Metabolism of the bovine cumulus-oocyte complex and influence on subsequent developmental competence

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The two types of cells that make up the cumulus-oocyte complex (i.e. the oocyte and cumulus cells) have very different metabolic demands, with glucose occupying a central role in metabolic activity. Cumulus cells have a significant requirement for and utilise high levels of glucose, yet appear to have little need for oxidative metabolism. In contrast, oocytes have a requirement for oxidative metabolism, although limited glucose metabolism may also be an important aspect of meiotic and developmental competence. Nevertheless, because of the metabolic and communication link between the cumulus and the oocyte, glucose availability and metabolism within the cumulus can have a significant impact on oocyte meiotic and developmental competence. In particular, the role of the hexosamine biosynthesis pathway within cumulus cells appears critical for the supply of substrate from glucose for extracellular matrix production, yet if overstimulated can significantly decrease developmental competence of the oocyte. Current static systems for in vitro maturation are clearly incompatible with meeting substrate demands, especially glucose. In the future, in vitro maturation will include a more dynamic approach, which will adjust nutrient components to meet the changing functional requirements of cumulus-oocyte complexes during the final process of maturation.

Introduction

Understanding oocyte meiotic and developmental competence and unravelling the factors that mediate their fulfilment remain as great challenges in the field of reproductive biology, although recent attempts to describe the molecular basis of oocyte competence have taken significant steps forward (Pan *et al.* 2005; Reis *et al.* 2006). In contrast, the benefits that will result from a greater understanding of oocyte meiotic and developmental competence have not altered. We are still inept in our ability to exploit fully the genetic value held within every female gamete, which could be far greater by capturing the significant numbers of oocytes within an individual female, each capable of producing a viable offspring. We are also still inept in our ability to store oocytes in any developmental state so as to efficiently derive viable embryos when needed.

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Oocyte developmental competence and oocyte capacitation

Oocyte developmental competence is the term that refers to the biochemical and molecular state that allows a mature oocyte to support development of the embryo and ultimately a healthy offspring at term. We still have a very poor understanding of what constitutes oocyte developmental competence, including the role that the environment surrounding an oocyte plays in its progress towards acquisition of developmental competence. The difficulty of studying the oocyte within the developing follicle remains the major barrier to rapid progress in this area. It is clear that oocytes gradually and sequentially acquire meiotic and developmental competence during the course of folliculogenesis. During the early stages of antral follicle growth, non-rodent oocytes are actively synthesizing RNA and as antral follicles progress towards ovulatory size, the synthetic activity of the oocyte is gradually reduced until the oocyte reaches a quiescent state (de Smedt *et al.* 1994; Fair *et al.* 1995). This latter phase of oocyte development has been termed "oocyte capacitation" (Hytell *et al.* 1997) as it is during this phase of oogenesis that the oocyte acquires the cytoplasmic machinery necessary to fully support preimplantation embryo development (Brevini-Gandolfi & Gandolfi 2001). The somatic cells of the follicle, and the cumulus cells in particular, certainly play a key role in the acquisition of developmental competence in vivo. However, we have only a limited understanding of the nature and diversity of compounds that transfer between the cumulus cells and the oocyte via gap junctions during this final phase of follicular development (Albertini *et al.* 2001) and we still have no idea of how dynamic this process is and whether or not dynamic changes to levels of transferring molecules impacts the process of developmental competence. We also have a poor understanding of the role that granulosa and cumulus cells have in modifying the extracellular environment that surrounds the oocyte; speculation with little data is usually the norm here (e.g. nutrients) and little consideration is taken for the influence of events such as gap junction breakdown and cumulus expansion have on nutrient availability to the oocyte.

Our laboratory has taken an interest in the metabolism of the cumulus-oocyte complex (COC) while undergoing in vitro maturation (IVM) and what role this may have on oocyte developmental competence, especially in the bovine. It is noteworthy that oocyte IVM involves the artificial removal of oocytes, generally from mid-sized antral follicles, that presumably have not completed oocyte capacitation. Incubating COCs in vitro leads effectively to a precocious spontaneous meiotic maturation, that occurs in the absence of these crucial events and environments that are required for complete cytoplasmic maturation of the oocyte. Many may argue that this is a poor model for studying the far more complex interactions that take place within the follicle during final antral follicle development. Whilst this is reasonable, the fact remains that oocyte IVM technologies, especially in domestic animals, must deal with the very mixed population of oocytes collected from follicles at varying stages of development and atresia. Our efforts are directed at trying to understand the complex metabolic interplay between oocytes and cumulus cells as they undergo maturation in vitro, in an attempt to develop IVM systems that actually confer developmental competence on oocytes that would otherwise undergo natural demise in vivo by subordination and atresia.

Here we review the progress we, and others, have made in our understanding of the metabolic conditions that influence both meiotic and developmental competence during IVM of bovine COCs in recent times. Other reviews in the area that precede this and may be of useful reference include Sutton *et al.* (2003b) and Krisher (2004).

Energy demands by the oocyte – developmental competence

Mammalian oocytes are essentially reliant on oxidative phosphorylation for the generation of ATP (Rieger and Loskutoff 1994; Steeves and Gardner 1999). Not surprisingly, the level of ATP within a mature oocyte appears now as a key indicator of developmental competence (Hashimoto *et al.* 2000a; Stojkovic *et al.* 2001). Van Blerkom (2004) has long argued the importance of mitochondria within human oocytes in the development of competence. This has only recently been examined in several other species, e.g. mouse (Thouas *et al.* 2004) and bovine (Stojkovic *et al.* 2001; Tarazona *et al.* 2006), which are all in agreement on the importance of mitochondria. However, there is also evidence that the activity of the glycolytic pathway within the oocyte following maturation also relates to developmental competence (Krisher and Bavister 1999; Steeves and Gardner 1999), despite the low levels of glycolytic activity within bovine oocytes (Rieger and Loskutoff 1994; Cetica *et al.* 2002).

Concentration of substrates in follicular fluid

Several recent publications have described levels of energy substrates available to immature, compact ruminant COCs from antral follicles prior to the LH surge (Table 1). Concentrations of glucose and pyruvate have been measured at approximately 2 - 3 mM and 0.4 mM respectively in these studies. Although studies have found lactic acid is also present within follicular fluid, care must be used in interpreting this, as most data has been derived from ovaries collected in an abattoir in which the time from slaughter to fluid collection will affect lactic acid levels.

Table 1. Glucose and carboxylic acid concentrations (Mean ± S.E.) in follicular fluid from various sizes of follicles (small = ~ 3mm, medium = ~ 5mm, large = ~ 8mm)

	Small	Medium	Large	
Glucose (mM)	2.01±0.10	2.85±0.16	3.75±0.18	Leroy *et al.* (2004)
	1.4±0.2	2.2±0.3	2.3±0.2	Sutton McDowall *et al.* (2005)
	3.5±0.5	-	3.9±0.4	Iwata *et al.* (2004).
Pyruvate (mM)	-	-	-	"
	0.4±0.1	0.4±0.1	0.4±0.0	"
	-	-	-	"
Lactate (mM)	14.4±0.35	9.4±0.35	5.6±0.37	"
	3.0±0.7	6.4±1.7	3.2±0.6	"
	-	-	-	"

Less is known regarding other substrates. Recently, Leroy *et al.* (2004) measured, amongst other parameters, levels of ß-hydroxybutyrate, urea and non-esterified fatty acids within bovine follicular fluid. Berg and colleagues (Berg *et al.* 2003) have reported that O_2 concentrations within dominant follicles in vivo are around 67 – 86 mmHg, (i.e. around 9 - 12% O_2), which seems higher than many other species (reviewed by Sutton *et al.* 2003b).

The uptake of oxygen by bovine COCs

Although oxygenation of the follicle has been linked with oocyte developmental competence, (Van Blerkom 1998; Berg *et al.* 2003), there is little known regarding the oxygen consumption by intact COCs from any mammalian species and what role this plays in oocyte growth and

development. Sutton and colleagues (Sutton *et al.* 2003a) measured oxygen uptake by bovine COCs over the course of a 24 h in vitro maturation period (in the presence of FSH). On a per ng DNA basis, COCs increase their oxygen consumption from approximately 50 pmol/h at the start of maturation, when the COC is compact, to 90 pmol/h for fully expanded COCs, which equates to from around 1.5 to 2.5 nl/COC/h. This total uptake of oxygen is remarkably low for a complex of cells such as the COC and is comparable to about double the respiration rate of a bovine blastocyst (0.9 nl/h, Thompson *et al.* 1996). Mouse COCs consume an average of 4 nl/h O_2 during maturation (Downs *et al.* 1997), which is higher, but within a similar range as the bovine COC. These values led us to ask what level of O_2 actually reaches the oocyte within the cumulus complex? In particular, we wanted to know if there is significant loss of oxygen within the COC as O_2 diffuses towards the oocyte, especially in immature, compact COCs. We developed a mathematical model (Clark *et al.* 2006), which assumes the COC is spherical, but accounts for a non-linear consumption of oxygen dependent on the external concentration. Our model also accounts for the two layers of cells, the cumulus layer and the oocyte and differs from other such models (e.g. Byatt-Smith *et al.* 1991). The model revealed that, surprisingly, the loss of oxygen across the compact bovine COC is negligible (less than 4% of the external concentration), even if hypoxic levels of oxygen are utilised. Thus the level that reaches the oocyte is effectively that which surrounds the COC in the follicular antrum. This has led us to postulate that the structure and metabolic activity of antral follicles is such that follicular oxygen is preserved for the oocyte, as oocytes are dependent on oxidative phosphorylation for ATP production (Rieger and Loskutoff 1994) and have little capacity for glycolysis (Cetica *et al.* 2002).

The central role and importance of glucose to oocyte meiotic and developmental competence

Uptake of glucose by the COC

The bovine COC from antral follicles consumes substantial quantities of glucose - 23 times more uptake on a per volume basis compared to oocytes (Thompson *et al.* 2005), pointing to the importance of glucose to COC metabolism (Fig. 1). Indeed, under standard IVM conditions (TCM199 medium containing 5.6 mM glucose), where the COC density is 1:5 μl medium, we have shown that over 70% of glucose will be metabolised and this may well impact glucose uptake and metabolism kinetics (Sutton-McDowall *et al.* 2004). Over the course of FSH-stimulated in vitro maturation, the uptake rate in TCM199-based medium is initially 23 pmol/ngDNA/h, increasing to 40 pmol/ngDNA/h. This is entirely due to uptake by the cumulus cells, as the bovine oocyte itself has a very low capacity for glucose uptake (Rieger and Loskutoff 1994; Zuelke and Brackett 1992). There is evidence that ruminant granulosa cells contain both GLUT1 and the insulin-sensitive GLUT4, which may explain the high uptake rates (Williams *et al.* 2001).

The induction of cumulus expansion increases glucose uptake by approximately 25% and is associated with an increase in hyaluronic acid synthesis via the hexosamine biosynthesis pathway (Sutton-McDowall *et al.* 2004). Using a non-metabolised fluorescent glucose analogue, we have observed the temporal uptake of glucose within the immature compact and fully expanded COC (Thompson *et al.* 2005). Although preliminary at this stage, in immature COCs, the non-metabolised glucose forms a gradient of fluorescence through the cumulus cell layers towards the oocyte. Within 40-60 min of exposure, significant accumulation occurs within the corona radiata. From approximately 40 min, fluorescence is observed within the oocyte. As this is a non-metabolised glucose, the question that remains to be answered is: Does this reflect the transfer of unmetabolised glucose or some metabolite of glucose? How much actual glu-

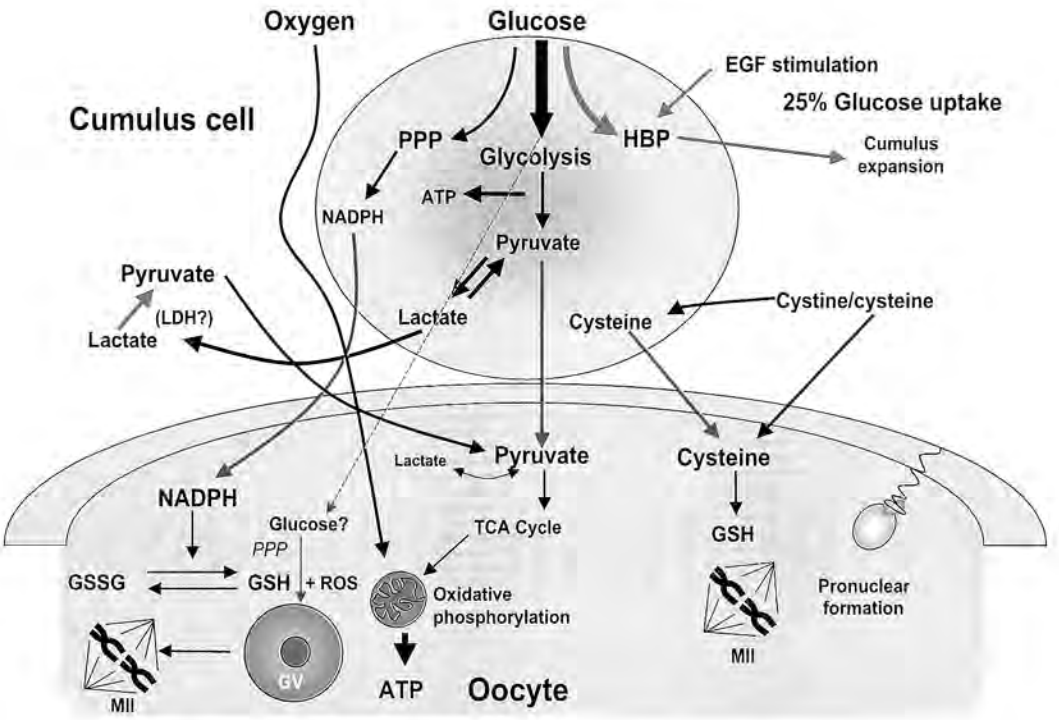

Fig. 1. Schematic representation of the metabolism of glucose and carboxylic acids, plus the amino acid cysteine in the cumulus oocyte complex during maturation. Direction of arrows represents the direction of substrate transfer and density of arrows suggests possible activity of pathways. Colours of arrows (other than black) represent stage of maturation, with blue specific for substrate transfer occurring prior to gap junction breakdown and red specific for activity following cumulus expansion. Abbreviations: ATP = Adenosine triphosphate; EGF = Epidermal Growth Factor; GSH = glutathione (reduced); GSSG glutathione (oxidised); GV = Germinal vesicle; HBP = Hexosamine biosynthesis pathway; LDH = Lactate dehydrogenase; MII = Metaphase II; PPP = Pentose Phosphate Pathway; NADPH = Nicotinamide adenine dinucleotide phosphate; ROS = reactive oxygen species.

cose reaches the oocyte within the immature compact cumulus complex? To answer this, we are developing a much more sophisticated mathematical model to understand the dynamics of facilitated transport, diffusion and metabolism across entities such as the COC. Nevertheless, Saito and others (Saito *et al.* 1994) have shown that in the mouse both glucose and glucose-6-phosphate levels are high in the immature oocyte derived from preovulatory follicles, falling dramatically with (presumably) the loss of cumulus cell gap-junction connection, strongly suggesting that prior to gap junction breakdown, both glucose and glucose metabolites are transferred from cumulus cells into the oocyte. In expanded COCs, over the same time course, it is clear that cumulus cells play little role in glucose transport into the oocyte as no fluorescence appears to enter the oocyte, similarly to that described in the mouse (Saito *et al.* 1994). This further supports the work of others that glucose uptake is very low in mature bovine oocytes (Rieger and Loskutoff 1994).

The effect of glucose on meiotic and developmental competence of the bovine oocyte

Several studies have demonstrated that the presence of glucose during spontaneous in vitro maturation is necessary for complete meiotic maturation to occur (Hashimoto *et al.* 2000b). However, recent studies have revealed that glucose availability (i.e. concentration vs. depletion as a result of uptake over the course of maturation) can have significant affects on the progression of maturation (Iwata *et al.* 2004; Sutton-McDowall *et al.* 2005). Indeed, the use of sequential IVM systems, where fresh medium is used to "replenish" glucose levels during the maturation, can alleviate poor meiotic responses in media with low levels of glucose. Nevertheless, delayed maturation appears to have little consequence in terms of developmental competence (Iwata *et al.* 2004; Sutton-McDowall *et al.* 2006). In contrast, also using a sequential maturation media, Atef and colleagues (Atef *et al.* 2005) found exposure to FSH for 6 hours during IVM was beneficial to development when using a low (1.5 mM) glucose concentration maturation medium (Synthetic Oviduct Fluid) compared with continuous exposure (no change in medium) or 2 h of FSH (and exchange at that time). We suggest these latter two treatments also induce significant glucose depletion at the volume per COC that was utilized (5 μl/COC). Depletion of glucose may prevent oocytes from maintaining glucose-6-phosphate levels (Saito *et al.* 1994), which is known to have a stimulatory effect on meiotic progression via activity of the pentose phosphate pathway (Downs *et al.* 1998).

The hexosamine biosynthesis pathway and oocyte competence

In most somatic cells the hexosamine biosynthesis pathway (HBP, Fig. 2) is a minor glucose metabolic pathway, accounting for 1-3% of glucose metabolism (Marshall *et al.* 1991). A major role of the HBP is the generation of N-linked acetyl sugars for the production of glycosaminoglycans. For example, the end product of the HBP is UDP-N-acetylglucosamine (UDP-N-GlcNAc), which is then synthesized into hyaluronic acid by the action of the enzyme, hyaluronic acid synthase (Fig. 2). The HBP is considered a self-regulating pathway; the rate-limiting enzyme is also the first step of the pathway, glutamine-fructose-6-phosphate transaminase (GFPT). Inhibition of GFPT activity is regulated by the pathway's end product, UDP-N-GlcNAc (McKnight *et al.* 1992). Cumulus cells have a great capacity for hyaluronic acid synthesis as a result of initiation of expansion by epidermal growth factor like factors in vivo (Park *et al.* 2004) or by FSH/EGF in vitro. As previously mentioned, glucose uptake increases 25% with the initiation of cumulus expansion (Sutton-McDowall *et al.* 2004), which appears to be entirely due to up regulation of the HBP pathway. How such a dramatic increase in activity is initiated remains to be determined, but it appears that factors regulating cumulus expansion do so by regulating GFPT activity.

An alternate fate for UDP-N-GlcNAc is O-linked glycosylation, via O-linked glycosyltransferase, an X-linked enzyme (O'Donnell *et al.* 2004). The importance of O-linked glycosylation within protein signalling systems has only recently come to light, with many major signalling proteins now recognized as being regulated by O-linked glycosylation (e.g. Sp1, Sp3, CREB, p53, in addition to many others). In particular, serines and threonines that are normally phosphorylated can also be O-linked glycosylated, thereby changing the activity (either up or down-regulated) of phosphorylated signalling proteins (Wells *et al.* 2003; Zachara and Hart 2004). One of the more widely studied effects of HBP up-regulation leading to increased O-linked glycosylation is insulin signalling through the insulin receptor substrate-1 (Andreozzi *et al.* 2004), where perturbed phosphorylation of the insulin receptor substrate significantly down-regulates the protein kinase B pathway.

Glucosamine is a sugar related to glucose and can be transported into cells by members of the facilitated glucose transporter family, similarly to glucose (albeit with different kinetics). However, unlike glucose, there is only one metabolic fate for glucosamine, phosphorylation to

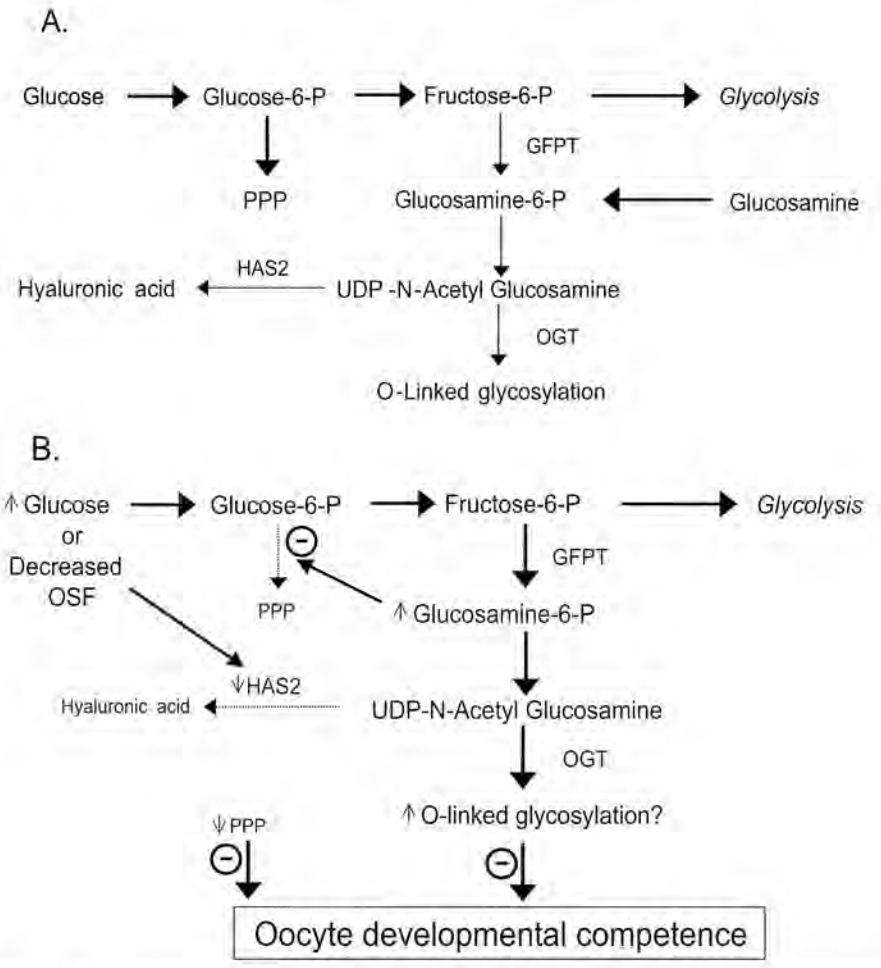

Fig. 2. A. Glucose metabolism, with particular focus on the hexosamine biosynthesis pathway. B. The effect of reduction of oocyte secreted factors or hyperglycaemia on developmental competence. An increase in the hexosamine biosynthesis pathway activity or diversion of UDP-N-acetylglucosamine towards O-linked glycosylation (either by hyperglycaemia or by inhibition of cumulus expansion) causes a reduction in developmental competence of the oocyte. Abbreviations: GFPT = glutamine:fructose-6-phosphate transaminase; HAS2 = hyaluronic acid synthase; OSF = oocyte secreted factors; PPP = Pentose phosphate pathway.

glucosamine-6-phosphate (Fig. 2) and thus bypasses the rate-limiting enzyme of the hexosamine pathway. The consequence is that UDP-N-GlcNAc levels increase significantly, which can lead to increased O-linked glycosylation. Glucosamine is therefore regarded as a hyperglycaemic mimetic.

Glucosamine addition to a bovine IVM medium significantly reduced the level of glucose incorporated within the expanding cumulus mass (Sutton-McDowall *et al.* 2004), signifying the role the hexosamine pathway plays in extracellular matrix formation. Surprisingly, although not significantly affecting meiotic maturation or early cleavage rates following fertilization, glucosamine addition during IVM also substantially reduced subsequent developmental com-

petence (Sutton-McDowall *et al.* 2006), with almost complete inhibition at levels of 2.5 mM glucosamine (when incubated with 5.6 mM glucose). This loss of developmental competence was somewhat reversed when inhibition of O-linked glycosyltransferase in the presence of glucosamine was performed (Sutton-McDowall *et al.* 2006). However, we have not yet fully determined if the glucosamine effect is mediated through cumulus cell signalling, or if it has a direct affect on the oocyte, although immunohistochemical staining of O-linked glycosylation was largely isolated to within cumulus cells, indicating the former rather than the latter.

Hashimoto and others (Hashimoto *et al.* 2000b) have also found that under hyperglycaemic conditions, developmental competence was compromised. This fits well with our model (Fig. 2B), where either hyperglycaemic conditions or compromised cumulus expansion mechanisms leads to an inhibition of developmental competence. However, this inhibition was overcome and, furthermore, developmental competence improved when oxygen concentration was reduced from atmospheric levels (20.9%) to 5% O_2 (Hashimoto *et al.* 2000a). Indeed, only when hyperglycaemic conditions were utilized, was low oxygen found to benefit developmental competence (Hashimoto *et al.* 2000a). This interaction between oxygen and glucose may possibly be explained by the involvement of the transcription factor, hypoxia inducible factor (HIF). Lowering pO_2 may activate HIF to increase glycolytic enzyme activities, which are regulated by HIF (Iyer *et al.* 1998), increasing the capacity to metabolise glucose via glycolysis, thus reducing the option for glucose to be utilised for the HBP and O-linked glycosylation (see Fig. 2B). HIF is also believed to mediate selected FSH-induced responses (Alam *et al.* 2004), so that low oxygen levels may increase the effectiveness of FSH within the maturation system.

Role of carboxylic acids

Most of the glucose taken up by an immature bovine COC is accounted for by the production of lactic acid by cumulus cells and efflux into the surrounding environment (Sutton *et al.* 2003a). Initially in IVM, a positive correlation exists between lactic acid production and glucose uptake, which then weakens as maturation proceeds (Sutton *et al.* 2003a). This, as well as the unchanging lactic acid production over the course of FSH-stimulated cumulus expansion and glucose uptake, provides further evidence that the increasing glucose uptake that occurs during maturation is directed into pathways other than glycolysis (Sutton *et al.* 2003a). Exogenous pyruvate uptake by the FSH stimulated bovine COC occurs, even in the presence of glucose (Sutton *et al.* 2003a). A role for exogenous pyruvate, however, is largely unknown, especially in the presence of adequate glucose. Glycolysis is particularly active in cumulus cells and they have significant levels of several isoforms of lactate dehydrogenase (LDH, the close to equilibrium enzyme for pyruvate conversion to lactate) (Cetica *et al.* 1999; 2002). Therefore it remains to be shown that there is a need for exogenous pyruvate, at least for much of the first nine hours of bovine IVM, where gap-junction communication is patent (Thomas *et al.* 2004). On the other hand, one can imagine that following initiation of cumulus expansion (and hence rapid loss of the oocyte's source of glycolytic metabolites), direct exposure of the oocyte to adequate levels of pyruvate for the latter period of maturation could be necessary for the oocyte. Interestingly, exogenous pyruvate appeared to have no influence on developmental competence, whereas exogenous lactate did (Rose-Hellekant *et al.* 1998). One possible explanation may be the high levels of lactate present in the medium as maturation proceeds and the release of LDH from apoptotic cumulus cells, especially those on the outer edge of the COC (Hussein *et al.* 2005), providing a source of pyruvate for the oocyte towards the end of maturation.

Amino acids

The role of cysteine (and related sulphated amino acids, such as cysteamine), along with glutamine and proline as substrates for glutathione, is now well characterised as promoting developmental competence in bovine oocytes, and oocytes from other species (de Matos *et al.* 1995; de Matos and Furnus 2000). Inclusion of these sulphated amino acids during IVM and the subsequent increase in oocyte glutathione levels is thought to reduce the level of reactive oxygen species production within oocytes, which has beneficial effects on subsequent developmental competence (de Matos and Furnus 2000). Consequently, cysteamine is now a standard additive in most IVM culture systems. In addition, glutamine is known to be stimulatory for cumulus expansion (Rose-Hellekant *et al.* 1998), most likely through its involvement in hyaluronic acid synthesis through the hexosamine biosynthesis pathway (see above). Zuelke and Brackett (1993) also found that under the influence of luteinizing hormone, glutamine oxidation increased within the COC, although the relevance of this observation remains obscure.

Lipids

Little new work has emerged on the role of lipids during oocyte IVM, with the exception of the efforts of Leroy and co-workers (Leroy *et al.* 2005), who have an interest in the hypothesis that non-esterified fatty acids within follicular fluid play a role in oocyte competence during periods of negative energy balance in high performing dairy cows. Their recent work has revealed that addition of stearic and palmitic acids, but not oleic acid, had negative affects on oocyte developmental competence when included during IVM (Leroy *et al.* 2005).

Conclusions

The bovine cumulus oocyte complex contains two cell types that appear to have antagonistic nutritional demands. Cumulus cells have a great need and appetite for glucose. Too little glucose during maturation appears to have significant adverse effects on oocyte meiotic competence, whilst too much appears to affect oocyte developmental competence. The former may involve glucose and glucose-6-phosphate within the oocyte itself, the latter appears to be mediated through the hexosamine biosynthesis pathway and links the process of supplying substrate for cumulus expansion with oocyte developmental competence. However, achieving consistent glucose concentrations under static IVM conditions is problematic due to the high uptake rate. In contrast, the demand for oxygen and oxidative substrates appear low.

Oocytes, on the other hand, require oxygen and oxidative phosphorylation, and although some glycolytic activity occurs, we question its significance prior to cumulus expansion and loss of gap-junction communication.

The anatomical design of the cumulus oocyte complex fits these antagonistic demands, as oxygen appears to readily diffuse through the cumulus layer to the oocyte, with little being consumed by the cumulus cells themselves. In contrast, glycolytic activity within cumulus cells is such that some glucose appears to reach the immature oocyte whilst the cumulus layer remains compact. This changes once cumulus expansion occurs, so that little glucose enters the oocyte.

Achieving such a delicate balance in substrate supply during oocyte in vitro maturation will remain difficult whilst the current static culture systems employed continue to be utilized. Novel culture systems, utilizing principles of perfusion, microfluidics and small culture chambers with large media reservoirs - the WOW system (Vajta *et al.* 2000), will facilitate more

purpose-built conditions that satisfy the complex demands required for successful in vitro maturation of oocytes.

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