Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation *in vitro*

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Few studies demonstrate at a biochemical level the metabolic profile of both cumulus cells and the oocyte during maturation. The aim of the present study was to investigate the differential participation of enzymatic activity in cumulus cells and in the oocyte during *in vitro* maturation (IVM) by studying the activity of enzymes involved in the control of amino acid metabolism, alanine aminotransferase (ALT) and aspartate aminotransferase (AST); and the tricarboxylic acid (TCA) cycle, isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH). No NAD-dependent isocitrate dehydrogenase (NAD-IDH) activity was recorded in cumulus–oocyte complexes (COCs). ALT, AST, NADP-dependent isocitrate dehydrogenase (NADP-IDH) and MDH enzymatic units remained constant in cumulus cells and oocytes during IVM. Specific activities increased in oocytes and decreased in cumulus

cells as a result of IVM ($P < 0.05$). Similar activity of both transaminases was detected in cumulus cells, unlike in the oocyte, in which activity of AST was 4.4 times greater than that of ALT ($P < 0.05$). High NADP-IDH and MDH activity was detected in the oocyte. Addition of alanine, aspartate, isocitrate + NADP, oxaloacetate or malate + NAD to maturation media increased the percentage of denuded oocytes reaching maturation ($P < 0.05$), in contrast to COCs in which differences were not observed by addition of these substrates and co-enzymes. The activity of studied enzymes and the use of oxidative substrates denotes a major participation of transaminations and the TCA cycle in the process of gamete maturation. The oocyte thus seems versatile in the use of several oxidative substrates depending on the redox state.

Introduction

Current knowledge of the requirements of the bovine oocyte necessary for reaching normal *in vitro* maturation (IVM) and the metabolic profile of the oocyte is insufficient. As meiosis readily occurs *in vitro*, metabolic studies on the maturation process are scarce compared with those carried out on embryo development. However, the oocyte cytoplasm is known to provide the appropriate metabolic machinery for the production of energy required for cellular function during maturation, fertilization and early embryo development (Gardner, 1998). Application of knowledge gained about cytoplasmic changes during maturation may result in improved IVM and overall *in vitro* embryo production (Bavister *et al.*, 1992; Krisher and Bavister, 1998; Sutton *et al.*, 2003c).

Appropriate oocyte maturation depends largely on the presence of surrounding cumulus cells that perform nutritive and regulatory functions during this process, as removal of cumulus cells before maturation results in compromised maturation, fertilization and embryo

development compared with removal of cumulus cells after IVF (Zhang *et al.*, 1995; Cetica *et al.*, 1999a; Fatehi *et al.*, 2002). Several authors have suggested that the fate of glucose within cumulus cells is primarily the glycolytic pathway (Zuelke and Brackett, 1992; Cetica *et al.*, 1999b, 2002; Sutton *et al.*, 2003a). Cumulus cells would thus be in charge of producing metabolites derived from glucose degradation to be used as oxidative substrates by the oocyte (Buccione *et al.*, 1990; Brackett and Zuelke, 1993; Cetica *et al.*, 1999b). In rodents, oocytes free of somatic cells underwent spontaneous maturation when cultured in the presence of pyruvate or oxaloacetate, but failed to mature with glucose, lactate or phosphoenolpyruvate, indicating that oocyte maturation is sustained by the tricarboxylic acid (TCA) cycle (Biggers *et al.*, 1967). It was postulated that pyruvate, glycine and glutamine uptake by bovine oocytes during maturation is one of the main pathways to obtain energy via the TCA cycle (Zuelke and Brackett, 1993; Rieger and Loskutoff, 1994). The presence of certain amino acids in maturation media increases the percentage of successful IVF cycles and the number of bovine embryos that reach the blastocyst stage. It was observed that a combination of glucose and amino acids in a given maturation medium free of proteins improved embryo development,

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whereas pyruvate combined with glucose or glutamine in the same medium led to a low percentage of embryo production (Krisher and Bavister, 1998; Rose-Hellekant *et al.*, 1998).

The study of key enzyme activities responsible for controlling the rate of a metabolic pathway may be used to determine the participation of a given metabolic route in the cells under study. So far, no studies have been carried out to determine the activity of enzymes involved in the metabolism of amino acids and the TCA cycle in bovine cumulus-oocytes complexes (COCs) during IVM.

The first step in amino acid catabolism consists of the elimination of the alpha amino nitrogen atom by transamination, after which the resulting carbon skeleton is degraded to obtain energy. As the reactions catalysed by transaminases are reversible, they may catalyse amino acid reactions of catabolism or biosynthesis. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are present in most mammalian tissues and play a key role in amino acid metabolism (Torchinsky, 1987). No isoenzymes have been described for ALT, but two isoforms have been identified for AST, a cationic or mitochondrial isoform and an anionic or cytosolic isoform (Schwartz, 1971).

Metabolites arising from carbohydrates, lipids and proteins are oxidized via the TCA cycle. During the course of the cycle, reduction equivalents are produced by specific dehydrogenases, which are re-oxidized in the respiratory chain generating ATP in the process of oxidative phosphorylation. Isocitrate dehydrogenase (IDH) is considered the main key enzyme of the TCA cycle; three isoenzymes, two mitochondrial and one cytosolic have been described. The mitochondrial isoenzymes use NAD and NADP as electron acceptors, unlike the cytosolic isoform that requires only NADP. Enzyme activity depends on ATP:ADP and NAD(P):NAD(P)H ratios (Hansford, 1980; Clarenburg, 1992). Malate dehydrogenase (MDH) performs a key function in the passage of reduction equivalents through the internal mitochondrial membrane as a result of the presence of cytosolic and mitochondrial isoenzymes. Oxaloacetate and NADH are impermeable to this membrane, unlike malate which can enter mitochondria (Clarenburg, 1992). Kitto (1969) observed that the cytosolic isoenzyme is inhibited by high malate concentrations and the mitochondrial isoenzyme is inhibited by high oxaloacetate concentrations.

Few studies demonstrate at a biochemical level the metabolic profile of both cumulus cells and the oocyte during maturation. More thorough knowledge of the activity of key enzymes that control the flow of metabolites in COCs will contribute to elucidate their nutritional requirements during IVM. Cetica *et al.* (2002) determined the activity of key enzymes that regulate glycolysis, the pentose phosphate pathway and lipolysis. The aim of the present study was to investigate the differential participation of enzymatic activity in cumulus

cells and in the oocyte during IVM, by studying the activity of enzymes involved in the control of amino acid metabolism (ALT and AST) and the TCA cycle (IDH and MDH).

Materials and Methods

Materials

All chemicals were purchased from Sigma Chemical Company (St Louis, MO), except for medium 199 (Earle's salts, L-glutamine, 2.2 mg sodium bicarbonate l⁻¹), which was obtained from GIBCO BRL (Grand Island, NY).

Recovery and classification of COCs

Bovine ovaries were obtained from an abattoir within 30 min after the animals were killed and were stored at 30°C during the 2 h journey to the laboratory. Ovaries were washed in physiological saline containing 100 000 iu penicillin l⁻¹ and 100 mg streptomycin l⁻¹. COCs were recovered by aspiration of antral follicles (2–5 mm in diameter) and classified according to cumulus morphology under a stereomicroscope. Only oocytes completely surrounded by compact and thick cumulus were used.

In vitro maturation of oocytes

COCs were cultured in medium 199 supplemented with 10% (v/v) steer serum and 50 mg gentamicine sulphate l⁻¹ under paraffin oil at 39°C for 22–24 h in an atmosphere of 5% CO₂ with humidified air.

Meiotic maturation was evaluated using 10% of samples. Oocytes were placed in a hypotonic medium of 10 g sodium citrate l⁻¹ at 37°C for 15 min, fixed on a slide with acetic acid-alcohol (Tarkowski, 1966), stained with 5% (v/v) Giemsa for 15 min and observed under a light microscope at × 100 and × 400. Oocytes were considered mature when metaphase II chromosome configuration was present.

Denuded oocytes

Immature or matured oocytes were completely denuded by vortex agitation in sterile physiological saline for 1 min and then collected with a pipette to separate them from cumulus cells. Denuded oocytes were washed twice in the same medium to eliminate residual cumulus cells.

Preparation of enzymatic extracts

Immature or *in vitro*-matured COCs and denuded oocytes were suspended in distilled water and frozen-thawed twice to produce cold osmotic shock, centrifuged

at 10 000 g for 20 min at 4°C, and enzyme activities were determined in supernatants. Residual cumulus cells suspended in physiological saline were processed in the same way. Extracts were prepared so that the final amount of each enzyme ensured linear behaviour during activity measurement, thus enabling absorbance per min variation to be calculated.

Determination of alanine and aspartate aminotransferase activities

Transaminase activities were determined in extracts of immature or *in vitro*-matured COCs ($n=70$) and from denuded oocytes ($n=250$). ALT activity was measured in a spectrophotometer at 340 nm for 3 min at 37°C in 100 mmol Tris-HCl buffer l⁻¹, pH 7.3; 0.18 mmol NADH l⁻¹; 500 mmol L-alanine l⁻¹; and 15 mmol α-ketoglutarate l⁻¹ in the presence of (1) ≥ 1200 U lactate dehydrogenase l⁻¹ (Bergmeyer *et al.*, 1986a). AST activity was measured in a spectrophotometer at 340 nm for 3 min at 37°C in 80 mmol Tris-HCl buffer l⁻¹, pH 7.8; 0.18 mmol NADH l⁻¹; 240 mmol L-aspartate l⁻¹; and 12 mmol α-ketoglutarate l⁻¹ in the presence of (1) ≥ 600 U lactate dehydrogenase l⁻¹ and (2) ≥ 420 U malate dehydrogenase l⁻¹ (Bergmeyer *et al.*, 1986b). An enzymatic unit of ALT or AST is defined as the quantity of enzyme that catalyses the oxidation of 1 μmol NADH min⁻¹.

Determination of isocitrate dehydrogenase activity

IDH activity was determined in extracts of immature or *in vitro*-matured COCs ($n=50$) and from denuded oocytes ($n=100$). NADP-IDH activity was measured in a spectrophotometer at 340 nm for 10 min at 37°C in 70 mmol Tris-HCl buffer l⁻¹, pH 7.5; 1 mmol MnCl₂ l⁻¹; 8 mmol MgCl₂ l⁻¹; 1.5 mmol isocitrate l⁻¹; 10 mmol citrate l⁻¹; and 0.5 mmol NADP l⁻¹. NAD-IDH activity was measured in the same conditions but NADP was replaced with 2 mmol NAD l⁻¹, pH 7.1, with the addition of 2.0 mmol ADP l⁻¹ (Alp *et al.*, 1976).

An enzymatic unit of IDH is defined as the quantity of enzyme that catalyses the reduction of 1 μmol NAD(P) min⁻¹.

Determination of malate dehydrogenase activity

MDH activity was determined in extracts of immature or *in vitro*-matured COCs ($n=20$) and from denuded oocytes ($n=50$). Activity was measured in a spectrophotometer at 340 nm for 1.5 min at 30°C in 90 mmol glycine buffer l⁻¹, pH 10.0; 1.0 mol L-malate l⁻¹; and 12.3 mmol NAD l⁻¹ (Kitto, 1969). An enzymatic unit of MDH is defined as the quantity of enzyme that catalyses the reduction of 1 μmol NAD min⁻¹.

Determination of total proteins

Total protein concentrations were determined in enzymatic extract supernatants using the method described by Lowry *et al.* (1951).

Oxidative substrates and co-enzymes added to the maturation medium

COCs or denuded oocytes were matured under the described conditions, with addition of oxidative substrates and co-enzymes corresponding to the enzymes under study: (a) 10 mmol L-alanine l⁻¹; (b) 10 mmol L-aspartate l⁻¹; (c) 1.5 mmol isocitrate l⁻¹ + 10 mmol citrate l⁻¹; (d) 1.5 mmol isocitrate l⁻¹ + 10 mmol citrate l⁻¹ + 12.5 mmol NAD l⁻¹; (e) 1.5 mmol isocitrate l⁻¹ + 10 mmol citrate l⁻¹ + 12.5 mmol NADP l⁻¹; (f) 1 mmol oxaloacetate l⁻¹; (g) 1 mmol malate l⁻¹; (h) 1 mmol malate l⁻¹ + 12.5 mmol NAD l⁻¹; (i) 12.5 mmol NAD l⁻¹; and (j) 12.5 mmol NADP l⁻¹.

The percentage of meiotic maturation was evaluated in each case.

Experimental design and statistical analysis

Enzymatic activity was expressed as enzymatic units and specific activity. Enzymatic units were expressed in units per COC, denuded oocyte or cumulus. Determination of total proteins in enzymatic extracts enabled the comparison of enzymatic activity between denuded oocytes and cumulus cells expressed in units per μg protein (specific activity). Data were expressed as means ± SEM. Intra-assay and interassay variability were estimated according to Rodbard (1974) and expressed as co-efficient of variation.

The enzymatic activity of each enzyme in COCs matured *in vitro* was determined by comparing immature and matured COCs using Student's *t* test. Activities in denuded oocytes and cumulus cells arising from

Table 1. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), NADP-dependent isocitrate dehydrogenase (NADP-IDH) and malate dehydrogenase (MDH) activities in immature and *in vitro*-matured cumulus-oocyte complexes (COCs)

Enzyme	COCs			
	Units per COC		Units μg^{-1} protein	
	Immature	Matured	Immature	Matured
ALT	$(1.22 \pm 0.13) 10^{-4,a}$	$(1.02 \pm 0.08) 10^{-4,a}$	$(1.47 \pm 0.14) 10^{-4,b}$	$(0.38 \pm 0.02) 10^{-4,c}$
AST	$(1.21 \pm 0.13) 10^{-4,a}$	$(1.17 \pm 0.10) 10^{-4,a}$	$(1.46 \pm 0.14) 10^{-4,b}$	$(0.43 \pm 0.03) 10^{-4,c}$
NADP-IDH	$(8.21 \pm 0.48) 10^{-5,a}$	$(8.69 \pm 0.52) 10^{-5,a}$	$(9.65 \pm 0.56) 10^{-5,b}$	$(3.48 \pm 0.21) 10^{-5,c}$
MDH	$(2.03 \pm 0.19) 10^{-4,a}$	$(2.08 \pm 0.13) 10^{-4,a}$	$(2.54 \pm 0.23) 10^{-4,b}$	$(0.83 \pm 0.05) 10^{-4,c}$

Activities are measured in units per COC as specific activity (units μg^{-1} protein). Values are means $\pm$ SEM of 11–13 replicates.

^aValues with the same superscript are not significantly different.

^{bc}Values with different superscripts are significantly different ($P < 0.05$).

immature and matured COCs were compared in a factorial design to evaluate the enzymatic activity contribution by either type of COC cell.

Statistical analyses of meiotic maturation rates were made by chi-squared analysis.

A P value < 0.05 was considered significant.

Results

In 10% of samples used to evaluate meiotic maturation of COCs, the percentage of IVM ranged from 79.0% to 84.4%. Activities expressed in enzymatic units remained constant during COC IVM in all enzymes studied, in contrast to the decrease observed in specific activity ($P < 0.05$; Table 1).

ALT and AST units remained constant in the oocyte and cumulus cells during IVM; the cumulus cells reached on average 36-fold and ninefold greater ALT and AST units than the oocyte, respectively ($P < 0.05$). AST activity in the oocyte was approximately 4.4 times greater than that recorded for ALT ($P < 0.05$), unlike in cumulus cells, in which similar activity was maintained for both enzymes (Fig. 1). Specific activities of both transaminases increased in the oocyte and diminished in cumulus cells during IVM ($P < 0.05$); however, ALT activity was invariably higher in cumulus cells and AST activity was higher in the matured oocyte compared with matured cumulus cells ($P < 0.05$; Fig. 1).

On adding substrates corresponding to ALT or AST to the maturation medium, the percentage of COCs reaching meiotic maturation in the presence of alanine or aspartate did not differ from that of controls, but increased with both substrates for the denuded oocytes ($P < 0.05$; Fig. 2).

No NAD-IDH activity was recorded in immature and *in vitro*-matured COCs under the assay conditions used. NADP-IDH units remained constant in the oocyte and cumulus cells during IVM; the cumulus cells reached on average 14-fold more enzymatic units than the oocyte ($P < 0.05$). Specific activity increased in the

oocyte ($P < 0.05$) and diminished in cumulus cells during IVM ($P < 0.05$), reaching similar activities in the matured stage (Fig. 3).

On adding substrates, co-enzymes or both corresponding to NADP-IDH and NAD-IDH to the maturation medium, the percentage of COCs reaching meiotic maturation was not different for the different treatments. However, the percentage of denuded oocytes reaching meiotic maturation increased versus the control in the presence of isocitrate + citrate + NADP ($P < 0.05$), but remained unchanged when isocitrate + citrate, isocitrate + citrate + NAD, NAD or NADP was added (Fig. 4).

MDH units remained constant in the oocyte and in cumulus cells during IVM; the cumulus cells reached on average 22-fold more enzymatic units than the oocyte ($P < 0.05$). Specific activity increased in the oocyte and diminished in cumulus cells during *in vitro* maturation ($P < 0.05$), reaching the same activities in the matured stage (Fig. 5).

On adding substrates, co-enzymes or both corresponding to enzyme MDH to the maturation medium, the percentage of COCs reaching meiotic maturation was not different for the different treatments. However, the percentage of denuded oocytes reaching meiotic maturation increased with regard to the control in the presence of oxaloacetate or malate + NAD ($P < 0.05$), but remained unchanged in the presence of malate or NAD (Fig. 6).

Repeatability of data for enzymatic units and specific activity were similar in COCs, denuded oocytes and cumulus cells for each enzyme. For ALT, intra-assay co-efficient of variation = 20–41% and interassay co-efficient of variation = 0–20%; for AST, intra-assay co-efficient of variation = 15–40% and interassay co-efficient of variation = 0–22%; for NADP-IDH, intra-assay co-efficient of variation = 13–26% and interassay co-efficient of variation = 0–19%; and for MDH, intra-assay co-efficient of variation = 11–42% and interassay co-efficient of variation = 0–19%.

Fig. 1. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in denuded oocytes ($\square$) and cumulus cells ($\blacksquare$) from immature and *in vitro*-matured cumulus-oocyte complexes. Activities are measured (a,c) in units per denuded oocyte or cumulus and (b,d) as specific activity (units μg^{-1} protein). Values are means $\pm$ SEM of 10–12 replicates. ^{a–d} Bars with different superscripts are significantly different ($P < 0.05$); *significantly different from corresponding ALT activity ($P < 0.05$). There are significant differences between denuded oocytes and corresponding cumulus cells for both enzymes ($P < 0.05$).

Discussion

It is well documented that cumulus cells supply oxidative substrates to the oocyte during maturation (Buccione *et al.*, 1990; Brackett and Zuelke, 1993; Cetica *et al.*, 1999b). The present study determined the activity of ALT, AST, IDH and MDH in extracts from oocytes and cumulus cells. The specific oxidative substrate was added for each studied enzyme, replacing the function of cumulus cells during denuded oocyte maturation, to confirm that the enzyme is participating in the maturation process.

ALT, AST, NADP-IDH and MDH activities expressed as units per COC remained unchanged during maturation because both denuded oocyte and cumulus units did not vary during this process. It has been reported that the amount of protein present in bovine cumulus cells increases gradually during the 24 h of IVM, in part due to the increase in the number of cumulus cells (Wu

et al., 1996; Cetica *et al.*, 2001). However, a slight but significant decrease in total protein concentration in bovine denuded oocyte extracts was recorded (Cetica *et al.*, 2001), as well as in protein synthesis rate (Wu *et al.*, 1996; Gandolfi *et al.*, 1998). These findings explain the decrease in cumulus cells and the increase in denuded oocyte specific activity recorded for the studied enzymes in the course of maturation. It may therefore be inferred that the enzymatic activity of cumulus cells would be gradually lost when cumulus expansion occurs during maturation. The studied enzymes would not be involved in the detected loss of oocyte proteins, indicating that the gamete maintains its enzymatic machinery even when dissociated from cumulus cells during maturation, as previously observed for other enzymes (Cetica *et al.* 2001, 2002).

Glycolysis is considered to be the predominant metabolic pathway for glucose utilization in cumulus

Fig. 2. Meiotic maturation rate in (a) cumulus–oocyte complexes (COCs; ■) and (b) denuded oocytes (□) cultured with different supplements; $n = 167$ – 186 for each treatment. ^{ab}Bars with different superscripts are significantly different ($P < 0.05$).

cells (Zuelke and Brackett, 1992; Cetica *et al.*, 1999b, 2002; Sutton *et al.*, 2003a), with raised activity of phosphofructokinase, the main enzyme regulating the rate of the pathway (Cetica *et al.*, 2002). In contrast, denuded oocytes display very low levels of glucose uptake, glycolytic activity, glucose oxidation (Zuelke and Brackett, 1992; Rieger and Loskutoff, 1994; Saito *et al.*, 1994) and low phosphofructokinase activity (Cetica *et al.*, 2002). Hence, it was suggested that the fate of glucose within cumulus cells is mainly the production of pyruvate or lactate, both preferential oocyte substrates oxidized in the TCA cycle (Cetica *et al.*, 1999b; Eppig *et al.*, 2000; Khurana and Niemann, 2000).

Similarly high ALT and AST activities were recorded in cumulus cells, comparable to that reported for phosphofructokinase. Such raised transaminase activity could be linked to the synthesis of amino acids in these cells with a high rate of protein synthesis or oxidative substrate production to be supplied to the oocyte. It has been suggested that in rodents, amino acids may be utilized by oocytes as an energy source with cumulus cells, and play a major role in amino

acid flux into the oocyte (Colonna and Mangia, 1983). Transaminase activity in the oocyte was increased with regard to phosphofructokinase, and was on average 2.8 and 12.4 times greater for ALT and AST, respectively. In turn, AST activity in the oocyte was significantly greater than that of ALT, reaching on average 4.4-fold greater activity. These results may indicate a leading function of aspartate in oocyte energy metabolism due to the conversion to oxaloacetate to be used in the TCA cycle or for participation in the malate–aspartate shuttle system in the oxidation of reduction equivalents (Fig. 7), for example those generated partly by lactate dehydrogenase-1 isoenzyme in lactate oxidation (Cetica *et al.*, 1999b).

An increased percentage of meiotic maturation of oocytes denuded in the presence of alanine or aspartate in the maturation medium confirms their use as oxidative substrates for the oocytes or the participation of these compounds in the synthesis of protein factors involved in the control of maturation. Alanine and aspartate are among the amino acids classified as non-essential by Eagle (1959). In fact, non-essential amino acids should be components of bovine maturation and embryo culture media in order to achieve a high percentage of successful IVF cycles and embryos reaching the blastocyst stage (Gardner, 1998; Rose-Hellekant *et al.*, 1998; Lim *et al.*, 1999).

When IDH activity was studied in bovine COCs, NADP-dependent isoenzyme activity was observed, but not activity of the NAD-dependent isoform. This finding need not rule out NAD-IDH activity, but rather implies that due to its scarce quantity it proves undetectable under assay conditions, in which sample concentration is a limiting factor. However, meiotic maturation data from denuded oocytes appear to confirm these results, because an increase was observed only in maturation percentage in the presence of isocitrate + citrate + NADP; when isocitrate + citrate + NAD was added there was no change. In the absence of NADP, isocitrate + citrate failed to increase the maturation percentage, demonstrating that the co-enzyme must be present so that substrates can be used by the oocyte.

It should be highlighted that in both IDH enzymatic activity determination and IVM assays, high citrate concentrations were added because it acts as an enzyme activator or it displaces the reversible reaction of the (1) aconitase toward isocitrate formation to ensure its uptake by (2) IDH, rather than due to the presence of aconitase.

NADP-IDH units in cumulus cells are 1.5-fold lower than those reported for phosphofructokinase (Cetica *et al.*, 2002), in agreement with the observation that glycolysis is the main pathway for glucose utilization in cumulus cells, the level of oxygen uptake proving

Fig. 3. NADP-dependent isocitrate dehydrogenase (NADP-IDH) activity in denuded oocytes (□) and cumulus cells (■) from immature and *in vitro*-matured cumulus-oocyte complexes (COCs). Activity is measured (a) in units per denuded oocyte or cumulus and (b) as specific activity (units μg^{-1} protein). Values are means $\pm$ SEM of 10–13 replicates. ^{a–d}Bars with different superscripts are significantly different ($P < 0.05$); *significantly different from NADP-IDH activity of corresponding denuded oocyte ($P < 0.05$).

Fig. 4. Meiotic maturation rate in (a) cumulus-oocyte complexes (COCs; ■) and (b) denuded oocytes (□) cultured with different supplements; $n = 168$ –202 for each treatment. ^{ab}Bars with different superscripts are significantly different ($P < 0.05$).

very low (Sutton *et al.*, 2003b). In contrast, enzyme activity in the oocyte is on average 4.8 times higher than in phosphofructokinase, denoting a greater relative importance of the TCA cycle with regard to glycolysis in the female gamete (Fig. 7).

In several mammalian tissues and cells, considerably greater activity of NADP-IDH with regard to NAD-IDH (ranging from ten- to 400-fold) has been reported,

indicating that both enzymes are particularly involved in isocitrate dehydrogenation for energy production when the TCA cycle flow approaches peak value. These tissues and cells are characterized by possessing high activity of the catabolically oriented cycle in order to sustain a constant energy supply, as heart, red skeletal muscle (Alp *et al.*, 1976) and spermatozoa (Brooks, 1978). The oocyte during maturation and the embryo during development may also respond to these metabolic features. In addition to its role in cumulus cells and oocytes, NADP-IDH could participate in the generation of NADPH for reductive synthesis, supplementing that produced by glucose-6-phosphate dehydrogenase, the key enzyme in the control of the pentose pathway (Cetica *et al.*, 2002).

Although MDH is not considered an enzyme that controls the flow of the TCA cycle, it nonetheless plays a key role in the operation of the TCA cycle because it catalyses a reaction distant from the equilibrium that regenerates oxaloacetate to initiate the cycle (Clarenburg, 1992). Enzyme activity in the oocyte was high and could be linked not only with the cycle for oxidative energy supply but also with the malate-aspartate shuttle system for transport of reduction equivalents through the internal mitochondrial membrane. Combined MDH and AST activities, the levels of which are markedly similar, support the above proposal of the presence of this shuttle system in the bovine oocyte (Fig. 7). In tissues such as heart, liver and kidney, with a high capacity to metabolize lactate, the shuttle system described is that of malate-aspartate (Dawson, 1979; Lehninger *et al.*, 1997). Co-enzyme NADH, produced when lactate is oxidized to pyruvate by the action of lactate dehydrogenase, is re-oxidized by the participation of enzymes MDH and AST. The oocyte would also respond to these features with raised activity of lactate dehydrogenase-1 isoenzyme, MDH and AST.

Fig. 5. Malate dehydrogenase (MDH) activity in denuded oocytes (□) and cumulus cells (■) from immature and *in vitro*-matured cumulus-oocyte complexes (COCs). Activity is measured (a) in units per denuded oocyte or cumulus and (b) as specific activity (units μg^{-1} protein). Values are means $\pm$ SEM of 10–11 replicates. ^{a–d}Bars with different superscripts are significantly different ($P < 0.05$); *significantly different from MDH activity of corresponding denuded oocyte ($P < 0.05$).

Fig. 6. Meiotic maturation rate in (a) cumulus-oocyte complexes (COCs; ■) and (b) denuded oocytes (□) cultured with different supplements; $n = 160$ –191 for each treatment. ^{a,b}Bars with different superscripts are significantly different ($P < 0.05$).

However, MDH activity recorded in cumulus cells could participate in malate supply to the oocyte as an oxidative substrate starting from products derived from glucose oxidation by the glycolytic pathway and also re-oxidize co-enzyme NAD to ensure the continuity of glycolytic flow (Fig. 7), probably supplementing lactate

dehydrogenase activity previously recorded in bovine cumulus cells (Cetica *et al.*, 1999b).

The percentage of denuded oocytes reaching meiotic maturation increased in the presence of oxaloacetate, as also observed in mice (Biggers *et al.*, 1967), indicating that this compound can be used by the oocyte as an oxidative substrate. The presence of malate in the maturation medium failed to increase the number of mature oocytes, requiring addition of NAD to improve the percentage of oocytes reaching maturation. In all likelihood, this takes place due to oxaloacetate formation starting from malate when the supply of NAD is sufficient.

Increased percentage of denuded bovine oocytes reaching meiotic maturation in media supplemented with citrate + isocitrate and with malate by addition of oxidized co-enzymes NADP and NAD, respectively, was also previously observed for lactate with the addition of NAD (Cetica *et al.*, 1999b). This beneficial effect was not recorded in the meiotic maturation of COCs, indicating that cumulus cells play a key regulatory role in the redox state of the oocyte. These cells could supply the oocyte with co-enzymes or their precursors or contribute to maintain redox equilibrium for normal gamete function. In rodents, it has been found that co-enzyme concentrations present in the oocyte are low (Wales, 1975) and there is ribonucleoside uptake by the oocyte mediated by cumulus and granulosa cells (Heller and Schultz, 1980; Heller *et al.*, 1981; Brower and Schultz, 1982). In ovine embryos, NAD:NADH and pyruvate:lactate ratios may determine the cytosol redox state, thus altering the mitochondrial redox state and the normal operation of the TCA cycle (Lieber, 1991; Thompson *et al.*, 1993).

To date, there is minimal information regarding the metabolic profile of COCs during maturation. The present data extend knowledge of the utilization of amino

Fig. 7. Proposed model of the main pathways for amino acid and carboxylic acid oxidation in cumulus-oocyte complexes. PFK: phosphofructokinase; LDH: lactate dehydrogenase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; IDH: isocitrate dehydrogenase; MDH: malate dehydrogenase; OA: oxaloacetate; α -KG: α -ketoglutarate; TCA: tricarboxylic acid.

acids and carboxylic acids within the whole complex. It is possible that further investigations in the control of the enzymatic activity related to COC metabolism may aid in the optimization of the culture conditions during IVM and the subsequent *in vitro* culture.

In summary, raised transaminase activities detected in cumulus cells indicate an important dynamism in the metabolism of amino acids. Alanine and aspartate are involved in bovine oocyte IVM. The participation of NADP-IDH and MDH and the use of TCA cycle intermediaries (oxaloacetate, isocitrate, malate) would confirm the aerobic activity of the oocyte. High MDH and AST activities support the proposal of the presence of the malate-aspartate shuttle system in the bovine oocyte. To date, the oocyte is believed to rely on cumulus cells to metabolize initially glucose to pyruvate as the preferred oxidative substrate of the oocyte. However, the present data indicate that the oocyte proves more versatile in the use of diverse oxidative substrates depending on its redox

state. The appropriate balance among lactate, pyruvate or alanine; malate, oxaloacetate or aspartate and NAD(P) or NAD(P)H would therefore be decisive conditions for the use of these oxidative substrates by the oocyte during IVM.

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