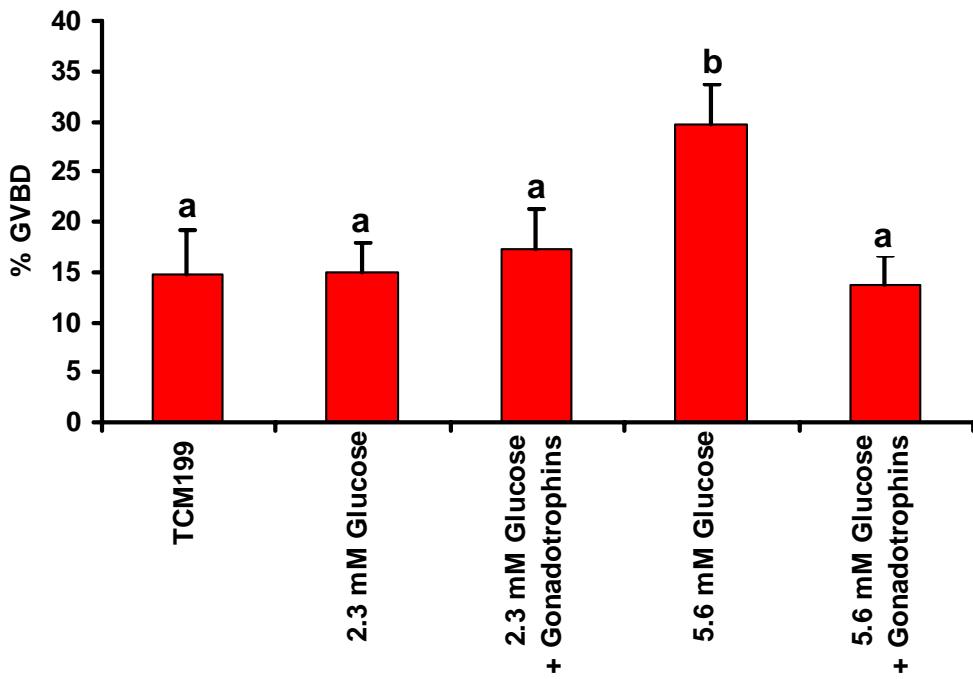


**Figure 5.** The effect of gonadotrophins (FSH and hCG) for the first 16 h of IVM on meiotic progression under variable glucose concentrations. The proportion of oocytes that progressed to metaphase II (MII) after culture in □ Tissue Culture Medium 199 (TCM199), ■ Syntetic Follicular Fluid Media (SFFM) + 2.3 mM glucose - gonadotrophins, ▨ SFFM + 2.3 mM glucose + gonadotrophins, ▢ SFFM + 5.6 mM glucose - gonadotrophins and ▢ SFFM + 5.6 mM glucose + gonadotrophins for initial 16 h of culture. COCs were transferred into either TCM199 or SFFM + 5.6 mM glucose + gonadotrophins for the final culture period. All SFFM treatments contained 5 mM glucosamine. Different superscripts are significantly different (<sup>ab, cd</sup> P < 0.05).

+ 5.6 mM glucose + gonadotrophins (Figure 6), there was a significant interaction between 5.6 mM glucose in the absence of gonadotrophins, leading to 2-fold more COCs cultured in this medium undergoing GVBD compared to the other treatments (Figure 6,  $P < 0.05$ ).

## Discussion

Follicular fluid analyses (described in Chapter 4) revealed that there is 2.4-fold lower glucose concentration in follicular fluid compared with TCM199 (the most commonly used medium for bovine IVM). Glucose is an important metabolite and it is utilised by the COC via numerous pathways during maturation (Rose-Hellekant *et al.* 1998; Krisher and Bavister 1999; Lim *et al.* 1999; Khurana and Niemann 2000; Zheng *et al.* 2001). A major role of glucose during oocyte maturation is ATP and carboxylic acid production via glycolysis, particularly in the initial stages of IVM (Rieger and Loskutoff 1994). We have previously demonstrated that towards the end of IVM, a significant proportion of glucose consumed by COCs is utilised for extracellular matrix synthesis during FSH-stimulated cumulus expansion (Chapter 3). As such, COCs in high-density IVM culture systems are at risk of glucose starvation towards the end of IVM. The addition of glucosamine to IVM media leads to decreased glucose consumption, since glucosamine is preferentially used for matrix synthesis over glucose. Therefore, I hypothesised that the addition of glucosamine to IVM media would be a viable means to decreasing glucose concentrations in media to more physiological levels, while still allowing for adequate mucification, since the use of low glucose concentrations without glucosamine during IVM has previously resulted in suboptimal cumulus expansion (Rose-Hellekant *et al.* 1998). The present study has demonstrated that COCs cultured in SFFM + 2.3 mM glucose + glucosamine had comparable cumulus expansion to COCs cultured in TCM199; hence under low glucose conditions, glucosamine supplementation supplies the COC with adequate substrates for mucification. In the presence of low glucose (SFFM + 2.3 mM), significantly less oocytes completed nuclear maturation compared to oocyte cultured in higher glucose (SFFM + 5.6 mM and TCM199) and this effect was independent of glucosamine supplementation.



**Figure 6.** The influence of glucose and gonadotrophins (FSH and hCG) on meiotic resumption (germinal vesicle bre-akdown, GVBD) after 6 h of culture. Different superscripts are significantly different (<sup>ab</sup> P < 0.05). TCM199 = Tissue Culture Medium 199, 2.3 mM Glucose = Synthetic Follicular Fluid Medium (SFFM) + 2.3 mM glucose, 2.3 mM Glucose + Gonadotrophins = SFFM + 2.3 mM glucose + gonadotrophins, 5.6 mM Glucose = SFFM + 5.6 mM glucose, 5.6 mM Glucose + Gonadotrophins = SFFM + 5.6 mM glucose + gonadotrophins. All SFFM treatments contained 5 mM glucosamine.

Downs et al (Downs *et al.* 1998) reported a link between increased glucose concentrations in media and meiotic resumption in mouse oocytes, via increasing the flux of glucose through the pentose phosphate pathway. A product of this pathway, phosphoribosylphosphate is an important substrate for the synthesis of purine nucleotides via the de novo and salvage pathways (Downs 1997). Increased activities of both pathways, in particular de novo synthesis, are important for FSH-stimulated meiotic maturation (Downs 1997).

The influence of a sequential culture system on nuclear maturation was examined since the proportion of oocytes reaching MII appeared to plateau after 24 h and 30 h in SFFM + 2.3 mM glucose medium and this may have been due to substantial depletion of glucose from media to levels detrimental to oocyte maturation, especially in low glucose concentrations. The media changes coincided approximately with the resumption of meiosis, (~6 h in bovine oocytes; Wehrend and Meinecke 2001) and the MI to MII transition. In a static culture system, over the 24 h maturation period physiological (follicular) glucose concentrations (2.3 mM) fail to support normal rates of nuclear maturation, whereas unphysiological concentrations (5.6 mM) are required to support completion of maturation at levels similar to TCM199 within this time period. However, SFFM + 5.6 mM glucose minus gonadotrophins for the initial 6 h of culture promoted rapid GVBD, introduction of gonadotrophins at 6 h of maturation led to a MI to MII transition block at 24 h and 30 h. This was a surprising and unexpected result. Previous work performed in our laboratory has demonstrated that culturing COCs in TCM199 without gonadotrophins stimulates rapid GVBD compared to TCM199 + gonadotrophins (Thomas *et al.* 2004). This was attributed to gonadotrophins delaying the onset of GVBD (Thomas *et al.* 2004), most likely by raising intracellular cAMP levels. However, here we reveal that the effect is likely mediated by high glucose concentrations (as found in TCM199), as incubation in low glucose (2.3 mM) in the absence of FSH and hCG does not promote this precocious rate of GVBD. Our results suggest that glucose concentration plays not only a role in the rate of meiotic completion, but also a fundamental role in regulating initiation of GVBD in bovine oocytes and that gonadotrophins influence this effect.

The addition of gonadotrophins to a high glucose maturation system, 6 h following initiation of maturation, severely inhibits the MI to MII transition. The activity of maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) are known to be vital for the progression of oocyte meiosis (Wehrend and Meinecke 2001; Jones 2004). Glucose-induced precocious GVBD with delayed gonadotrophin addition most likely causes an asynchrony between meiotic progression and FSH-regulated MPF levels, leading to a MI block. This effect appears to be dependent on both the timing of gonadotrophin reintroduction (i.e. between 6-16 h) and the concentration of glucose, as the effect is not observed in the presence of low glucose. Furthermore the MI to MII block is lost if gonadotrophins are either present or absent for most of the maturation period. Although the mechanisms by which glucose concentration regulates meiotic progression remains unclear, these results suggest that significant perturbation of meiosis can be achieved simply by altering glucose concentrations. To our knowledge, with acknowledgement to the work of Downs and colleagues in the mouse, this is the first time that glucose concentration has been reported to significantly regulate meiotic progression in oocytes of a species other than the mouse.

The mechanisms controlling oocyte nuclear maturation have been well documented in numerous species (Eppig and Downs 1984; Thibault *et al.* 1987; Eppig 1996; Sirard *et al.* 1998; Sirard 2001; Combelles *et al.* 2002). In rodent and bovine models, it has been shown that molecules that elevate or inhibit the hydrolysis of intra-oocyte cAMP, such as purines (i.e. adenosine and hypoxanthine), phosphodiesterase inhibitors (rolipram and milrinone) and dbcAMP, arrest or delay meiotic resumption. Molecules that have successfully been used to control meiosis in rodent oocytes, such as purines (Eppig *et al.* 1985; Downs and Eppig 1987; Tornell *et al.* 1990), are not as effective in controlling meiosis in oocytes from ruminants (Sirard and First 1988; Miyano *et al.* 1995). Such studies have used TCM199 as the base medium, which contains higher glucose concentration than in bovine follicular fluid. The combination of lower, physiological glucose levels plus inhibitors may facilitate better control of meiosis in bovine oocytes.

In summary, this study has demonstrated that oocytes matured in media based on physiological ion and energy substrate conditions, in particular decreased glucose

concentrations, have perturbed completion of nuclear maturation compared to oocytes cultured in TCM199. This is despite COCs undergoing normal cumulus expansion, even in the presence of reduced glucose levels. The use of different glucose concentrations and gonadotrophin supplementation in a sequential culture system manipulated the kinetics and the capacity of oocytes to undergo and complete nuclear maturation. Further investigation of the impact of sequential systems on meiotic progression and subsequent developmental competency is warranted.

## Acknowledgements

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# **Chapter 6**

**The effect of glucosamine supplementation during in vitro maturation on the developmental capacity of bovine oocytes**

## **Abstract**

Glucosamine has previously been shown to decrease the uptake of glucose by bovine cumulus oocyte complexes (COCs), presumably by providing an alternative substrate for extracellular matrix synthesis during cumulus expansion. While earlier studies demonstrated that glucosamine has no effect on oocyte nuclear maturation, its influence on cytoplasmic maturation and developmental capacity are unknown. The aim of this study was to determine the effect of glucosamine during in vitro maturation (IVM) on subsequent embryo development. Bovine COCs were matured in Tissue Culture Medium 199 (TCM199) or Synthetic Follicular Fluid Medium with 5.6 mM glucose (SFFM) ± glucosamine. Glucosamine supplementation led to significantly reduced blastocyst development in a dose-dependent manner ( $P < 0.05$ ). Whilst IVM media containing 0.5 mM glucosamine reduced blastocyst development ~2-fold, compared to medium lacking glucosamine, concentrations of 2.5 and 5 mM effectively prevented blastocyst development (0.8 ± 0.8 and 4.9 ± 2.5% blastocyst development, respectively). The detrimental effects of glucosamine during IVM specifically affected embryo developmental competence, as cleavage rates were comparable to oocytes that were cultured in the absence of glucosamine. Perturbed embryo development coincided with glucosamine reducing protein synthesis within oocytes during IVM, implying a possible cause of reduced developmental competence. However, the addition of EGF and glucosamine during IVM led to protein synthesis levels significantly higher than oocytes cultured without glucosamine ( $P < 0.05$ ). This study demonstrated that glucosamine supplementation during IVM is detrimental to oocyte protein synthesis and embryo development. The influence of combined EGF and glucosamine supplementation oocyte developmental capacity has yet to be determined.

## **Introduction**

Although pyruvate is the preferred energy substrate of the oocyte (Biggers *et al.* 1967), glucose is the major substrate used by cumulus bovine oocyte complexes (COCs) during in vitro maturation (IVM, Chapter 2), and its addition to culture medium leads to increased developmental capacity and embryo development (Rose-Hellekant *et al.* 1998; Lim *et al.* 1999; Zheng *et al.* 2001).

While a large proportion of glucose consumed by COCs is metabolised via the glycolytic pathway to lactate, in the latter period of oocyte maturation, a considerable proportion is used as a substrate for extracellular matrix synthesis during cumulus expansion (Chapter 3). Glucose concentration can also influence progression of nuclear maturation via the pentose phosphate pathway and de novo purine synthesis (Downs and Mastropolo 1994; Downs *et al.* 1998; Downs and Hudson 2000). Since glucose concentration has significant effects on oocyte maturation and subsequent developmental potential, it is of note that *in vivo*, concentrations of glucose in large antral follicles are 2.4-fold lower than that in commonly used IVM media.

Investigations in this thesis into glucose utilisation by bovine COCs demonstrated that the presence of glucosamine partially attenuated glucose depletion from maturation media (Chapter 3), since glucosamine is preferentially used for extracellular matrix synthesis. In the absence of glucosamine, a considerable proportion of glucose is depleted from media, despite initial concentrations being higher than follicular fluid concentrations. I therefore hypothesised that the addition of glucosamine to oocyte maturation media may permit glucose concentrations to be reduced to physiological level, while still providing COCs with adequate substrates to undergo cumulus expansion.

Synthetic Follicular Fluid Medium (SFFM) was developed, based on the ion and energy substrate composition of bovine follicular fluid, measured in Chapter 4. Two different glucose concentrations were tested, 2.3 mM glucose representing physiological concentrations and 5.6 mM glucose representing the concentration in TCM199. The addition of different glucose concentrations to SFFM had variable

effects on the completion of oocyte nuclear maturation, dependant on hormone supplementation and exposure time (Chapter 5). Furthermore, glucosamine had no effect on nuclear maturation. The aim of this study was to determine the effect of glucosamine supplementation during IVM on the embryo developmental capacity of bovine COCs.

## **Materials and method**

Unless specified, all reagents were purchased from Sigma (St Louis, MO, USA). Handling medium was HEPES-buffered Tissue Culture Medium 199 (TCM199; ICN Biochemicals, Irvine, CA, USA) supplemented with 0.5 mM sodium pyruvate and 4 mg/ml fatty acid free BSA (ICPbio Ltd, Auckland, NZ). Control medium for oocyte maturation was bicarbonate-buffered TCM199 supplemented with 0.5 mM sodium pyruvate and 4 mg/ml fatty acid free BSA (TCM199). Synthetic follicular fluid medium (SFFM) is a defined medium based on the composition of FF from antral follicles (Chapter 4) and contains 5.6 mM glucose, non-essential and essential amino acids (ICN Biochemicals), 4 mg/ml fatty acid free BSA and unless indicated, 5 mM glucosamine. All IVM treatments were supplemented with 0.1 IU/ml FSH (Puregon, Organon, Oss, Netherlands) and, 0.1 IU/ml hCG (Pregnyl, Organon).

### *Oocyte collection*

Bovine ovaries were transported from a local abattoir in warm saline (30-35°C). Follicles of non-atretic appearance and 3-8 mm in size were aspirated using an 18-gauge needle and 10 ml syringe. Cumulus oocyte complexes (COCs) with multiple intact cell layers and smooth, ungranulated ooplasm were collected from the total aspirates, washed twice in handling medium and once in corresponding maturation media.

### *Glucosamine supplementation during in vitro maturation*

To determine the influence of SFFM and glucosamine supplementation during IVM on oocyte developmental capacity, COCs were cultured in groups of 10 in 100 µl of

either 1) TCM199, 2) – Glc (SFFM) or 3) + Glc (SFFM + 5 mM glucosamine). Culture drops were overlaid with mineral oil and cultured in 6% CO<sub>2</sub> in humidified air at 39°C. After 24 h of culture, COCs were fertilized with either 1 x 10<sup>6</sup> or 5 x 10<sup>6</sup> spermatozoa/ml. The latter high spermatozoa concentration was used to assess if glucosamine supplementation altered matrix formation and required increased number of spermatozoa in the insemination period. Five replicate experiments were performed with 40 COCs used per treatment group.

#### *Glucosamine dose-response and embryo development*

To determine the effects of different glucosamine concentrations on oocyte developmental capacity, groups of 10 COCs were cultured in 100 µl of TCM199, SFFM, or SFFM supplemented with 0.5, 1, 2.5 or 5 mM glucosamine. Media drops were overlaid with mineral oil and COCs were cultured in 6% CO<sub>2</sub> in humidified air at 39°C. After 24 h, COCs were fertilized with 1 x 10<sup>6</sup> spermatozoa/ml. Five replicate experiments were performed with 30 COCs used per treatment.

#### *Oocyte protein synthesis during in vitro maturation in glucosamine and EGF supplemented media*

To determine whether glucosamine supplementation during IVM was detrimental to protein synthesis within the oocyte, 10 COCs were cultured individually in 10 µl pre-equilibrated drops of SFFM ± 5 mM glucosamine ± 10 ng/ml EGF. Media were also supplemented with 1 µM L-[2, 3, 4, 5, 6 – <sup>3</sup>H] phenylalanine (specific activity = 4.55 TBq/mol; Amersham Bioscience, Buckinghamshire, England). Drops were overlaid with mineral oil and COCs were cultured in 6% CO<sub>2</sub> in humidified air at 39°C. After 24 h of culture, COCs within each treatment group were pooled and were mechanically denuded in 50 IU/ml hyaluronidase. Denuded oocytes (DOs) were washed three times in HEPES-buffered TCM199 and transferred into a 1.6 ml Eppendorf tube in 5 µl of medium. Five µl of 4% BSA (w/v) solution and 100 µl of ice cold 20% trichloroacetic acid (TCA, w/v) was added to the Eppendorf tubes and incubated overnight at 4°C. The protein precipitate was washed with 100 µl of ice cold 5% TCA (w/v) and redissolved in 50 µl of 0.3 M NaOH. Aqueous phase scintillation cocktail (1 ml, Opti Phase Supermix cocktail, EG&G Wallac, Turku,

Finland) was added to each tube and  $^3\text{H}$  radioactivity was measured using a Microbeta Trilux counter (EG&G Wallac). Controls included BSA precipitate with no radiolabel, NaOH without BSA and samples with known amounts of radioactivity. Five replicate experiments were performed with 10 COCs used per treatment.

#### *In vitro fertilization and embryo culture*

Frozen-thawed semen from a single sire of proven fertility was prepared and used as described by (Thompson *et al.* 2000). Briefly, two straws (0.25 ml) of semen stored in liquid nitrogen were rapidly thawed and semen was layered on top of a discontinuous (45%: 90%) Percoll gradient (Amersham Pharmacia Biotech, Sweden). The gradient was centrifuged at room temperature for 20-25 mins at 700 g. The supernatant was removed and the spermatozoa pellet was washed with 500  $\mu\text{l}$  Bovine Vitro Wash (Cook Australia, Eight Mile Plains, Qld, Australia) and centrifuged for 5 mins at 200 g. The spermatozoa was resuspended with IVF medium (Bovine Vitro Fert, Cook Australia, supplemented with 0.01 mM heparin, 0.2 mM penicillamine and 0.1 mM hypotaurine), and added to the insemination drops at a final concentration of either  $1 \times 10^6$  or  $5 \times 10^6$  spermatozoa/ml. Ten COCs were inseminated for 24 h in 100  $\mu\text{l}$  drops of IVF medium, overlaid with mineral oil, at 39°C in 6%  $\text{CO}_2$  in humidified air.

Presumptive zygotes were transferred to Bovine Vitro Wash, mechanical denuded of their cumulus vestments using a finely drawn glass pipette and washed twice in Bovine Vitro Cleave (Cook Australia). Five presumptive zygotes were transferred into 20  $\mu\text{l}$  micro drops of pre-equilibrated Bovine Vitro Cleave overlaid with mineral oil and cultured for five days (Day 1 to Day 5) at 39°C in 7%  $\text{O}_2$ , 6%  $\text{CO}_2$ , balance  $\text{N}_2$  atmosphere. On Day 5, embryos were transferred to 20  $\mu\text{l}$  micro drops of pre-equilibrated Bovine Vitro Blast overlaid with mineral oil and cultured to Day 8.

### *Statistical analyses*

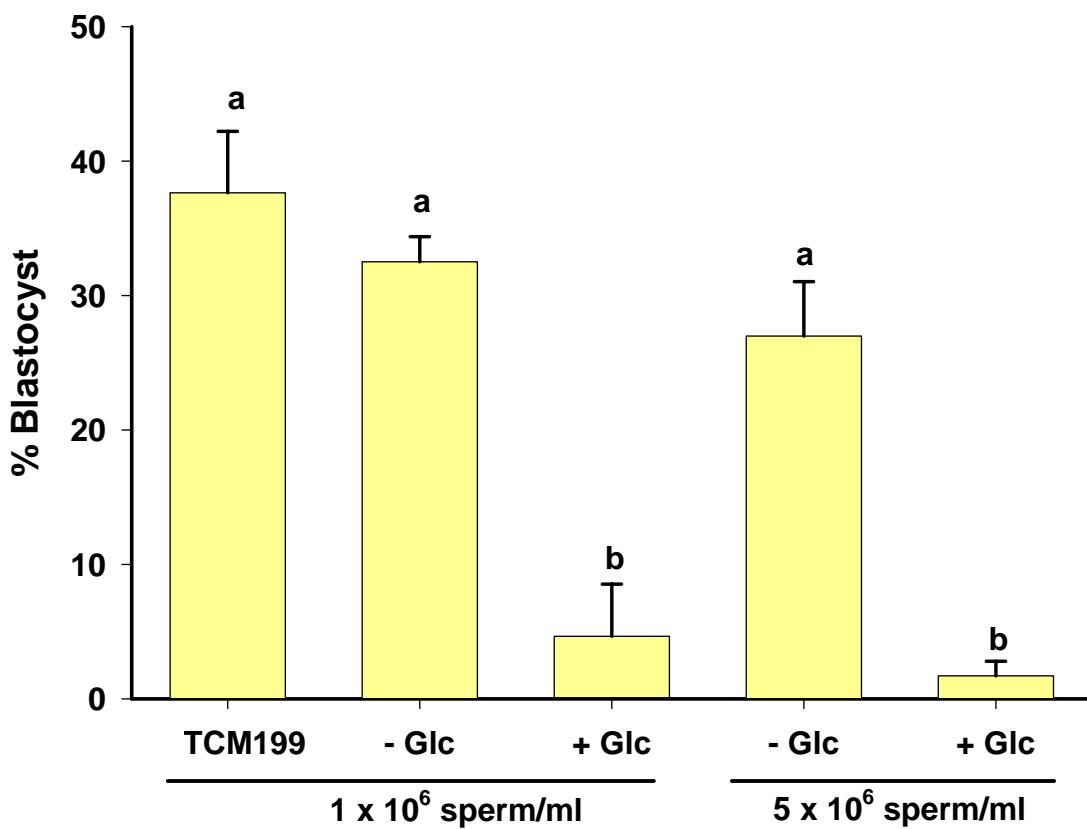
The frequencies of embryo development were arcsine transformed and radioactivity counts from the protein synthesis assay were expressed as fold change relative to the control sample. Data were analysed using analysis of variance (ANOVA) followed by Tukey test and all statistical analyses were performed using SigmaStat version 2.0 computer software (SPSS Inc, Chicago, IL, USA). Probabilities less than 0.05 were considered significantly different.

## **Results**

### *Glucosamine supplementation during in vitro maturation*

To investigate the influence of glucosamine supplementation during IVM on subsequent embryo development, COCs were cultured in SFFM + 5.6 mM glucose ± 5 mM glucosamine. After 24 h, COCs were fertilized with two different spermatozoa concentrations as preliminary experiments demonstrated low cleavage rates occurred when COCs, matured in the presence of glucosamine, were fertilized in the presence of standard spermatozoa concentrations (data not shown).

There were no significant differences in cleavage rates of embryos between all treatment groups (Table 1. P > 0.05). However significantly less oocytes matured in SFFM + glucosamine and fertilized in  $5 \times 10^6$  sperm/ml reached the 4-cell embryo stage, than any other treatment group. On Day 8 of embryo culture, oocytes that were matured in SFFM – glucosamine had a similar proportion of cleaved zygotes reaching the blastocyst stage compared to oocytes matured in TCM199 (Figure 1, P > 0.05). Significantly less blastocysts developed from oocytes that were matured in SFFM + 5.6 mM glucose + 5 mM glucosamine (Figure 1, main effect: - glucosamine 32% vs. + glucosamine 4%, P < 0.001). The concentration of spermatozoa used to fertilize the oocytes did not effect blastocyst development.



**Figure 1.** Embryo development of bovine oocytes matured in media ± glucosamine and fertilized with different spermatozoa concentrations. Values are expressed as the proportion of cleaved embryos that developed to the blastocyst stage. TCM199 = Tissue Culture Medium 199, - Glc = Synthetic Follicular Fluid Medium - glucosamine, + Glc = Synthetic Follicular Fluid Medium + 5 mM glucosamine. Different superscripts are significantly different (<sup>ab</sup> P < 0.05).

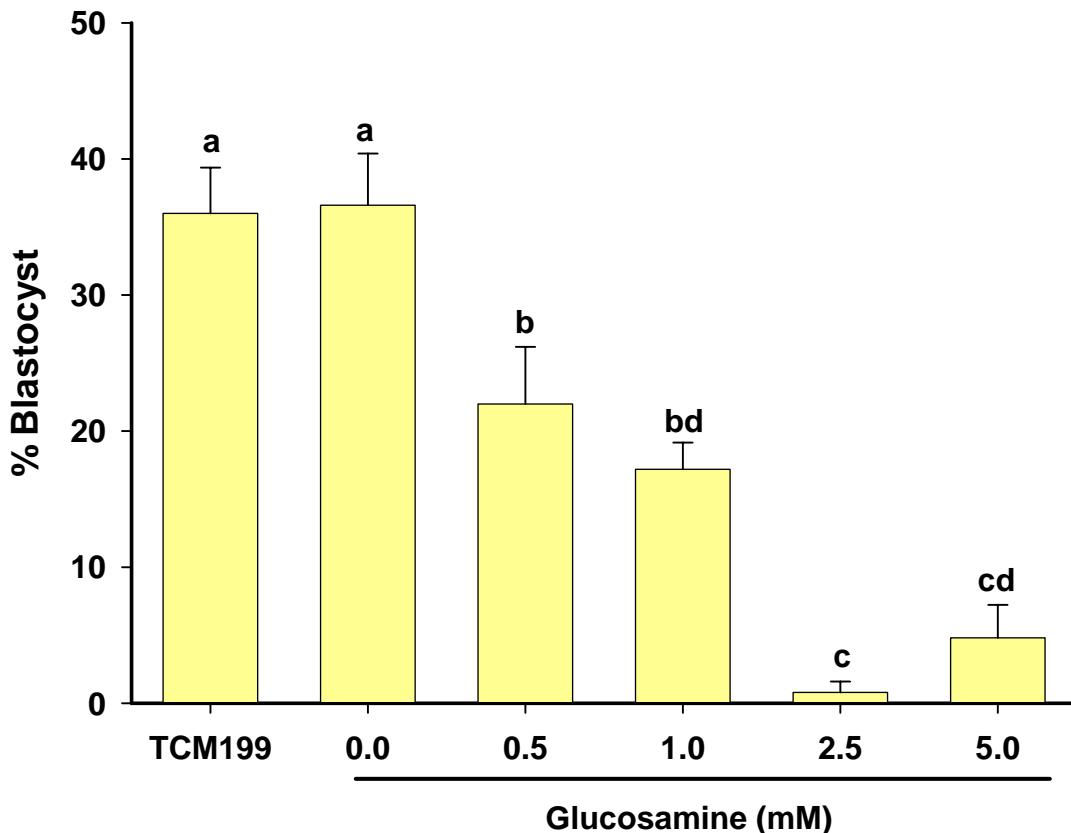
**Table 1.** The influence of glucosamine supplementation during in vitro maturation and sperm concentrations during in vitro fertilization on embryo development.

Culture Media	Sperm (per ml)	% Cleaved/Total (n)	% $\geq 4$ Cells/Cleaved (n)	% Morulae and Blastocyst/Cleaved (n)	% Blastocyst/Cleaved (n)
TCM199	<b>1 x 10<sup>6</sup></b>	79.3 ± 3.0 <sup>a</sup> (148/186)	87.8 ± 4.7 <sup>b</sup> (131/148)	72 ± 10.3 <sup>d</sup> (109/148)	37.6 ± 4.6 <sup>f</sup> (56/148)
- Glc	<b>1 x 10<sup>6</sup></b>	75.7 ± 7.8 <sup>a</sup> (146/192)	85 ± 3.8 <sup>b</sup> (124/146)	61.8 ± 8.3 <sup>d</sup> (91/146)	32.5 ± 1.9 <sup>f</sup> (50/146)
+ Glc	<b>1 x 10<sup>6</sup></b>	67.5 ± 5.7 <sup>a</sup> (121/179)	85.4 ± 2.1 <sup>b</sup> (104/121)	34.3 ± 6 <sup>e</sup> (44/121)	4.7 ± 3.9 <sup>g</sup> (6/121)
- Glc	<b>5 x 10<sup>6</sup></b>	71.9 ± 4.7 <sup>a</sup> (136/188)	82.4 ± 2.9 <sup>b</sup> (112/136)	51.3 ± 7.8 <sup>d</sup> (70/136)	27 ± 4.1 <sup>f</sup> (37/136)
+ Glc	<b>5 x 10<sup>6</sup></b>	64.5 ± 5.3 <sup>a</sup> (125/193)	72.5 ± 2.3 <sup>c</sup> (91/125)	33.7 ± 9.6 <sup>e</sup> (39/125)	1.7 ± 1.1 <sup>g</sup> (3/125)

TCM199 = Tissue Culture Medium 199, Glc = Synthetic Follicular Fluid Medium ± glucosamine. Different superscripts within columns are significantly different (<sup>a, bc, de, fg</sup> P < 0.05)

#### *Glucosamine dose-response and embryo development*

The influence of maturing COCs in SFFM + 5.6 mM glucose with different concentrations of glucosamine on subsequent embryo development was determined. Embryo cleavage rates ranged from 68.3 ± 2.6 to 82.5 ± 3.3% and there was no variation detected between treatment groups for the proportion of cleaved zygotes and embryos  $\geq 4$  cells (Table 2. P > 0.05). The proportion of cleaved zygotes that reached the morulae and blastocyst stage was similar between COCs cultured in TCM199 and SFFM + 5.6 mM glucose (Figure 2, P > 0.05). However, the addition of 0.5 mM and 1 mM glucosamine to SFFM approximately halved blastocyst rates compared to oocytes matured in media without glucosamine (P < 0.05), with the addition of 2.5 and 5 mM glucosamine to IVM being the most detrimental to subsequent embryo development (2.5 mM = 0.8 ± 0.8% and 5 mM = 4.9 ± 2.5% blastocysts).



**Figure 2.** Dose effect of glucosamine during in vitro maturation on embryo development, expressed as the proportion of cleaved embryos that developed to the blastocyst stage. TCM199 = Tissue Culture Medium 199, 0 to 5 represent the concentration (mM) of glucosamine added to Synthetic Follicular Fluid Medium during maturation. Different superscripts are significantly different (<sup>abcd</sup> P < 0.05).

**Table 2.** Glucosamine dose-response during in vitro maturation and the effect on embryo development.

Culture Media	% Cleaved/Total (n)	% ≥ 4 Cells/Cleaved (n)	% Morulae and Blastocyst/Cleaved (n)	% Blastocyst/Cleaved (n)
<b>TCM199</b>	82.5 ± 3.3 <sup>a</sup> (152/183)	84.3 ± 5.8 <sup>b</sup> (131/152)	58.4 ± 6.8 <sup>c</sup> (90/152)	36.2 ± 3.3 <sup>e</sup> (55/152)
<b>0 mM</b>	78.7 ± 4 <sup>a</sup> (147/185)	87.2 ± 7.4 <sup>b</sup> (132/147)	59 ± 7.4 <sup>c</sup> (90/147)	36.9 ± 3.8 <sup>e</sup> (56/147)
<b>0.5 mM</b>	79 ± 6.3 <sup>a</sup> (142/178)	82.4 ± 3.4 <sup>b</sup> (119/142)	41.3 ± 8.2 <sup>c</sup> (58/142)	22.1 ± 4.3 <sup>f</sup> (29/142)
<b>1 mM</b>	78.5 ± 1.7 <sup>a</sup> (149/190)	84.7 ± 4.1 <sup>b</sup> (127/149)	23.4 ± 3 <sup>d</sup> (35/149)	17.1 ± 1.9 <sup>fg</sup> (25/149)
<b>2.5 mM</b>	70.6 ± 3.5 <sup>a</sup> (124/177)	77.7 ± 5.2 <sup>b</sup> (96/124)	10.5 ± 5.2 <sup>d</sup> (13/124)	0.8 ± 0.8 <sup>h</sup> (1/124)
<b>5 mM</b>	68.3 ± 2.6 <sup>a</sup> (117/170)	76.8 ± 9.7 <sup>b</sup> (94/117)	12.7 ± 3.1 <sup>d</sup> (15/117)	4.9 ± 2.5 <sup>hg</sup> (5/117)

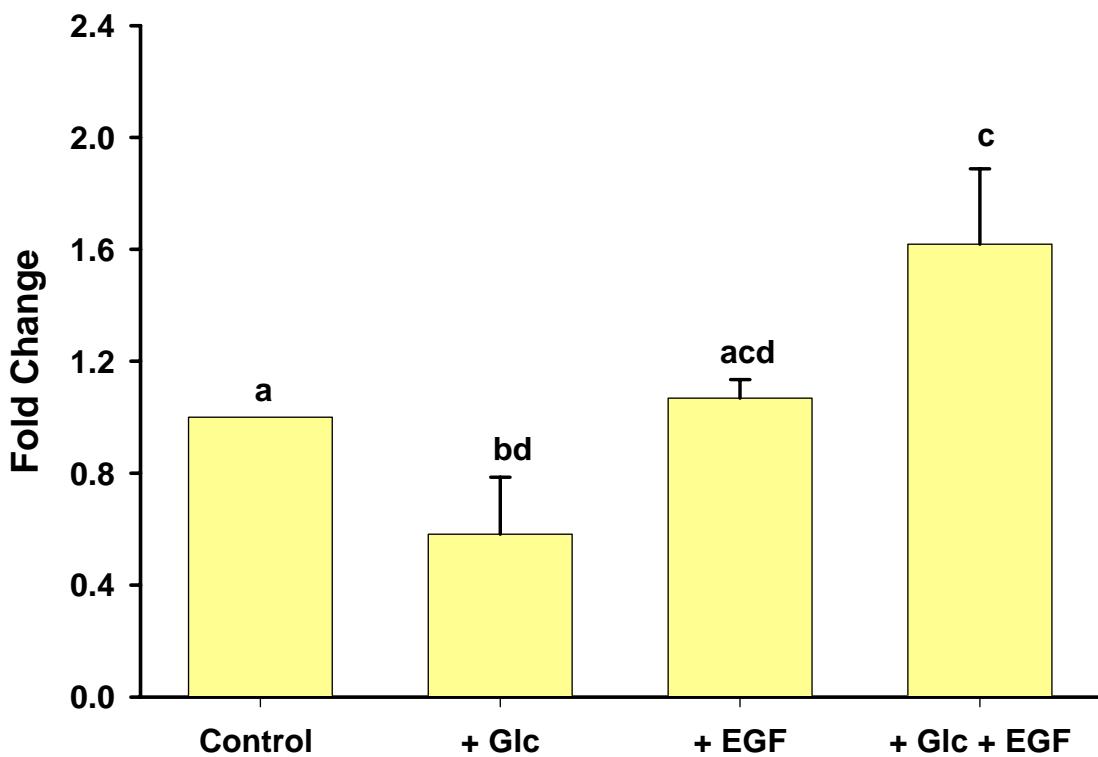
TCM199 = Tissue Culture Medium 199, 0-5 = the concentration of glucosamine added to Synthetic Follicular Fluid Medium. Different superscripts within columns are significantly different (<sup>a, b, cd, efgh</sup> P < 0.05).

### *Oocyte protein synthesis during in vitro maturation in glucosamine and EGF supplemented media*

The influence of glucosamine and EGF on protein synthesis within the oocyte was determined by measuring [2, 3, 4, 5, 6-<sup>3</sup>H] phenylalanine incorporation in the TCA-precipitation fraction. The presence of glucosamine during IVM lead to significantly less [2, 3, 4, 5, 6-<sup>3</sup>H] phenylalanine incorporation compared to control and EGF-treated COCs (Figure 3, main effect; P < 0.05). A significant interaction existed between EGF and glucosamine, where in the absence of EGF, glucosamine reduced [2, 3, 4, 5, 6-<sup>3</sup>H] phenylalanine incorporation (P < 0.05), but in the presence of EGF, this effect was reversed in which an increased level of incorporation was observed.

## Discussion

Glucosamine is an alternative substrate to glucose for extracellular matrix synthesis during cumulus expansion [Salustri, 1989 #282; Chen, 1990 #280] and its supplementation during IVM reduces glucose consumption by bovine COCs



**Figure 3.** Protein synthesis by bovine oocytes during in vitro maturation after culture in Synthetic Follicular Fluid Medium (SFFM) supplemented with glucosamine (Glc) and/or epidermal growth factor (EGF). Data is presented as the fold change in [2, 3, 4, 5, 6-<sup>3</sup>H] phenylalanine incorporation in the TCA-precipitation fraction compared to the Control (SFFM – Glc – EGF; mean ± SEM). Different superscripts are significantly different (<sup>abcd</sup> P < 0.05).

(Chapter 3). The aim of this study was to determine the effects of glucosamine supplementation on oocyte developmental capacity.

Despite the addition of glucosamine having no effect on oocyte nuclear maturation (Chapter 5), the developmental capacity of COCs was severely diminished, in a dose-dependent manner, as demonstrated by poor blastocyst yields. This is manifested as a detrimental effect on subsequent developmental competence following early embryo development, since cleavage rates were comparable to oocytes matured in TCM199 or SFFM without glucosamine. The majority of embryos arrested pre-compaction, coinciding with embryonic genome activation in bovine embryos (Kanka 2003) and may be related to the decreased protein synthesis seen after maturation in the presence of glucosamine.

The concentration of glucosamine that lead to the lowest blastocyst rate resulted in the highest cumulus expansion in rodent COCs [Chen, 1990 #280]. Although cumulus expansion is important for ovulation and post-ovulatory events (Dekel and Phillips 1979; Salustri, *et al.* 1989), it is possible that over stimulation of cumulus expansion by exogenous means may be harmful to oocyte developmental capacity.

Addition of glucosamine to COCs dramatically suppressed oocyte protein synthesis during maturation by 0.6-fold compared to the control group. Glucosamine exposure also perturbs protein synthesis in pancreatic  $\beta$ -cells (Andreozzi *et al.* 2004), and this is thought to be due to glucosamine supplementation in somatic cell cultures having similar effects as hyperglycaemic conditions, leading to the development of diabetic phenotypes (Wang *et al.* 1998; Weigert *et al.* 2001; Ravussin 2002). Colton et al (Colton *et al.* 2002) have investigated the influence of diabetic conditions on oocyte maturation and demonstrated that COCs derived from induced diabetic mice have accelerated resumption of both spontaneous and ligand-induced nuclear maturation, but decreased rates of completion of nuclear maturation (Colton, *et al.* 2002). Significantly less COCs derived from diabetic mice reached the blastocyst stage and glucose caused a dose-dependent decrease in blastocyst rates from COCs derived from control animals (Colton, *et al.* 2002). Glucosamine mimics hyperglycaemia by causing over-expression of the hexosamine pathway, resulting in down regulation of

pathways important for protein synthesis and anti-apoptotic signals, such as the phosphatidylinositol 3- kinase (PI3K)/Akt pathway (Andreozzi, *et al.* 2004).

EGF is a widely used IVM growth factor additive, and the rationale behind examining EGF supplementation here is that the EGF-family of growth factors have been shown to up regulate the PI3K/Akt pathway (Koyama *et al.* 2003). Hence EGF supplementation was hypothesized to overcome the glucosamine-induced inhibition of oocyte protein synthesis. While EGF alone had no effect on protein synthesis levels compared to controls, surprisingly the combination of glucosamine and EGF significantly increased protein synthesis. One can only speculate the mechanisms involved, although if protein synthesis is indicative of oocyte developmental capacity, glucosamine may still be of some value to IVM to reduce glucose concentrations in media, whilst EGF overcomes the detrimental effects of glucosamine-induced inhibition of PI3K/Akt signalling. Further investigations are required to determine the effects of the combined glucosamine/EGF supplementation during IVM on subsequent embryo developmental capacity.

In conclusion, despite glucosamine having no effect on oocyte nuclear maturation, supplementation during IVM leads to perturbed cytoplasmic maturation, demonstrated by decreased development to the blastocyst stage and decreased oocyte protein synthesis during IVM. The addition of EGF to media containing glucosamine led to an increase in protein synthesis. The influence of EGF and glucosamine supplementation during IVM on developmental capacity is yet to be determined.

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# **Chapter 7**

## **Final Discussion**

Substantial improvements have been made in the field of in vitro embryo production (IVP), with the design of in vitro embryo culture (IVC) systems based on physiological conditions and the metabolic requirements of the embryo. These improvements have lead to increased quality and quantity of embryos being able to be produced using this technology. Conversely, in vitro oocyte maturation (IVM) conditions have remained relatively unchanged over the past few decades; hence an area of IVP where potential improvements to efficiency may be made. The aim of this thesis was to design a novel culture system that would improve the developmental capacity of in vitro matured bovine oocytes. This was achieved by firstly examining the metabolic profiles of bovine cumulus oocyte complexes (COCs) during IVM and secondly by investigating the energy substrate and ionic composition of bovine follicular fluid (FF). These two approaches were then combined to develop a culture system that may improve IVM of bovine oocytes.

The metabolic profiles of intact bovine COCs throughout maturation were determined, focusing on the utilisation of oxygen, pyruvate, glucose and production of L-lactate. To date there is still minimal data available concerning the metabolic activities of intact COCs, regardless of species, with the majority of metabolic studies focusing on the oocyte and cumulus vestment as separate entities (Zuelke and Brackett 1992; Rieger and Loskutoff 1994; Krisher and Bavister 1999; Steeves and Gardner 1999; Khurana and Niemann 2000; Spindler *et al.* 2000). This is somewhat surprising since, during both in vivo and in vitro maturation, oocytes are reliant on the cumulus vestment for numerous functions, and oocytes matured in the absence of cumulus cells have compromised developmental capacity (Shioya *et al.* 1988; Madison *et al.* 1992; Lonergan *et al.* 1994; Goud *et al.* 1998). More specifically, oocytes rely on the surrounding cumulus vestment to utilise energy substrates, especially glucose, since the oocyte itself poorly utilises this substrate (Biggers *et al.* 1967; Rieger and Loskutoff 1994; Saito *et al.* 1994).

Bi-directional communication occurs between the oocyte and cumulus cells and oocytes secrete factors that are required for many cumulus cell functions, such as murine cumulus expansion (Buccione *et al.* 1990; Buccione *et al.* 1990; Vanderhyden *et al.* 2003). If oocyte-secreted factors influenced the metabolic activity of their surrounding cumulus vestment, then non-invasive measurements of

energy substrates in spent media could be used as a diagnostic for oocyte developmental capacity. Results from this study showed this not to be the case (Chapter 2), since cumulus cell complexes in which the oocyte had been microsurgically removed (oocytectomized complexes, OOX) had comparable oxygen, pyruvate, glucose and L-lactate utilisation as intact COCs and OOX co-cultured with denuded oocytes (DOs). The reliance of cumulus cells on oocyte-secreted factors may be a species-specific phenomenon, since bovine cumulus expansion can occur in the absence of the oocyte (Ralph *et al.* 1995). Furthermore, investigations into the metabolic activities of feline oocytes and cumulus cells revealed that while glucose metabolism is related to developmental capacity, the biochemical activities of cumulus cells are independent of oocyte developmental capacity (Spindler, *et al.* 2000).

To date there are no definitive, non-invasive measures for developmental potential of the immature oocyte, with development to the blastocyst stage the current best indicator of oocyte quality. Recent work performed in human embryos has demonstrated that there is a relationship between amino acid turnover and embryo developmental potential (Houghton *et al.* 2002). This possibility has yet to be explored in COCs.

Rather than treating the oocyte and cumulus vestment separately, the metabolic profiles of COCs as a whole were determined. Firstly it was ascertained that by 24 h IVM culture period, the uptake of oxygen, pyruvate and glucose by bovine COCs were approximately 2-fold higher than at the initiation of IVM. This agrees with previous studies (Steeves and Gardner 1999). Furthermore glucose, not pyruvate or oxygen, was the major energy substrate used by COCs when cultured in standard IVM culture conditions (Tissue Culture Medium 199, TCM199). Indeed, on a per volume of tissue basis, cumulus cells consume 23-fold more glucose than the oocyte (based on calculations of glucose consumption by oocytes as determined by Thompson *et al.* 1996). In contrast, the oocyte consumes 3-fold more O<sub>2</sub> than cumulus cells on a per volume of tissue basis.

At the initiation of IVM, a highly significant correlation existed between glucose consumption and L-lactate production, suggesting that the majority of glucose

consumed by COCs was metabolised via the glycolytic pathway. Although this relationship still existed half way through the maturation period (10-14 h), by 24 h a considerable proportion of the glucose consumed by COCs was not converted to lactate. It was hypothesised that during the latter periods of IVM, an increasing proportion of the consumed glucose was being utilised as a substrate for FSH-stimulated extracellular matrix (ECM) synthesis during cumulus cell mucification. Before glucose can be converted to hyaluronic acid (the major structural backbone of cumulus ECM), it must be converted to glucosamine-6-phosphate. Glucosamine, an amino carbohydrate, can also be converted to hyaluronic acid, by firstly being metabolised to glucosamine-6-phosphate. To determine whether glucose was being used for mucification, IVM media was supplemented with glucosamine and glucose consumption by COCs was determined (Chapter 3).

After 0-4 h of culture, total glucose consumption did not vary between non-stimulated and FSH-stimulated COCs, nor did glucosamine supplement have any effect. In contrast, from 20-24 h, COCs cultured in media containing glucosamine consumed significantly less glucose, regardless of the presence or absence of FSH. The role of glucose as a substrate in ECM synthesis was further validated by COCs cultured in the presence of glucosamine having significantly less [ $^{14}\text{C}$ ] glucose incorporation into ECM compared to COCs cultured without glucosamine. These results support the hypothesis that during IVM, glucose is utilised for ECM synthesis, as well as for energy production. Glucosamine supplementation reduces glucose consumed by COCs, hence can partially prevent significant glucose depletion from IVM culture systems.

The energy substrate and ionic composition of bovine follicular fluid (FF) were determined so a new IVM medium could be devised that more closely resembles physiological conditions (Chapter 4). The composition of FF did not vary with stage of oestrous cycle, although the concentrations of some ions and energy substrates did change with follicle size. In particular, glucose concentrations ranged from 1.4 mM to 2.3 mM in FF derived from antral follicles. This is considerably lower than that found in TCM199 (5.6 mM), the most commonly used bovine IVM base medium.

Synthetic Follicular Fluid Medium (SFFM) was created, based on the results obtained from the FF analyses. The composition of SFFM differed from TCM199 by containing either 2.3 mM glucose (concentrations seen in FF) or 5.6 mM glucose (concentrations seen in TCM199). The new media also contained cysteamine; which has been shown to be beneficial to oocyte developmental capacity by increasing production of glutathione (de Matos *et al.* 1995; de Matos *et al.* 1997; de Matos *et al.* 2002).

Downs and colleagues have demonstrated that in mice, glucose concentrations and its metabolism via the Pentose Phosphate Pathway (PPP) influences oocyte nuclear maturation (Downs 1997; Downs *et al.* 1998; Downs and Hudson 2000). Others have also shown that the concentration of glucose used during IVM can have varied effects on oocyte developmental capacity and subsequent embryonic development. High glucose levels have been shown to increase the rate of nuclear maturation, while concentrations as high as 28 mM decrease oocyte glutathione levels and increase reactive oxygen species (Hashimoto *et al.* 2000). In contrast, COCs cultured in media containing either very low levels or no glucose are associated with compromised developmental capacity (Rose-Hellekant *et al.* 1998). However, when a physiological level of glucose (2.3 mM) during IVM was assessed for meiotic maturation, significantly less oocytes within COCs reached metaphase II (MII) after 24 h and 30 h of culture compared to those cultured in 5.6 mM glucose (Chapter 4). This may have been a result of glucose being depleted to levels detrimental to COC maturation.

A significant deficiency in current IVM conditions is the static nature of the culture system, which allows accumulation of waste products as well as significant depletion of substrates from the medium, such as glucose (Chapter 3). In an attempt to reduce the need for high levels of glucose, supplementation of SFFM with glucosamine was proposed as being the method by which glucose concentrations could be decreased to physiological levels without depleting glucose to levels detrimental to oocyte developmental capacity. However, addition of glucosamine did not restore the depressed levels of meiotic maturation when oocytes are matured in medium with reduced (2.3 mM) glucose levels. To overcome this deficiency, an assessment of sequentially altering glucose concentrations to IVM was conducted in an attempt to

determine the period of time in which low glucose levels are critical for normal meiotic progression (Chapter 5). These studies revealed that COCs cultured in SFFM + 2.3 mM glucose and glucosamine had comparable cumulus expansion as COCs cultured in either TCM199 or SFFM + 5.6 mM glucose. Culturing COCs in different glucose concentrations influenced the MI to MII transition and nuclear maturation could further be manipulated by changing COCs into media containing different glucose concentrations and  $\pm$  gonadotrophins. However, no sequential culture system examined, rendered any further advantage in the kinetics of oocyte meiotic maturation in comparison to continuous culture in 5.6 mM glucose with gonadotrophins.

Recently, Ali et al (Ali and Sirard 2003) have shown that culturing bovine COCs in low glucose (1.5 mM glucose in SOF) and FSH for the initial 6 h of maturation (followed by low glucose without FSH for the final 18 h), significantly improved blastocyst yield compared to COCs cultured in low glucose + FSH for the initial 2 h or the entire 24 h of IVM. In contrast, I demonstrated that culturing COCs in 5.6 mM glucose and no gonadotrophins for the initial 6 h, followed by 5.6 mM glucose + gonadotrophins for the final 18 h, resulted in significantly less oocytes reaching MII after 24 h of culture. Although Ali et al (Ali and Sirard 2003) did not report the effect of their culture system on nuclear maturation, it is quite possible that this system is advantageous to oocyte developmental capacity due to the brief exposure of FSH limiting the requirement of glucose. The influences of gonadotrophins on nuclear maturation of COCs cultured in 5.6 mM glucose were unexpected and most likely the mechanisms causing the effects are complex in nature. Evaluating glucose depletion under FSH-free or limited conditions may reveal a significant advantage of such systems.

Despite no effect of glucosamine on the proportion of oocytes reaching MII after 24 h and 30 h of culture, development to the blastocyst stage after 8 days of culture was severely compromised by this treatment, in a dose-dependent manner. COCs matured in SFFM + 5.6 mM glucose, minus glucosamine, had similar blastocyst development as COCs cultured in TCM199, while glucosamine supplementation during IVM lead to a dose-dependent decrease in blastocyst yield. This result was surprising and demonstrates clearly that glucosamine during IVM is detrimental to oocyte

cytoplasmic maturation, as a large proportion of embryos arrested at the 8- to 16-cell stage, coinciding with bovine embryonic genome activation.

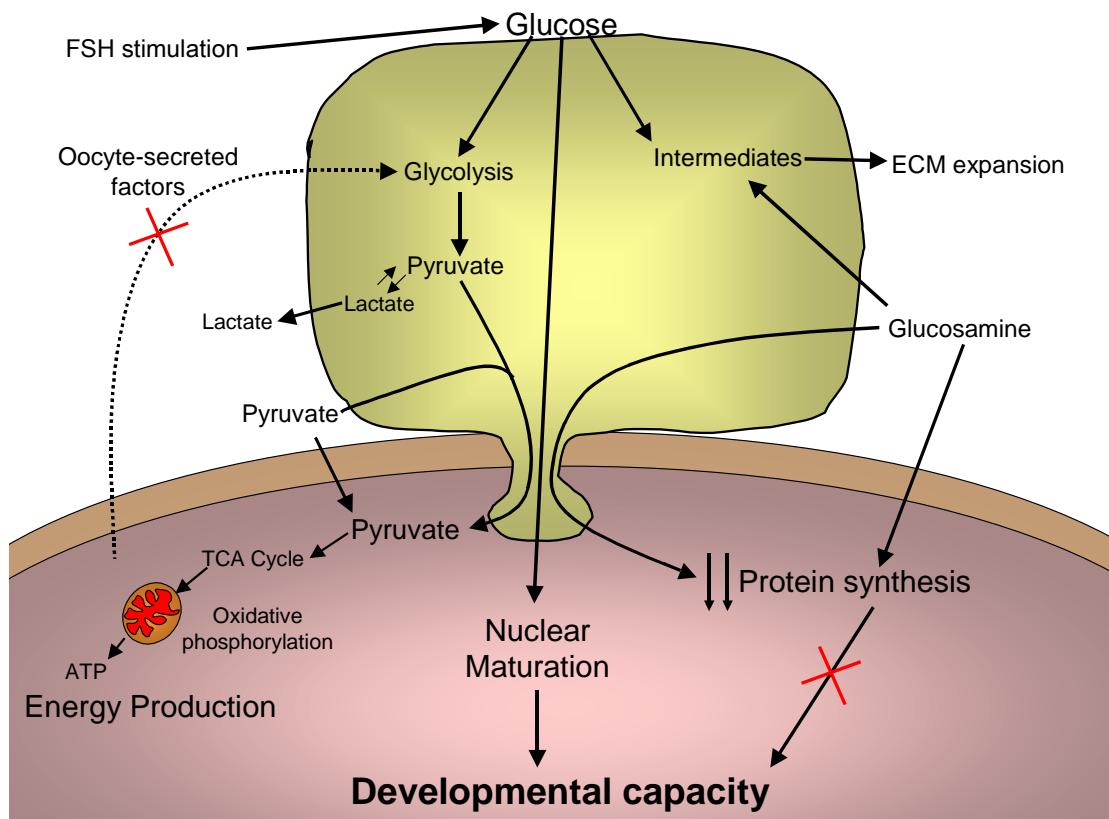
The mechanism for glucosamine-induced failure of oocyte cytoplasmic maturation is completely unknown and is difficult to interpret. Consequently, a new hypothesis (see below) has been developed involving glucosamine supplementation mimicking hyperglycaemic conditions and up regulation of the hexosamine pathway resulting in inhibition of protein synthesis. Protein synthesis during IVM was measured in COCs cultured in  $\pm$  glucosamine and  $\pm$  EGF (a promoter of downstream pathways that influence protein synthesis). Glucosamine supplementation led to a significant reduction in protein synthesis within the oocyte. Interestingly, the combination of glucosamine and EGF resulted in significantly higher oocyte protein synthesis; hence it is possible that in the presence of EGF, glucosamine could still be used to reduce glucose concentrations to physiological levels. Future research will be performed to investigate whether the increased protein synthesis by oocytes cultured in EGF and glucosamine is related to increased developmental capacity, hence embryo development.

The developmental capacity of bovine COCs cultured in media containing low glucose and no glucosamine needs to be investigated. Alternative mechanisms for culturing COCs in media containing low glucose needs to be devised to prevent glucose depleting to levels detrimental to oocyte maturation. A possible alternative could be the application of perfusion culture systems.

Oocyte maturation medium could be further improved by undertaking more detailed follicular fluid analyses, focusing on amino acid concentrations, hormones and growth factors. Recent work has demonstrated that bovine COCs derived from small (2-5 mm), atretic follicles have higher rates of development to the blastocyst stage compared to COCs originating from healthy follicles of the same size (Irving-Rodgers *et al.* 2004). The fluid composition from follicles using this classification may vary and aid further medium development studies.

## **Conclusion**

The oocyte is a unique cell type and it remains quiescent from the time of its formation in early fetal development to follicle activation and the initiation of follicle and oocyte growth decades later. Hence *in vivo*, the oocyte is exposed to a dynamic environment that is constantly changing throughout folliculogenesis and oogenesis. In contrast, *in vitro* oocyte culture conditions are static and are largely based on somatic cell cultures. Therefore the aim of this thesis was to study the follicular environment and metabolic profiles of bovine oocytes and utilise this knowledge to create an improved culture system for bovine IVM. The research presented in this thesis demonstrates that the energy substrate composition of follicular fluid varies between fluids from different follicle sizes. Also the metabolic activities of bovine COCs are not influenced by oocyte-secreted factors and vary during a 24 h IVM culture (Figure 1.). Exposure to different culture conditions, such as glucose concentrations and media densities, can alter the developmental capacity of bovine oocytes. New media based on the follicular environment was devised that manipulated nuclear maturation but compromised cytoplasmic maturation of the oocyte. Further research is required for *in vitro* oocyte maturation to become a more efficient and viable technique for assisted reproduction in livestock and humans.



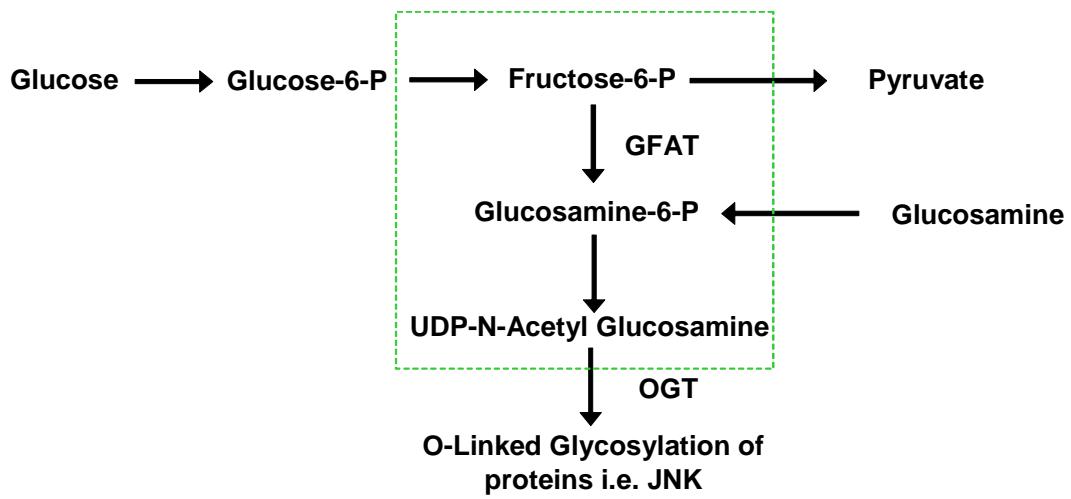
**Figure 1.** Energy metabolism by bovine cumulus oocyte complexes (COCs) during in vitro maturation (IVM).

## **Future Direction: Manipulation of Glucose Metabolic Pathways May Increase Oocyte Developmental Capacity During In Vitro Maturation**

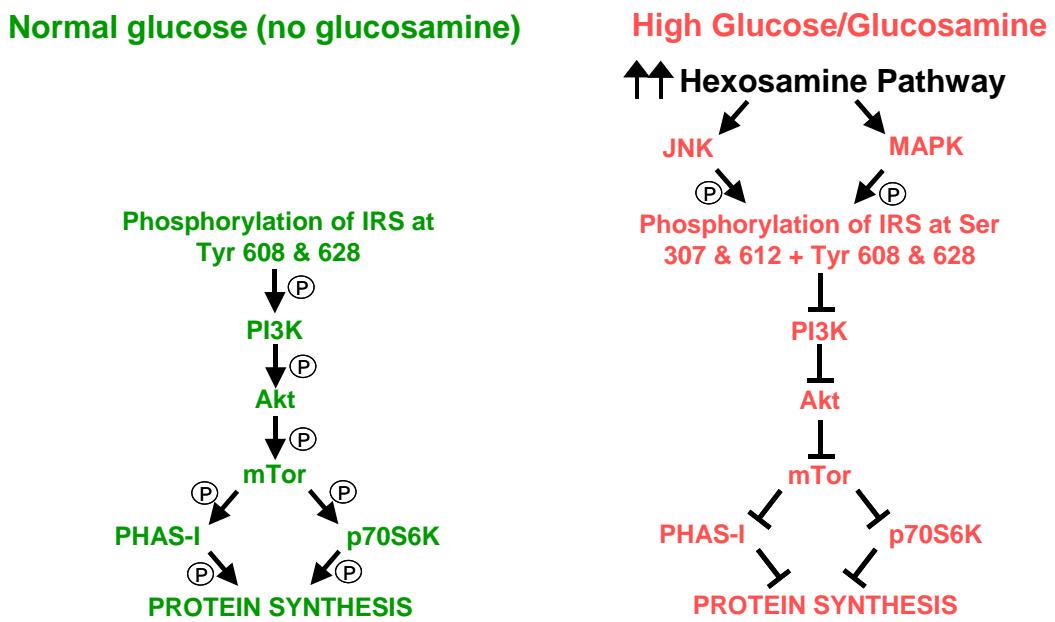
### *The hexosamine pathway*

The hexosamine pathway is a fuel-sensing pathway, and it is believed to play an important role in energy metabolism by regulating glucose uptake, insulin resistance (reviewed by Ravussin 2002) and leptin (Wang *et al.* 1998). The first step in the hexosamine pathway is the conversion of fructose-6-phosphate (a product of glycolysis) to glucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate limiting enzyme of the pathway. Glucosamine-6-phosphate is rapidly converted to UDP-N-acetyl-glucosamine, a substrate of O-linked N-acetyl glucosamine transferase (OGT), which is a key enzyme in O-linked glycosylation of proteins (Figure 2). Under normoglycemic conditions, only 1-3% of total glucose consumed is diverted to the hexosamine pathway (Marshall *et al.* 1991). In hyperglycemic conditions, there is an increased flux of fructose-6-phosphate into the hexosamine pathway, leading to abnormal phosphorylation of proteins. For example, c-Jun N-terminal kinase (JNK) and mitogen activating protein kinase (MAPK) activities are increased, resulting in extra phosphorylation of IRS-1 and its deactivation (Figure 3). This results in impaired activation of the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) pathways (Figure 3, Andreozzi *et al.* 2004). Both pathways are important in promoting protein synthesis by controlling downstream translation events; hence protein synthesis is down regulated. Increased Akt activity is also anti-apoptotic (Vara *et al.* 2004) and protects cells from glucose toxicity.

Interestingly, glucosamine mimics high glucose levels as it can enter the hexosamine pathway downstream from GFAT. It seems feasible that the supplementation of IVM culture medium with glucosamine, which perturbs protein synthesis within the oocyte, is caused by up regulation of the hexosamine pathway. It is also possible that culture conditions that promote ECM synthesis are detrimental to oocyte developmental capacity by increasing the presence of glucosamine in media.



**Figure 2.** The hexosamine pathway (enclosed within the box) links glycolysis with protein glycosylation. GFAT = glutamine:fructose-6-phosphate amidotransferase, OGT = O-linked N-acetyl glucosamine transferase.



**Figure 3.** The hexosamine pathway and protein synthesis in the presence of normal glucose or hyperglycemic and/or glucosamine supplementation. JNK: c-Jun N-terminal kinase, MAPK: Mitogen activated protein kinase, PI3K: phosphatidylinositol pathway, Akt: Protein kinase B pathway, PHAS: Phosphorylated heat and acid stable.

### *The pentose phosphate pathway and benfotiamine*

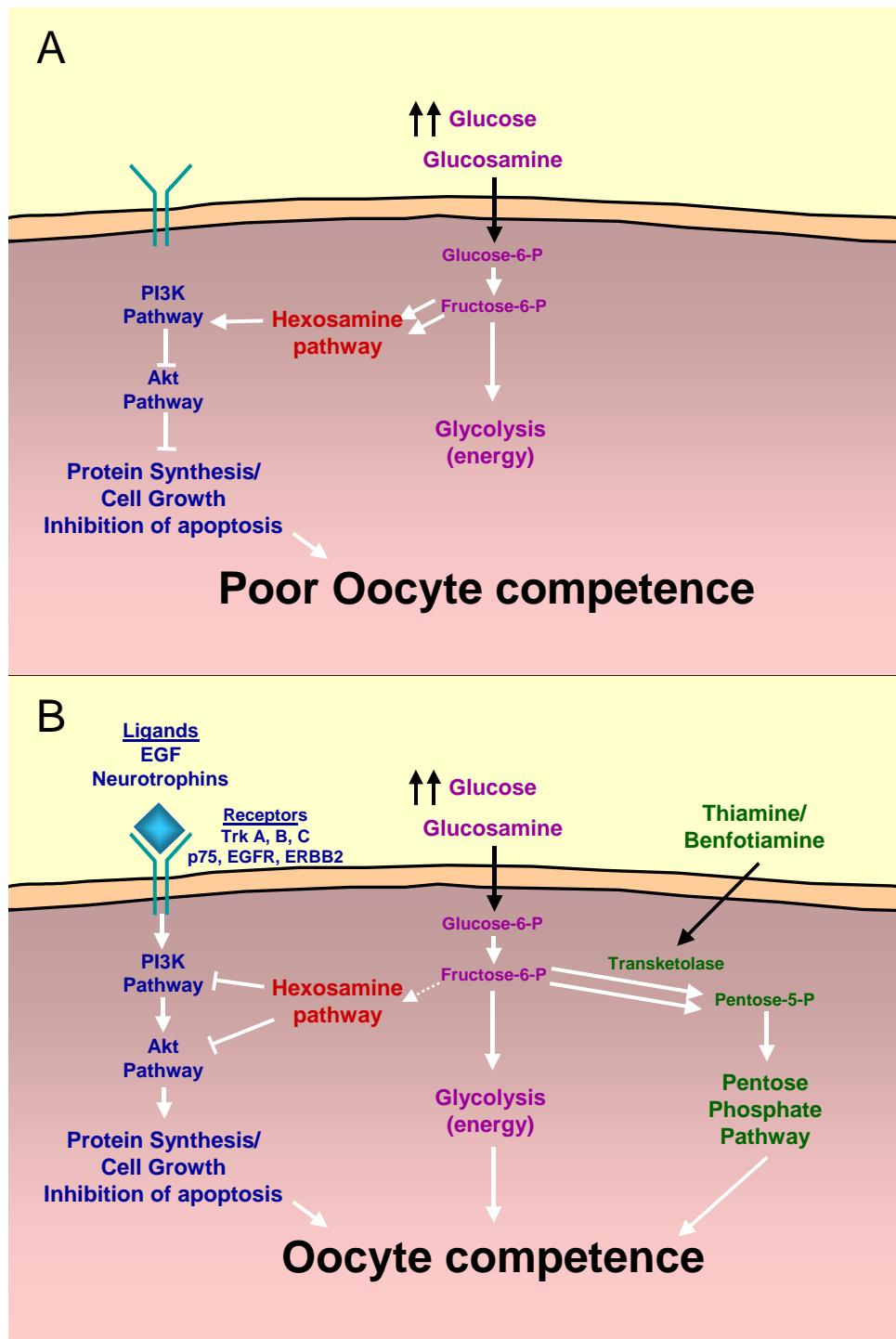
Previously increased flux through the Pentose Phosphate Pathway (PPP) has been shown to be beneficial to oocytes and embryo development, and this was thought to be due to the provision of substrates for de novo purine synthesis (Downs, *et al.* 1998; Downs and Utecht 1999; Downs and Hudson 2000) and NADPH, the latter of which is used to reduce GSSG to GSH. Recent studies using induced diabetic rats demonstrated that these animals have 25-30% reduction of transketolase activity compared to controls (Babaei-Jadidi *et al.* 2003). Transketolase is the rate-limiting enzyme of the non-oxidative stage of the PPP and converts fructose-6-phosphate to pentose-5-phosphate. Transketolase is thiamine-dependent (Vitamin B) and the addition of high-doses of thiamine and benfotiamine (S-benzoylthiamine) increased transketolase activity in diabetic rats to levels comparable to control animals (Babaei-Jadidi, *et al.* 2003). The relevance of thiamine and benfotiamine therapy to hyperglycemic conditions is that increased levels of fructose-6-phosphate produced via the glycolytic pathway can be diverted away from the hexosamine pathway and into the PPP (Pomero *et al.* 2001; Hammes *et al.* 2003), hence preventing inactivation of important down-stream pathways.

### *Hypothesis*

Glucosamine supplementation in IVM media is mimicking the effects of high glucose, therefore up regulating the activity of the hexosamine pathway, inactivating important downstream PI3K/Akt pathways, leading to perturbed oocyte protein synthesis and poor developmental competence (Figure 4A). I previously demonstrated that a considerable proportion of glucose is depleted from media during IVM (Chapter 3), hence given the high rate at which COCs consume glucose in a static culture system, it is quite possible that the levels of glucose required to elicit a similar response to glucosamine supplementation would have to be >30 mM. Glucosamine supplementation most likely has a more pronounced effect since it is utilised downstream from GFAT, the rate-limiting enzyme of the hexosamine pathway.

The adverse effect of glucosamine and increased hexosamine pathway activity could be alleviated in two ways. Firstly, adding a growth factor such as EGF or insulin to the culture system, could activate the PI3K/Akt pathways via the growth factor receptors such as Trk A, B, C, EGFR and ERBB2. Preliminary evidence exists for this in that the inhibition of protein synthesis by glucosamine was prevented by EGF inclusion (Chapter 6). Activation of these pathways would allow increased protein synthesis to occur within the oocyte, hence improving developmental capacity of bovine oocytes (Figure 4B). Secondly, increased activation of the PPP by the addition of benfotiamine may reduce the proportion of fructose-6-phosphate entering the hexosamine pathway and be beneficial to oocyte developmental capacity by increasing the production of NADPH and purines (Figure 4B).

In conclusion, supplementation of bovine IVM culture with glucosamine induces a diabetic phenotype, leading to perturbed protein synthesis, and hence compromised developmental capacity. Manipulation of PI3K/Akt pathways and PPP by adding growth factors such as EGF and neurotrophins, as well as the thiamine derivative benfotiamine, may be a novel mechanism through which oocyte developmental capacity could be increased.



**Figure 4.** The influence of glucosamine on oocyte developmental capacity. A) the proposed model by which glucosamine is detrimental to protein synthesis, hence developmental competence and B) the proposed model by which the adverse effects of glucosamine can be reversed.

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# **Appendices**

## Appendix 1: Additional Experiments

### The influence of BSA and FCS on oxygen consumption of cumulus oocyte complexes

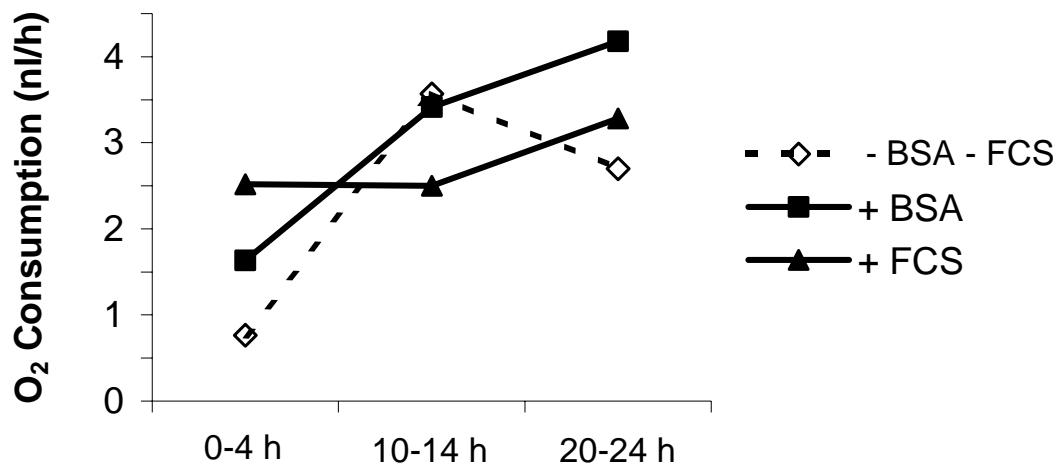
Preliminary experiments were performed to determine the effects of different macromolecule sources, specifically bovine serum albumin (BSA) and fetal calf serum (FCS), on O<sub>2</sub> consumption by bovine cumulus oocyte complexes (COCs). One of the most commonly used protein sources for in vitro oocyte maturation (IVM) is FCS, despite being highly undefined. Although high purity BSA, such as affinity column purified BSA, is still undefined, less variation is seen between different preparations, hence it is a more suitable protein source for semi-defined cultures (Bavister 1995 *Human Reproduction Update* **1** 91-148; Eckert and Niemann 1995 *Theriogenology* **43** 1211-1225; Mingoti *et al.* 2002 *Animal Reproduction Science* **69** 175-186). The aim of this experiment was to determine the different effects of FCS and BSA on oxygen consumption by COCs.

COCs were cultured individually in 10 µl drops of medium -BSA – FCS (TCM199 + pyruvate + FSH + hCG), + BSA or + FCS. At 0-4 h, 10-14 h and 20-24 h, COCs were removed from culture and assayed for O<sub>2</sub> consumption (Chapter 2 and Appendix 3). At the end of each assay spent media and COCs were recovered and stored at -80°C. Three replicate experiments were performed and a total of 45 COCs were assayed. Results were analysed using a one-way ANOVA, followed by a Tukeys test.

O<sub>2</sub> consumption over a 24 h culture period for the three treatments is shown in Figure 1 and revealed differing profiles for the three different treatments over a 24 h period. The COCs that were not exposed to BSA and FCS (-BSA –FCS) had the most varied consumption, the rate of consumption at the start of the IVM period was relatively low compared with the other groups ( $P < 0.001$ ) but increased to the similar rate of the +BSA group at 10-14 h. By the end of the culture, O<sub>2</sub> consumption by COCs cultured in – BSA – FCS had decreased again. The + BSA group had the most linear and constant increase in consumption rate, initial consumption was  $1.6 \pm 0.1$  nl/h,

increasing to  $4.2 \pm 1.2$  nl/h by 24 h. The consumption rate for COC incubated with FCS was the highest at the start of the culture period,  $2.3 \pm 0.1$  nl/h was more than double than seen for the control group ( $P < 0.001$ ). The rate remained unchanged until the end of the maturation period where it increased to  $3.3 \pm 0.6$  nl/h.

Culturing COCs in media without protein or with different protein sources resulted in different O<sub>2</sub> consumption profiles over a 24 h period. Although the relationship between O<sub>2</sub> metabolism and oocyte developmental capacity is unknown, all subsequent experiments were performed using BSA as the sole protein source.



**Figure 1.** O<sub>2</sub> consumption profiles over a 24 h IVM period for cumulus oocyte complexes cultured in media without a macromolecule (- BSA - FCS), or with a macromolecule, either +BSA or +FCS.

## **Creation and validation of a DNA quantification assay for individual cumulus oocyte complexes**

Previous experiments involving the measurement of metabolic parameters of individual COCs revealed that as culture time increases, the variation within treatment groups also increased. This may reflect variable sizes and cell numbers of complexes. Therefore, to standardize the metabolic measurements across treatments, COC DNA content was measured (i.e. ng of DNA). A new method of DNA quantification was required since commonly used methods such as Hoechst staining and UV absorbances at 260 nm were not sensitive enough to assay individual COCs. PicoGreen (Molecular Probes, Eugene, OR, USA) is a fluorescent nucleic acid dye that is able to detect small quantities of DNA (as low as pg quantities), and has minimal binding to single stranded DNA and RNA when in equimolar concentrations of double stranded DNA. A protocol for whole cell DNA extraction was developed, as well as validation of the PicoGreen assay for use with COCs.

COCs were collected in individual wells of 96-well plates and DNA was extracted using a lysis buffer, consisting of a Tris-EDTA buffer (TE buffer; 50 mM and 1 mM respectively pH 7.6) and 500 µg/ml of proteinase K. Fifty µl aliquots of the lysis buffer was added to individual wells and was incubated at 50°C until COCs were no longer visible (~ 3.5 h), followed by 80°C for 15 mins to deactivate the proteinase K. The lysates were treated with 10 µg/ml DNase-free RNase (Roche Diagnostics, Basel, Switzerland) for 30 mins at 40°C, since initial experiments demonstrated that there was a high degree of background fluorescence that was attributed to PicoGreen binding with RNA. Furthermore, dose-response experiments showed that 10 µg/ml DNase-free RNase was the optimal concentration. Excess liquid was evaporated and 100 µl of Tris-EDTA buffer was added to each well.

Immediately prior to measuring DNA content, a 50-fold dilution of the standard (lambda DNA) was made using the TE buffer. Standard and blanks (100 µl) were added to the microplate, with each standard added in duplicate.

<b>Final DNA</b>	<b>Volume of DNA stock</b>	<b>Volume of TE</b>
	( $\mu$ l)	( $\mu$ l)
200 ng	1000	0
160 ng	880	220
96 ng	600	400
48 ng	500	500
24 ng	500	500

A 200-fold dilution of PicoGreen dsDNA quantitation reagent (Molecular Probes, Eugene, OR, USA) was prepared in TE buffer and 100  $\mu$ l of this was added to each well (including wells used for the standard curve) and incubated, protected from light, for 2 to 5 mins at room temperature. The fluorescence of each sample was determined using a FLUOstar Galaxy microplate reader (BMG Labtechnologies Pty Ltd, Offenburg, Germany) with excitation set at 485 nm and emission at 520 nm. A standard curve was created using the readings from the lambda DNA.

With the use of the microplate reader, the inter-assay CV was <3% and results were only used when standard curves with  $r^2$  values  $\geq 0.95$  were achieved.

In conclusion, an assay was developed that allowed for the successful measurement of the DNA content of individual COCs, hence metabolic measurements could be expressed on a per DNA content basis.

## **Macromolecule supplementation and the relationship between DNA content and diameters of cumulus oocyte complexes**

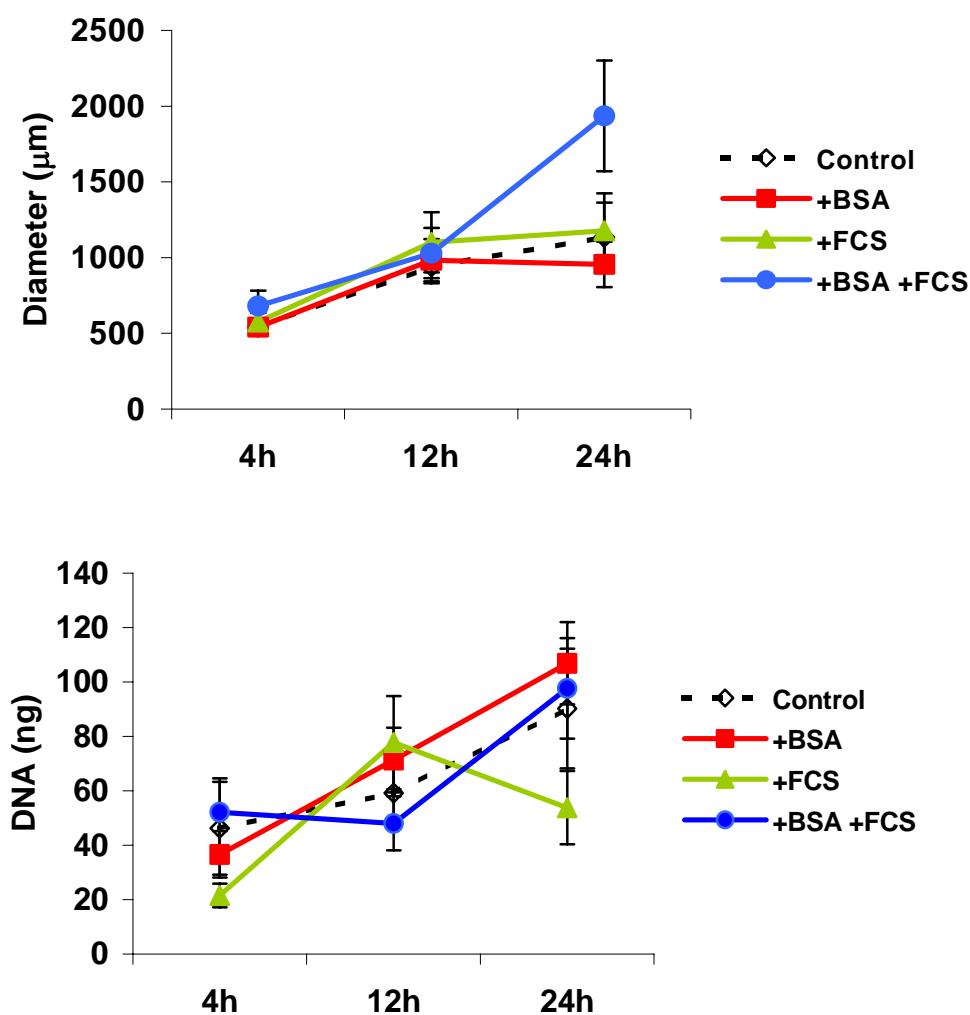
The aim of these experiments was to determine whether a positive correlation exists between DNA content and mean diameters of individual COCs. The existence of such a relationship would allow mean diameters to be used as a non-invasive predictor of DNA content, and hence used for the expression of metabolic measurements.

COCs were cultured in groups of 10 in 100 µl drops of IVM media (TCM 199 supplemented with FSH, hCG and  $\pm$  BSA,  $\pm$  FCS). At 4 h, 14 h or 24 h, the vertical and horizontal diameters of COCs were using a dissecting microscope and ocular graticule and COCs were stored in individual wells of a 96-well plate at -80°C for DNA quantitation. Three replicate experiments were performed and a total of 168 COCs were assayed. The influence of the treatments on DNA content and diameters were determined using a two-way ANOVA, followed by a Tukeys test and the potential relationships between DNA content and diameter were determined by regression analyses.

Linear regression analyses were performed for each treatment and time point to determine if there were any relationships between mean diameter and DNA content. After 4 h and 12 h of culture there were no significant differences in the mean diameters of COCs regardless of  $\pm$  BSA, or  $\pm$  FCS, despite there being variation in DNA content. Interestingly after 24 h, COCs cultured in media containing both BSA and FCS had 2-fold larger mean diameters compared to all other groups, despite DNA content being comparable to the control and + BSA groups (Figure 2). Linear regression analysis revealed that there was no relationship between DNA content and mean diameter after 4 h of culture in any of the media ( $P > 0.05$ ), although after 12 h, significant relationships between COCs DNA content and diameter was found for all treatment groups ( $P < 0.01$ ) with the exception of COCs cultured in + BSA + FCS. At 24 h of culture, significant relationships between DNA content and diameter

existed for COCs cultured in control, + BSA and + BSA + FCS media but not media containing FCS alone.

Interestingly, the type of macromolecule supplement used for bovine IVM can influence DNA content of COCs after as little as 4 h of culture. Furthermore, increased culture time had varied effects of the relationship between DNA content and mean diameters, dependant on whether media contained BSA or FCS. Therefore,



**Figure 2.** Mean diameters and DNA content of bovine cumulus oocyte complexes (COCs) cultured in media  $\pm$  BSA  $\pm$  FCS. Data points represent mean diameter or DNA  $\pm$  SEM.

it would appear as if these different treatments have independent effects on COC diameter and DNA content, which possibly reflect treatment differences in

extracellular matrix synthesis during cumulus expansion. Secondly, the PicoGreen dye may only be detecting healthy, not apoptotic cells within the COC, since the dye binds to double stranded DNA with a high affinity. One of the first stages of apoptosis is DNA fragmentation, which may be undetectable with PicoGreen quantification. Further studies, such as TUNEL staining, are required to validate this. In conclusion, supplementation of IVM media with different macromolecules has varied effects on DNA content, COC diameters, and the relationship between the two. The data revealed that the diameter of COC during maturation was a relatively poor indicator of cell number (as determined by DNA quantification), as different treatments affect either parameter (i.e. diameter and DNA content) independently of each other.

## **The influence of gonadotrophins on glucose utilisation and cumulus expansion**

To determine whether FSH and/or hCG influence cumulus expansion and glucose consumption, COCs were cultured individually in 10 µl drops of either Control (TCM199 + pyruvate + BSA), + FSH (control + 0.01 IU/ml FSH), + hCG (control + 0.1 IU/ml hCG) or + FSH + hCG (Control + FSH +hCG). Drops were overlaid with mineral oil and cultured at 39°C in 6% CO<sub>2</sub> in humidified air. At 0 or 20 h, COCs were transferred into fresh media and cultured in 2 µl drops for 4 h. The vertical and horizontal diameters of COCs were measured using a dissecting microscope and ocular graticule, after which COCs and spent media were stored separately in individual wells of 96-well plates at -80°C for DNA quantification and energy substrate analysis. Three replicate experiments were performed, with a total of 120 COCs assayed. Statistical differences were analysed using a two-way ANOVA, followed by a Tukeys test.

After 0-4 h there were no significant differences between the mean diameters of COCs cultured in ± FSH and/or hCG (Table 1, P > 0.05). By 20-24 h, the presence of FSH stimulated significantly more cumulus expansion compared to COCs cultured in the Control and + hCG media (Table 1, main effects; P < 0.001), and FSH stimulated glucose consumption after 0-4 h, regardless of hCG (Figure 3, main effect; P < 0.05). Glucose consumption by FSH-stimulated COCs was significantly higher than the control group but there was no effect of exposure to hCG. L-lactate production by COCs was also measured and a similar pattern to glucose uptake was seen, with +FSH stimulation leading to higher production compared to control and hCG alone at the beginning of maturation (Figure 4, main effects; P < 0.05). However by 20-24 h, while L-lactate production was substantially higher with FSH-stimulation compared to the control group, the addition of hCG led to similar levels of L-lactate being produced compared to + FSH and +FSH + hCG.

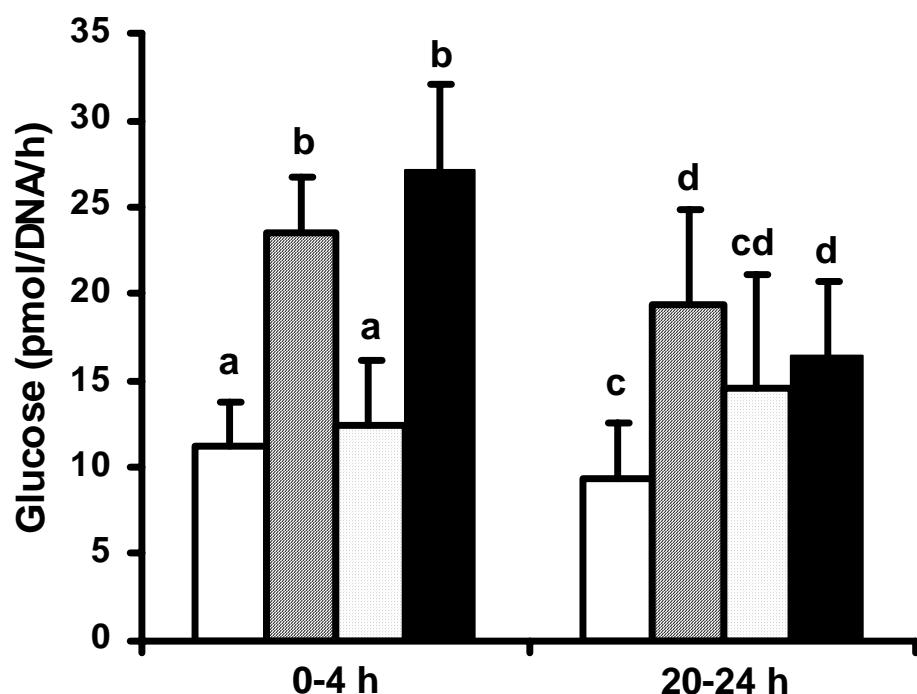
Since FSH alone had the most significant influence on cumulus-expansion and glucose consumption, subsequent experiments investigating the influence of

glucosamine on glucose consumption during cumulus-expansion were performed in media containing FSH and not hCG.

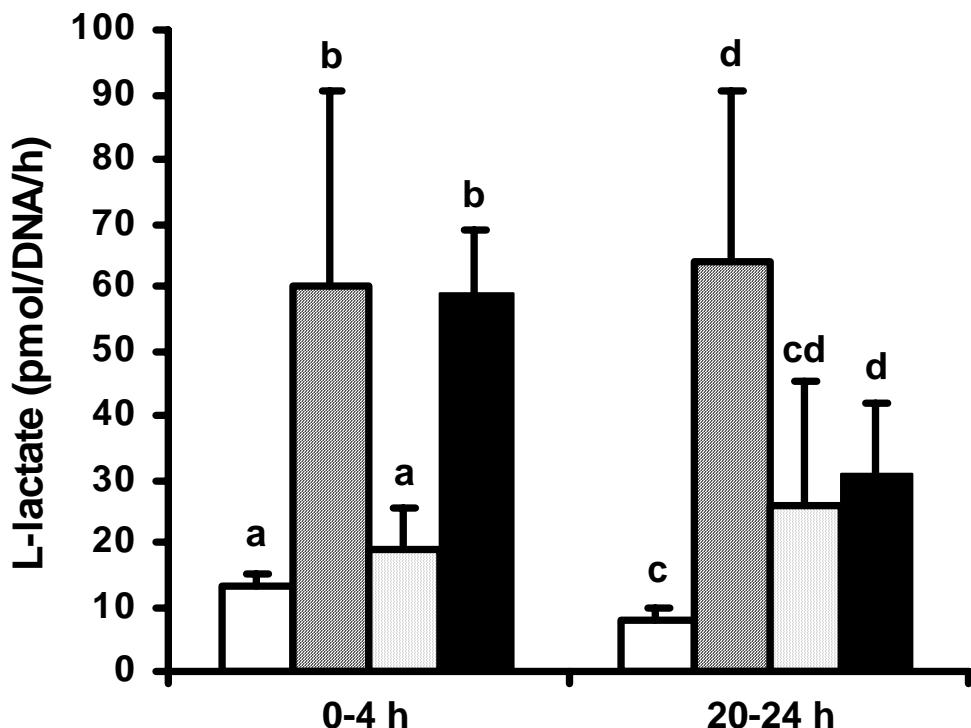
**Table 1.** The mean diameters of bovine cumulus oocyte complexes (COCs) after 0-4h or 20-24h of culture in either Control (TCM199 + pyruvate + BSA), + FSH (Control + FSH), + hCG (Control + hCG) or + FSH + hCG (Control + FSH + hCG).

Time	Culture				Diameter ( $\mu\text{m}$ )
	Control	+FSH	+hCG	+FSH +hCG	
0-4 h	306 $\pm$ 12 <sup>a</sup>	329 $\pm$ 17 <sup>a</sup>	308 $\pm$ 14 <sup>a</sup>	321 $\pm$ 17 <sup>a</sup>	
20-24 h	298 $\pm$ 14 <sup>b</sup>	574 $\pm$ 53 <sup>c</sup>	331 $\pm$ 18 <sup>b</sup>	668 $\pm$ 59 <sup>c</sup>	

Values represent means  $\pm$  SEM. <sup>bc</sup> Values within the same time period with different superscripts are significantly different ( $P < 0.05$ ).



**Figure 3.** The influence of FSH and/or hCG supplementation on glucose utilisation after 0-4h and 20-24 h of culture. □ Control (TCM199 + pyruvate + BSA), ■ + FSH (Control + FSH), ▨ + hCG (Control + hCG) and ▪ + FSH + hCG (Control + FSH + hCG). Bars represent means  $\pm$  SEM. <sup>ab</sup> and <sup>cd</sup> Bars with different superscripts are significantly different ( $P < 0.05$ ).



**Figure 4.** The influence of FSH and/or hCG supplementation on L-lactate production after 0-4h and 20-24 h of culture. □ Control (TCM199 + pyruvate + BSA), ■ + FSH (Control + FSH), ▨ + hCG (Control + hCG) and ▪ + FSH + hCG (Control + FSH + hCG). Bars represent means  $\pm$  SEM. <sup>ab</sup> and <sup>cd</sup> Bars with different superscripts are significantly different ( $P < 0.05$ ).

## Appendix 2. Culture Media

### Stock Solutions for IVM/IVF/IVC

Unless otherwise indicated, all chemicals and reagents were purchased for Sigma (St Louis, MO, USA).

#### **Stock A (x4)**

2337.6 mg NaCl  
59.64 mg KCl  
78.87 mg MgSO<sub>4</sub>•7H<sub>2</sub>O  
108.87 mg KH<sub>2</sub>PO<sub>4</sub>  
Dissolve in 100 ml of Milli Q

#### **Stock Heparin**

20 mg heparin  
Dissolve in 2 ml 0.9% sterile saline

#### **Stock B**

1.05 g NaHCO<sub>3</sub>  
5 mg Phenol Red  
Dissolve in 50 ml Milli Q

#### **Stock Hypotaurine (10 mM)**

10.9 mg hypotaurine  
Dissolve in 10 ml 0.9% sterile saline

#### **Stock C (x125)**

51 mg Na pyruvate  
Dissolve in 10 ml Milli Q

#### **Stock S (x10)**

5.5 g NaCl  
300 mg KCl  
36 mg NaH<sub>2</sub>PO<sub>4</sub>  
123 mg MgSO<sub>4</sub>.7H<sub>2</sub>O  
100 mg Kanamycin  
Dissolve in 100 ml Milli Q

#### **Stock Glu (x10)**

405 mg D-glucose  
Dissolve in 100 ml Milli Q

#### **Stock SH**

600 mg HEPES (free acid)  
650 mg HEPES (sodium salt)  
Dissolve in 25 ml Stock S

#### **Stock Glc (x10)**

539 mg glucosamine HCl  
Dissolve in 50 ml Milli Q

#### **Stock CA (x10)**

85.08 mg Ca [lactate]

Dissolve in 12 ml Milli Q

**N-Acetyl-L-cysteine (x100)**

16.32 mg N-Acetyl -L-cysteine

Dissolve in 10 ml Milli Q

**SPAD (x100)**

292 mg NaCl

300 mg CaCl<sub>2</sub>.2H<sub>2</sub>O

Dissolve in 10 ml Milli Q

**Cysteamine (x100)**

7.77 mg Mercatoethylaminse

Dissolve in 10 ml Milli Q

**TCM199 (x2)**

1 sachet TCM199 (ICN Biochemicals)

50 mg kanamycin sulphate

Dissolve in 500 ml Milli Q

Check osmolarity (~ 480)

**Stock H**

6 g HEPES (free acid)

6.5 g HEPES (sodium salt)

20 mg phenol red

Dissolve in 200 ml Milli Q

**Media for IVM**

**HEPES buffered TCM199 + BSA**

(H199 + BSA)

50 ml TCM199 (x2)

8 ml Stock H

2 ml Stock B

1 ml Stock C

Add MilliQ to make up to 100 ml and add 4 mg/ml fatty acid free BSA (ICPbio Ltd, Auckland, NZ).

Osmolarity = 270 Osm

**Bicarbonate buffered TCM199 + BSA**

(B199 + BSA)

50 ml TCM199 (x2)

10 ml Stock B

1 ml Stock C

Add MilliQ to make up to 100 ml and add 4 mg/ml fatty acid free BSA

Osmolarity = 270 Osm

### **Synthetic Follicular Fluid Medium**

(SFFM + 2.3 mM glucose)

25 ml Stock A

16 ml Stock B

800 µl Stock C

10 ml Stock CA

10 ml Stock Glc

10 ml Stock Glu

1 ml Glutamax I (Gibco Invitrogen Corporation, Carlsbad, CA, USA),

1 ml N-acetyl-cysteine

1 ml cysteamine

1 ml non-essential amino acids (100X, Gibco Invitrogen Corporation),

2 ml essential amino acids (50X, Gibco Invitrogen Corporation)

Add Milli Q to make up to 100 ml and add 4 mg/ml BSA.

Osmolarity = 294 Osm

### **Adaptations**

#### **SFFM + 5.6 mM glucose**

Add 24.34 ml of Stock Glu to the recipe above

#### **SFFM - Glucosamine (- Glc)**

Omit Stock Glc

## **Appendix 3. O<sub>2</sub> Consumption Assay**

The assay is based on the method originally described by (Houghton *et al.* 1996 *Molecular Reproduction and Development* **44** 476-485), which exploits the fluorescent properties of pyrene, the fluorescence of which increases with decreasing O<sub>2</sub> concentrations.

### **O<sub>2</sub> Assay Chambers**

Assay chambers were constructed using 5 µl PCR pipettes (Drummond Scientific Company, PA, USA) and stainless steel shafts that acted as plungers (Figure 5). One µl of 1 mM pyrene dissolved in mineral oil was drawn into a pipette, followed by 2 µl of HEPES buffered maturation media containing one complex (COC, OOX, OOX + DO or DO). An airtight seal was created by inserting the open end of the pipette into tubing, expelling a small amount of media and clamping the tubing. The plunger was then fixed in place with sealing wax.

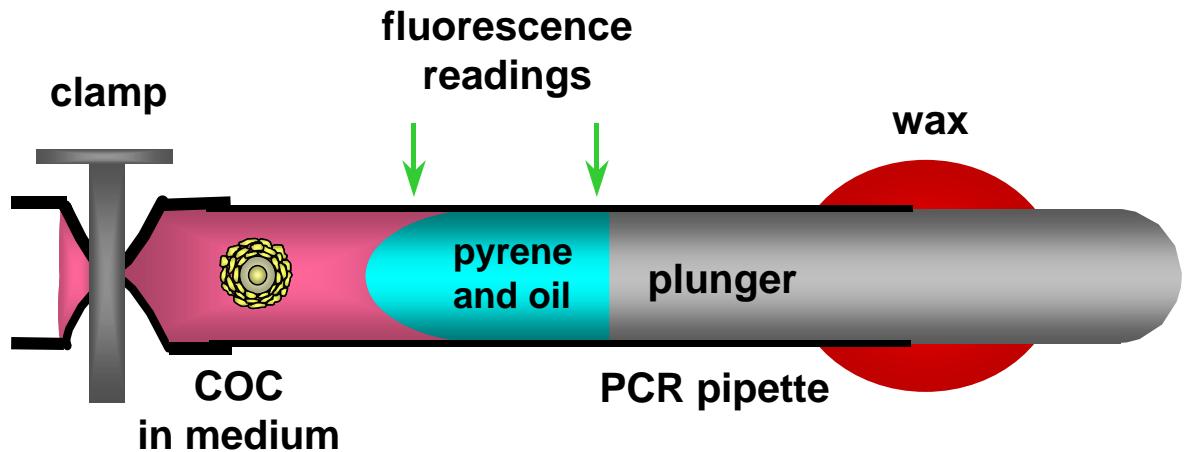
### **Controls**

0% and 20% O<sub>2</sub> control chambers were constructed in a similar manner; with 1 mM Oxyrase (Oxyrase Inc, Mansfield, OH, USA) in 60 mM glucose (equilibrated overnight to remove O<sub>2</sub>) replacing the media and positive controls (20% O<sub>2</sub>) were constructed with media alone.

### **Method**

The fluorescence emission of pyrene at the pyrene-media and the pyrene-plunger interfaces (Figure 5) were measured using a fluorophotometric-inverted microscope (Leica, Wetzlar, Germany). Over a 4 h period, measurements were taken half hourly. Chambers were maintained at 39°C in between measurements.

As the fluorescence readings were arbitrary units, data was entered into a computer model that describes the movement of O<sub>2</sub> from the pyrene into the media as a result of O<sub>2</sub> consumption by the complexes.



**Figure 5.** O<sub>2</sub> consumption assay chambers were constructed using 5 µl PCR pipettes. Each chamber contained an individual complex in media and pyrene in oil. An air tight seal was creating by fixing the open end of the pipette with tubing and a clamp and the stainless plunger was fixed in place with wax. Fluorescence readings were taken at the medium/pyrene and pyrene/plunger interfaces and were used to determine the concentration of O<sub>2</sub> consumed by the complex.

## **Appendix 4. Metabolism Assays**

### **Siliconised slides**

Assay slides were constructed by attaching four glass capillary tubes to a microscope slide with super glue, creating a dam (Figure 6). Approximately 500 µl of concentrated HCl was added to the dam for 30 s and air-dried. Sigmacote was added to the dam for a minimum of 1 min.

### **Nanopipettes**

Pairs of nanopipettes were used (Precision Glass Instruments, Adelaide, South Australia), at a ratio of 1:7-13 between small and large pipettes. New pipettes were immersed in concentrated HCl overnight and air-dried. Pipettes were siliconised by drawing up a small amount of Sigmacote and immersing the pipette for a minimum of 2 mins. Pipettes were allowed to air dry and then rinsed with Milli Q water.

### **EPPS buffer**

2.5 g EPPS (N-[2-Hydroxyethyl] piperazine-N'-[3-propane-sulfonic acid]) was added to 150 ml of Milli Q, pH was adjusted to 8.0 and Milli Q was added to make the total volume up to 200 ml. 10 mg kanamycin was added and the buffer was filter sterilised. The buffer was stored at 4°C.

### **Glycine buffer**

3.75 g Glycine, 2.6 g Hydrazine Sulphate and 0.1 g EDTA was suspended in a small amount of Milli Q, pH as adjusted to 9.4 and Milli Q was added to make the total volume up to 50 ml. The buffer was stored at 4°C.

### **Glucose cocktail**

3.7 ml EPPS buffer, 500 µl of 5 mM dithiothreitol, 500 µl of 37 mM MgSO<sub>4</sub>, 250 µl of 20 mM ATP (Roche Diagnostics, Basel, Switzerland), 750 µl of 20 mM NADP (Roche Diagnostics) and 275 µl hexokinase (Roche Diagnostics). Aliquots were stored at - 80°C.

### **L-lactate cocktail**

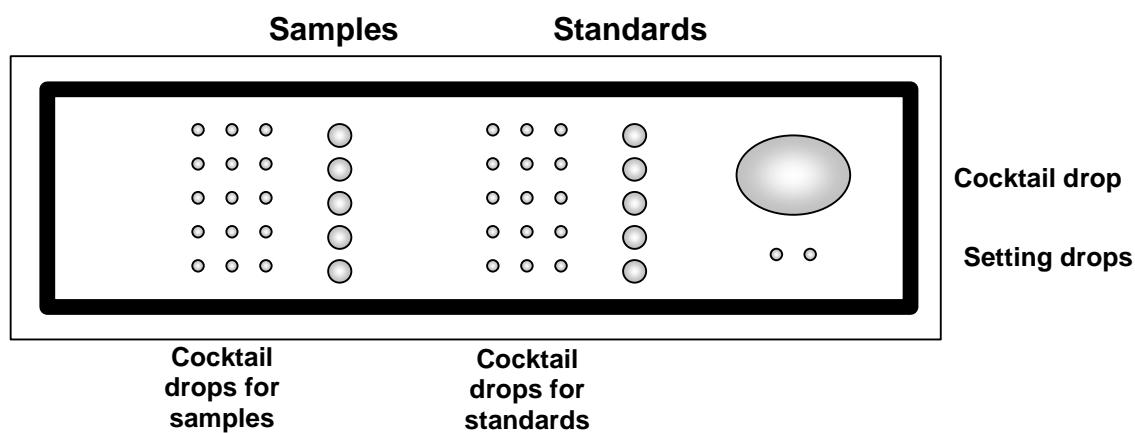
3 ml Glycine buffer, 2.54 ml Milli Q, 500 µl of 150.75 mM NAD (Roche Diagnostics) and 190 µl L-lactate dehydrogenase (Roche Diagnostics). Aliquots were stored at - 80°C.

### **Pyruvate cocktail**

3.5 ml EPPS buffer, 75 µl of 5 mM NADH and 100 µl L-lactate dehydrogenase. Aliquots were stored at - 80°C.

### **Method**

A large drop of metabolite cocktail (i.e. glucose, L-lactate or pyruvate) was added to a siliconised slide and overlaid with mineral oil (Figure 6).



**Figure 6.** Microscope slide set up for the metabolism assays

Test drops were created using the large nanopipette to make a drop of the assay cocktail, plus a drop of the highest standard using the small nanopipette. After the designated assay test (i.e. 40 mins for glucose, 20 mins for L-lactate and immediately for pyruvate assays), the fluorescence of the test drop was used to set the photometer.

Drops of the metabolite cocktail were created using the large nanopipette, allowing each drop to settle onto the slide. Three drops per sample/standard were made. The background fluorescence of each drop was determined.

The standards/samples were added to the large drops using the small nanopipette and after 20-40 mins, the fluorescence of each drop was read using the fluorophotometric-inverted microscope.

## **Appendix 5. Assessment of Nuclear Maturation**

### **Oocyte fix**

1 part 100% ethanol, 3 parts acetic acid

### **Orcein stain**

1g orcein was added to 45 ml acetic acid and dissolved with gentle heat. 55 ml water was added and the stain was filtered.

### **Destaining solution**

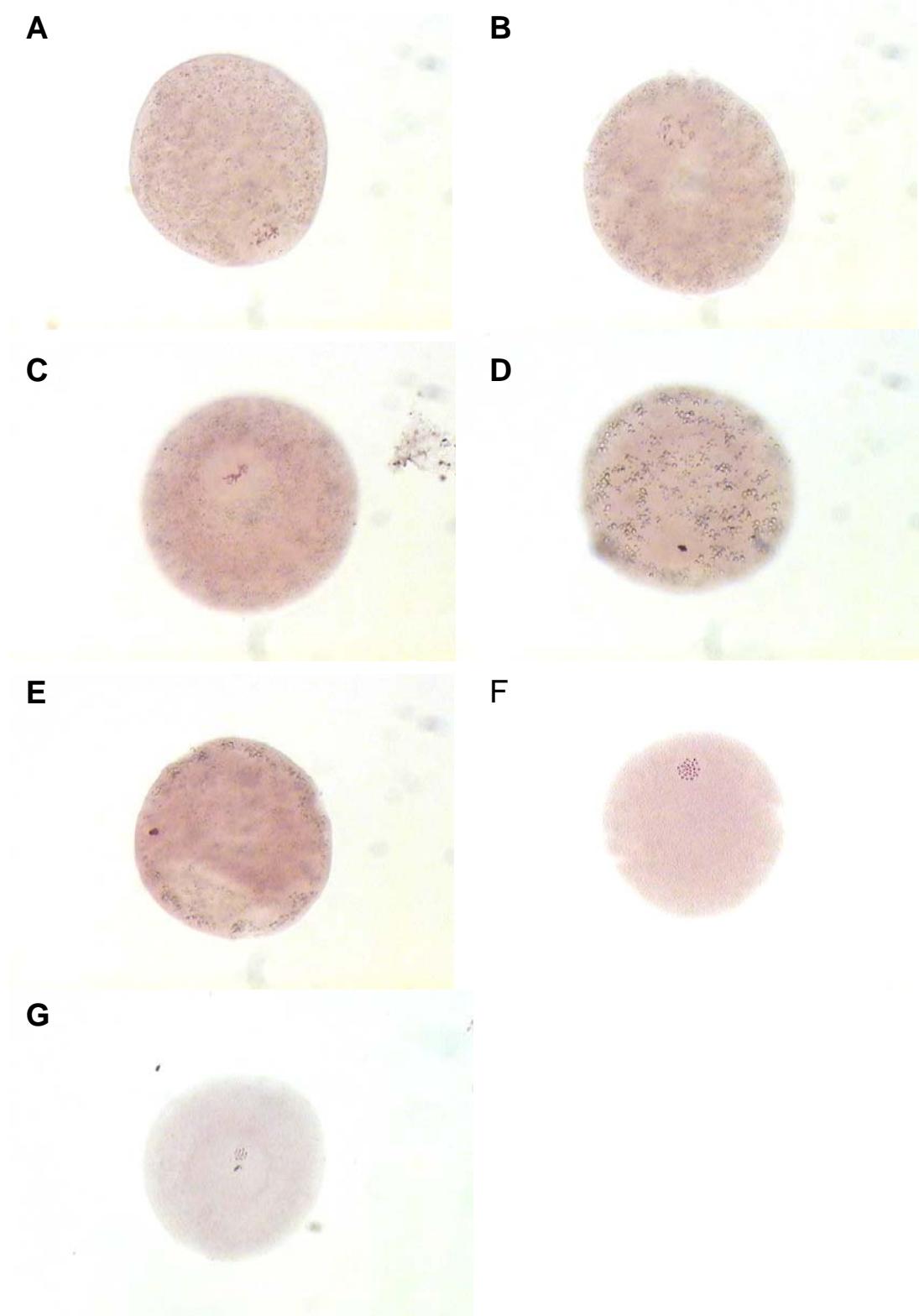
1 part glycerol, 1 part acetic acid, 3 parts water

### **Method**

Oocytes were mechanically denuded in 400 µl of culture media in 4-well dishes. Denuded oocytes were fixed by adding 400 µl of oocyte fix for a minimum of 48 h.

Fixed oocytes were transferred to a microscope slide and immobilised with a cover slip supported by petroleum jelly. Orcein dye was applied using capillary action and incubated at room temperature for 20-30 mins. Oocytes were treated with destaining solution and the cover slips fixed with nail polish.

Oocyte nuclear status was assessed at 400x using a phase contrast microscope. Oocytes were classified as being at germinal vesicle stage 1 (GV1), germinal vesicle stage 2 (GV2), germinal vesicle stage (GV3), germinal vesicle stage 4 (GV4), diakinesis (Germinal vesicle breakdown, GVBD), metaphase I (MI) or metaphase II (MII, Figure 7).



**Figure 7.** Stages of nuclear maturation. A) Germinal vesicle stage 1 (GV1), B) Germinal vesicle stage 2 (GV2), C) Germinal vesicles stage 3 (GV3), D) Germinal vesicle stage 4 (GV4), E) Diakinesis (Germinal vesicles breakdown, GVBD), F) Metaphase I (MI) and G) Metaphase II (MII).

## **Appendix 6. Published Version of Chapter 1**

**Sutton ML**, Gilchrist RB and Thompson JG 2003 Effects of in vivo and in vitro environments on metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity *Human Reproduction Update* **9** 35-48.

Sutton, M.L., Gilchrist, R.B. and Thompson, J.G. (2003) Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity.  
*Human Reproduction Update* v.9 (1) pp. 35-48, January/February 2003.

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1093/humupd/dmg009>

## **Appendix 7. Published Version of Chapter 2**

**Sutton ML, Cetica PD, Beconi MT, Kind KL, Gilchrist RB and Thompson JG 2003**

The influence of oocyte-secreted factors and culture conditions on the metabolic activity of bovine cumulus cell complexes *Reproduction* **126** 27-34.

Sutton, M.L., Cetica, P.D., Beconi, M.T., Kind, K.L., Gilchrist, R.B. and Thompson, J.G. (2003) Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes.  
*Reproduction* v.126 (1) pp. 27-34, July 2003.

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://dx.doi.org/10.1530/rep.0.1260027>

## **Appendix 8. Published Version of Chapter 3**

**Sutton-McDowall ML, Gilchrist GB and Thompson JG** 2004 Cumulus-expansion and glucose utilisation by bovine cumulus-oocyte complexes during in vitro maturation: the influence of glucosamine and follicle stimulating hormone  
*Reproduction* **128** 313-319.

Sutton-McDowall, M.L., Gilchrist, R.B. and Thompson, J.G. (2004) Cumulus expansion and glucose utilisation by bovine cumulus–oocyte complexes during in vitro maturation: the influence of glucosamine and follicle-stimulating hormone. *Reproduction* v.128 (3) pp. 313-319, September 2004.

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

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