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Effects of in-vivo and in-vitro environments on the metabolism of the cumulus–oocyte complex and its influence on oocyte developmental capacity

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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 competency, including: interactions between somatic cells of the follicle (in particular cumulus cells) and the oocyte; the composition of follicular fluid; and 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, and/or 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 differential influences on oocyte maturation and competency. 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.

Key words: culture conditions/follicular fluid/in-vitro maturation/metabolism/oocyte maturation

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 co-ordinated 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 gonadotrophic 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. Meiotic maturation following liberation of the oocyte from the follicle was first described during the 1930s (Pincus and Enzmann, 1935), but it was not until the mid-1960s that the potential for IVM as a step in the process of embryo production was recognized (Edwards, 1965). 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 with 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, for example 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 used 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 has clearly shown 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, and also the metabolism of the oocyte and the surrounding cellular vestment and relate these to developmental outcome and the current development of IVM 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 have demonstrated the importance of a bi-directional communication axis (Albertini *et al*., 2001). It is now becoming clear that oocyte paracrine signals are 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 specialized cumulus cells have distinctive trans-zonal cytoplasmic processes (TZP), which penetrate through the zona pellucida and abut the oolemma. Gap junctions at the ends of these TZP (and between cumulus cells) allow the transfer of low 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 from 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–TZP 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; Salustri *et al*., 1989; Chen *et al*., 1990). 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 with 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 with 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 500 000 in size to enter

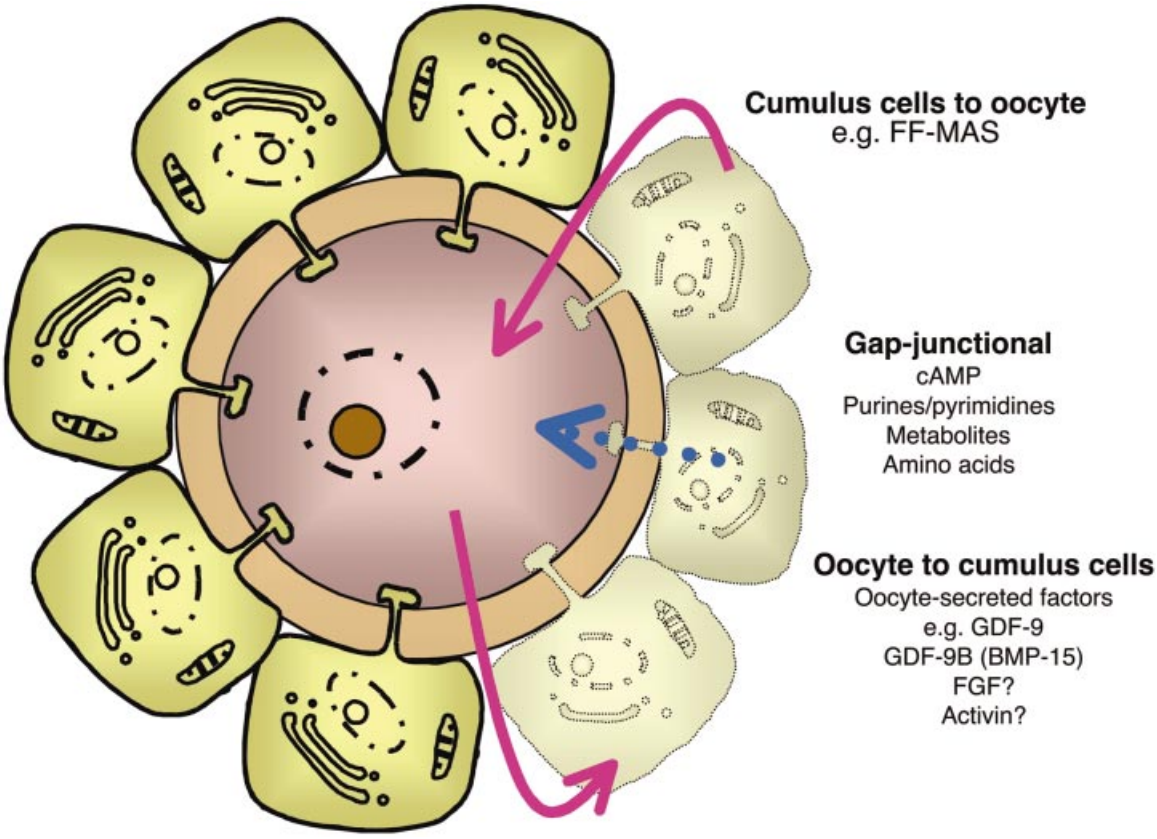

Figure 1. Oocyte–cumulus cell communication. Both paracrine (bold arrow) and gap-junctional (dashed arrow) communication between the oocyte and cumulus cells are required for normal oocyte and follicle development. Both communications pathways are bi-directional. Factors transmitted via these pathways 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.

the antrum (Gosden *et al*., 1988). The porous nature of the follicular epithelium results in FF composition being comparable with that of 'filtered' venous plasma (Table I).

Protein content

Mean protein concentration is significantly lower in bovine FF compared with blood serum, regardless of follicle size (Desjardins *et al*., 1966), and this is largely accounted for by the partial exclusion of most proteins with MW >250 000 (i.e. α_1-lipoprotein, α_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 mmol/l in plasma versus 0.062 mmol/l 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 µmol/l, 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 pre-ovulatory) are highly comparable with 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 pre-ovulatory follicles of hyperstimulated patients undergoing assisted reproduction treatments. One group (Leese and Lenton, 1990) reported that follicular lactate levels were 3- to 4-fold higher than serum levels (6.12 versus 1.5–2 mmol/l) 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 and 3.17 mmol/l 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

Table I. 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)	
						Small	Large	Small	Large
Na^+ (mmol/l)	149[b]	128[h] 139[b]	133.5[b]		132[b]	177.7[i]	109.2[i]	102.7[i]	88.1[i]
K^+ (mmol/l)	4.7[b]	15.9[h] 8.05[b]	4.9[b]		9.2[b]	10.2[i]	7.4[i]	11.4[i]	5.6[i]
Cl^- (mmol/l)	107[b]	97.3[b]	124.5[b]		149.5[b]				
Ca^{2+} (mmol/l)	2.29[b]	2.34[h] 2.3[b]	0.94[b]		3.1[b]	1.9[i]	2.1[i]	2.2[i]	1.8[i]
Mg^{2+} (mmol/l)	0.89[b]	0.75[b]	0.76[b]			0.90[i]	0.89[i]	1.3[i]	0.73[i]
Protein (g/100 ml)		6.84[h]	7.28[c]		7.08[a] 6.94[f]	247[j]	33[j]		
Albumin (mg/ml)			48.2[c]			43.4[i]	36[i]	54.1[i]	47.4[i]
Total aa (μg/ml)			236[c]						
Glucose (mmol/l)				3.44[d] 3.39[e]					
Lactate (mmol/l)				6.27[d] 3.17[e]					
pO_2 (mmHg)				59.8[g] 100.5[k]					
pCO_2 (mmHg)				46.9 [g] 34.8[k]					
pH				7.33 [g] 7.35[k]					
NH_4^+ (μmol/l)							134 [j]		

Superscripts indicate references. [a]Desjardins *et al*., 1966; [b]Gosden *et al*., 1988; [c]Velazquez *et al*., 1977; [d]Leese and Lenton, 1990; [e]Gull *et al*., 1999; [f]Andersen *et al*., 1976; [g]Fischer *et al*., 1992; [h]Schuetz and Anisowicz, 1974; [i]Wise, 1987; [j]Hammon *et al*., 2000; [k]Huey *et al*., 1999.
PCOS = polycystic ovary syndrome.

(Mangia *et al*., 1976). A positive correlation between glucose utilization and lactate production exists, and it is postulated that as the follicle grows then energy requirements increase with decreasing O_2 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_2 tension (59.8 mmHg in FF versus 102 mmHg in maternal blood) and higher CO_2 tension (46.9 mmHg in FF versus 38.3 mmHg in blood), resulting in a lower pH of FF compared with blood (7.33 and 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_2 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 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_2 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_2 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 that 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 its 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 to be 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 (MAS), that are intermediates in the cholesterol biosynthetic pathway. Follicular fluid MAS (FF-MAS) and testicular MAS (T-MAS, first purified from testicular tissue) are present in the FF of pre-ovulatory follicles in micromolar concentrations (Byskov *et al*., 2002). Their potential roles in the regulation of oocyte maturation are discussed later.

Table II. The composition of commercially supplied media commonly used for in-vitro oocyte maturation

Compound (mmol/l)	Medium					
	TCM199	Waymouth MB 752/1	Ham's F-12	MEM	DMEM	HECM
$CaCl_2$	1.802	0.82	0.23	1.36	1.36	1.9
$MgSO_4$	0.788	3.96	0.58	0.79	0.79	
KCl	5.367	2.01	3	5.37	5.37	3
NaCl	116.359	102.67	130.05	116.36	109.51	113.8
$NaHCO_3$		26.66	14	26.19	44.04	25
Na_2HPO_4	1.017	2.5	1.18	1.17	1.04	
DL-alanine	0.561		0.1			
L-arginine	0.332	0.36	1	0.6	0.4	
DL-aspartic acid	0.451	0.45	0.1			0.01
Asparagine						0.01
L-cysteine	6.98×10^{-4}	0.51	0.22			0.01
L-cystine	0.083	0.06		0.1	0.2	
DL-glutamic acid	0.908	1.02	0.1			0.01
L-glutamine	0.684	2.4	1	2	4	0.2
Glycine	0.666	0.67	0.1		0.4	0.01
L-histidine	0.104	0.78	0.17	0.2	0.2	0.01
Hydroxy-L-proline	0.0763					
DL-isoleucine	0.305	0.19	0.03	0.4	0.8	
DL-leucine	0.915	0.38	0.1	0.4	0.8	
L-lysine	0.479	1.64	0.25	0.5	1	0.01
DL-methionine	0.201	0.34	0.03	0.1	0.2	
DL-phenylalanine	0.303	0.3	0.03	0.2	0.4	
L-proline	0.348	0.43	0.3			0.01
DL-serine	0.476		0.1		0.4	0.01
Taurine						0.5
DL-threonine	0.504	0.63	0.1	0.4	0.8	
DL-tryptophan	0.0979	0.20	0.01	0.05	0.08	
L-tyrosine	0.256	0.26		0.23	0.46	
DL-valine	0.427	0.56	0.1	0.4	0.8	
Glucose	5.55	27.75	10	5.55	24.97	
DL-lactate						4.5
Pyruvate					1	
Glutathione	1.62×10^{-4}	0.16				
Hypoxanthine	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.

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, 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). It has been argued (Hunter *et al*., 1997) 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 entry of the oocyte into the Fallopian tubes. However, the 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 is higher than that within the ovary; Grøndahl *et al*., 1996; Hunter *et al*., 1997, 2000), 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 min 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 II.

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 with oocytes matured in Waymouth MB 752/1, Ham's F-12 (3% and 1% respectively; Rose and Bavister, 1992) or Ménézo'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 αMEM (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 (Tyrode's with lactate, pyruvate and polyvinyl alcohol) 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 test thoroughly the 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, 1997), while modified Connaught Medical Research Laboratories medium (CMRL-1066) is the most commonly used rhesus oocyte IVM medium (Schramm and Bavister, 1994, 1996; Schramm *et al*., 1994).

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, led to improved blastocyst development compared with 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, 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*., 2001b).

The formulation of IVM media specifically based on the composition of FFs 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, for example sheep oviduct fluid (SOF; Tervit *et al*., 1972), HTF (Quinn *et al*., 1985) and G1/G2; human tubal and uterine fluids (Gardner *et al*., 1996), MTF; mouse tubal fluid (Gardner and Leese, 1990) and PL_3 (based on bovine blood and sheep oviductal fluid; Park and Lin, 1993). IVM efficiency may be improved with the design of an IVM medium along similar principles.

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 in-vitro embryo culture (IVC) media. Numerous protein supplements are used (Fukui and Ono, 1989; Wiemer *et al*., 1991) such as fetal calf serum (FCS), estrous cow serum, estrous gilt serum, anestrous 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 pyrolidone (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 with that of 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 (LH, FSH and estradiol), growth factors (epidermal growth factor) and other beneficial factors (β-mercaptoethanol, hypotaurine) can increase blastocyst development to rates comparable with 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.

FF 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 from which the fluid originated, nor are 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 with 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 with 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 competency 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 COC metabolism

As the earliest stages of embryonic development are largely dependent 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 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 have demonstrated that cattle IVM under low (<10% O_2) 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_2 (5%), in conjunction with high (20 mmol/l) glucose was associated with improved developmental capacity (Hashimoto *et al*., 2000a). Furthermore, 5% O_2 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 utilization 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,b; Cetica *et al*., 2002), but few data exist which compare the relative changes in substrate uptake during the course of oocyte maturation. Following maturation, glucose utilization 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 utilization, 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 with that in 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. The PPP is also linked to other cellular enzymatic processes, through the reduction of $NADP^+$ to NADPH, a key regulator substrate in essential metabolic programmes such as GSH reduction (see 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 ethosulphate and pyrroline-5-carboxylate leads 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. The PPP appears to be involved in FSH-induced meiotic maturation in murine oocytes, while FSH stimulates the utilization 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 in cattle (Krisher and Bavister, 1998; Rose-Hellekant *et al*., 1998) and rhesus monkeys (Zheng *et al*., 2001a). However, high concentrations of glucose during IVM have adverse effects on subsequent embryo development; this is thought to be due to an increased generation of reactive oxygen species (ROS) and decreased intracellular GSH pools (Hashimoto *et al*., 2000b). Oocytes derived from adult and pre-pubertal cattle differ in their metabolic profiles, in

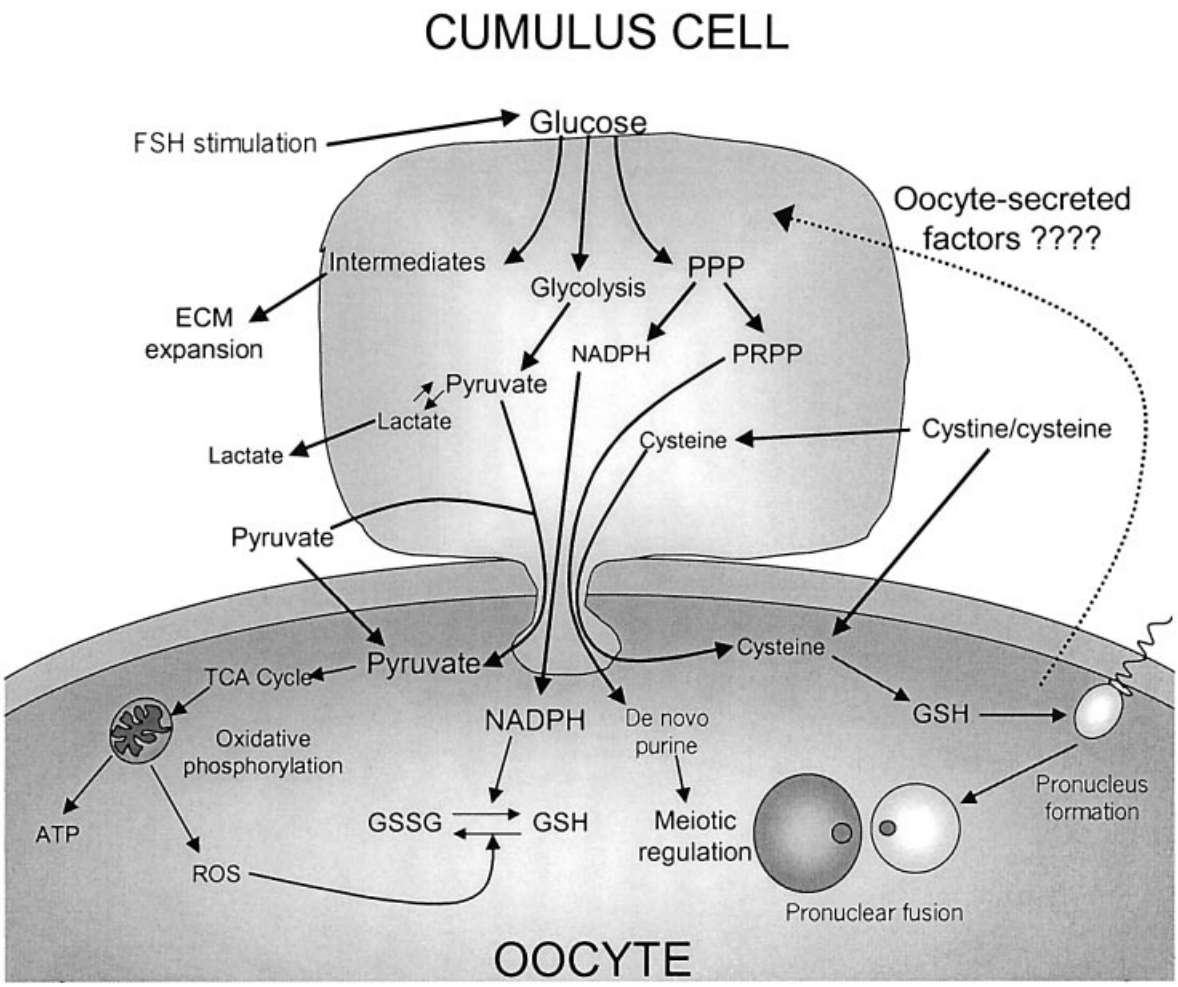

Figure 2. Proposed model of the metabolic interactions and activity of cumulus cells and the oocyte. Numerous energy substrates are supplied to the cumulus–oocyte complex (COC) by the surrounding fluid, including glucose, pyruvate, lactate and amino acids. Glucose can be utilized via three major pathways: (i) glucose oxidation (the combination of glycolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation); (ii) the pentose phosphate pathway (PPP); or (iii) it can be converted to intermediates and utilized for extracellular matrix (ECM) expansion. 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 oxidized 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 one of the products of the pathway, phosphoribosylpyrophosphate (PRPP), being used by the oocyte for purine synthesis. Purines are involved in the regulation of nuclear maturation. PPP is also involved in general cytoplasmic homeostasis since $NADP^+$ 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 are no data available concerning their effects on the metabolism of cumulus cells. GSSG = oxidized GSH; ROS = reactive oxygen species.

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 with an 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 with 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 utilize 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 utilize glucose is positively correlated with subsequent embryo developmental potential, and may be used 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^+ is required for lactate utilization to occur (Cetica *et al*., 1999). This result is somewhat surprising as NAD^+ is not considered cell-permeable, and suggests leakage of LDH 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 with DOs, but the LDH-1 isoform activity is greater in DOs than cumulus cells; hence, lactate can be utilized within the oocyte under appropriate REDOX conditions (Cetica *et al*., 1999). Pyruvate consumption by murine COCs was directly correlated to oocyte maturation; hence, an increasing requirement for pyruvate occurs as the oocyte matures (Downs *et al*., 2002).

Purines/pyrimidines

Reversible inhibition of oocyte nuclear maturation during IVM may allow for improved oocyte cytoplasmic maturation and enhanced embryo developmental competency (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, an interaction has been demonstrated between purines and glucose metabolism via the PPP within the murine oocyte (Downs and Mastropolo, 1994; Downs *et al*., 1998; Downs and Utecht, 1999; Downs and Hudson, 2000). 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, generally purines are 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 utilized by oocytes as an energy source with cumulus cells, and play an important role 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 with defined media without amino acids and macromolecule supplementation (Watson *et al*., 2000).

LH stimulates cumulus cells to convert glutamine to α-ketoglutarate, which is then oxidized through the tricarboxylic acid (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 oxidized to cystine. It has been shown (de Matos *et al*., 1997) 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 with 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 β-mercaptoethanol results in elevated oocyte GSH levels by reducing oxidized cystine to constituent cysteine in the medium (de Matos *et al*., 1995, 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, 1996). Furthermore, addition of cysteamine to IVC media leads to increased development of 6- to 8-cell bovine embryos to blastocysts, compared with 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

Few studies have been conducted to determine 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 material (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 (see below).

Effect 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 that 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, 1997). Furthermore, the addition of FSH to IVM media leads to a 50% increase in the resumption of meiosis in bovine COCs compared with unstimulated complexes or hCG stimulation (van Tol *et al*., 1996). Whether sterols such as FF-MAS play any role at all 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 result in reduced rates of MII COCs (Downs and Hudson, 2000). Glucose utilization 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 has little effect on the resumption of meiosis (Downs *et al*., 1996) compared with 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 the PPP (Downs and Utecht, 1999). Although FSH increases the activities of both the PPP and glycolysis in murine COCs (Downs and Utecht, 1999), LH significantly increases the glycolytic pathway in bovine COCs and decreases PPP activity compared with 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, because of the potential to greatly reduce dependency on gonadotrophin stimulation. 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 competency and metabolism. In particular, a close association between intracellular GSH levels and subsequent embryo developmental potential has been demonstrated in several species. Less well characterized 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 investigations of Downs and colleagues, few studies have been 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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