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Glycolytic pathway activity: effect on IVM and oxidative metabolism of bovine oocytes

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Abstract. The aim of the present study was to determine the effect of altering glycolytic pathway activity during bovine IVM on the meiotic maturation rate, oxidative activity, mitochondrial activity and the mitochondrial distribution within oocytes. Glycolytic activity was manipulated using two inhibitors (ATP, NaF) and a stimulator (AMP) of key enzymes of the pathway. Inhibition of glucose uptake, lactate production and meiotic maturation rates was observed when media were supplemented with ATP or NaF. The addition of AMP to the maturation medium had no effect on glucose uptake, lactate production or meiotic maturation. In the absence of gonadotrophin supplementation, AMP stimulated both glucose uptake and lactate production. However, AMP also decreased cytoplasmic maturation, as determined by early cleavage. During IVM, oocyte oxidative and mitochondrial activity was observed to increase at 15 and 22 h maturation. Inhibiting glycolysis with ATP or NaF led to a reduced oxidative and mitochondrial pattern compared with the respective control groups. Stimulation of the pathway with AMP increased oxidative and mitochondrial activity. A progressive mitochondrial migration to the central area was observed during maturation; oocytes treated with ATP, NaF or AMP showed limited migration. The present study reveals the effects of altering glycolytic pathway activity in cumulus–oocyte complexes, revealing the link between glycolysis of the cumulus–oocyte complex and the oxidative and mitochondrial activity of the oocyte.

Additional keywords: cumulus–oocyte complex, glycolysis, maturation, mitotracker green, redox sensor red.

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Introduction

Once the germinal vesicle (GV) oocyte is released from the ovarian antral follicle, it will mature spontaneously *in vitro* under appropriate conditions. The maturation process that is manifested initially by GV breakdown (GVBD) also involves cumulus expansion and cytoplasmic maturation. In cattle oocytes, an adequate concentration of glucose in the maturation medium is necessary for this maturation process, as well as for subsequent embryo development (Rose-Hellekant *et al.* 1998; Lim *et al.* 1999; Khurana and Niemann 2000). Indeed, manipulation of glucose concentrations during maturation can affect the kinetics of bovine oocyte meiosis (Sutton-McDowall *et al.* 2005).

As in other cell types, cumulus–oocyte complexes (COCs) metabolise glucose via glycolysis, the pentose phosphate pathway and the hexosamine biosynthesis pathway (Downs and Utecht 1999; Sutton *et al.* 2003; Gutnisky *et al.* 2007), as well as the polyol pathway (for a review, see Sutton-McDowall *et al.* 2010). However, cumulus cells have a great capacity for glycolysis, and this represents the predominant pathway for glucose metabolism in COCs. It has been suggested that cumulus cell glycolytic activity is high in order to generate ATP and produce pyruvate, lactate, malate and/or oxalacetate, which are readily used as oxidative substrates by the oocyte (Brackett and Zuelke 1993; Cetica *et al.* 1999, 2002, 2003). In contrast, oocytes appear to lack the capacity to undertake significant glucose metabolism (Zuelke and Brackett 1992; Sutton *et al.* 2003; Dumollard *et al.* 2007). In agreement with these observations, cumulus cells express a high-affinity glucose transporter, namely Glut4 (Roberts *et al.* 2004), whereas the oocyte does not.

For mice, a high glucose concentration in the maturation medium inhibits maturation by increasing intracellular ATP levels (Downs and Mastropolo 1994). It has also been proposed that the metabolic activity of the oocyte increases in the latter half of the meiotic maturation process, coinciding with a rise in

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lactate production, which has been proposed as an indicator of oocyte quality (Preis *et al.* 2005).

In somatic cells, the major regulatory point of the glycolytic pathway is the enzyme phosphofructokinase 1 (EC 2.7.1.11), with AMP and ATP having important positive and negative allosteric regulating roles, respectively (Schirmer and Evans 1990; Clarenburg 1992). Sodium fluoride (NaF) is also a well-known inhibitor of the pathway, inactivating the glycolytic enzyme enolase (EC 4.2.1.11; Mayes and Bender 2004). The intermediary metabolism of glucose also produces the reducing equivalent, NADH. This metabolite is mainly synthesised by cumulus cells in the glycolytic pathway by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and by the oocyte via the reaction catalysed by α-ketoglutarate dehydrogenase and malate dehydrogenase. In addition to being used as a cofactor for anabolic pathways, NADH is a key redox molecule (Dumollard *et al.* 2007), important in both cytosolic and mitochondrial redox regulation. The redox state describes the sum of interactions between oxidised and reduced forms of a variety of molecules, including NAD(P) : NAD(P)H, FAD : $FADH_2$, reduced glutathione : oxidised glutathione (for reviews, see Harvey *et al.* 2002; Herrick *et al.* 2006).

Oocyte maturation also includes cytoplasmic changes, such as the synthesis and accumulation of mRNA, proteins, transcription factors and the redistribution of organelles, especially mitochondria. Mitochondrial activity is essential for oocyte competence, and the ATP content of oocytes generated from the reducing equivalents derived from carboxylic acid metabolism through the tricarboxylic acid (TCA) cycle is highly correlated with oocyte competence (Stojkovic *et al.* 2001; Dumollard *et al.* 2007).

Mitochondrial distribution differs within *in vitro* maturing oocytes from different species. In mouse oocytes, mitochondria aggregate around the nucleus from the GV stage through to GVBD. With the movement of the nucleus throughout maturation, mitochondria concentrate around the nucleus (Nishi *et al.* 2003). In pig oocytes, mitochondria accumulate in the perinuclear area during meiotic progression from GVBD to anaphase I. In matured oocytes, mitochondrial foci are formed and move to the inner cytoplasm (Sun *et al.* 2001). Conversely, in bovine oocytes after 12–18 h IVM, the mitochondria move from a cortical location to an evenly distributed pattern (Hyttel *et al.* 1986). Furthermore, there appears to be an association between the ATP content of the oocyte and redistribution of the mitochondria; specifically, for oocytes with low ATP content, mitochondrial migration does not occur (Stojkovic *et al.* 2001).

Although it was proposed that glycolytic end products produced by cumulus cells are metabolised by the bovine oocyte during IVM, there are no studies that have evaluated the effect of glycolytic activity in COCs on oocyte oxidative metabolism and maturational capacity. Thus, the main aim of the present study was to investigate the glycolytic pathway activity during cattle oocyte IVM, analysing the effects of two inhibitors (ATP, NaF) and a stimulator (AMP) of the key enzymes of glycolysis on the maturation rate, oxidative and mitochondrial activity and mitochondrial distribution in oocytes.

Materials and methods

Materials

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

Recovery of COCs

Bovine ovaries were collected at an abattoir within 30 min of death and kept warm (30°C) during the 2-h journey to the laboratory. Ovaries were washed with physiological saline containing 100 000 IU L^{-1} penicillin and 100 mg L^{-1} streptomycin. The COCs were recovered by aspiration of antral follicles (2–5 mm in diameter) and only oocytes completely surrounded by a compact and multilayered cumulus oophorus were used.

IVM of COCs

The COCs were cultured in Medium 199 (Earle's salts, L-glutamine, 100 mg L^{-1}; sodium bicarbonate, 2.2 mg L^{-1}; GIBCO, Grand Island, NY, USA) supplemented with 5% (v/v) fetal bovine serum (FBS; GIBCO), 0.2 mg L^{-1} porcine FSH (Folltropin-V; Bioniche, Belleville, Ontario, Canada), 2 mg L^{-1} porcine LH (Lutropin-V; Bioniche) and 50 mg L^{-1} gentamicin sulfate under mineral oil at 39°C for 22 h in an atmosphere of humidified 5% CO_2 in air.

To study the effects of different enzyme modulators of glycolysis on COC glycolytic pathway activity and oocyte meiotic maturation, COCs were cultured individually in 20-μL drops of maturation medium supplemented with increasing concentrations of ATP (1, 10, 20 and 40 mM), NaF (2, 3, 4 and 5 mM) or AMP (1, 10, 20 and 40 mM) under the conditions described above.

To investigate the effects of manipulating COC glycolytic pathway activity on subsequent oxidative activity, mitochondrial activity, mitochondrial distribution and oocyte nuclear morphology in oocytes, COCs were cultured in groups of 50 in 500-μL drops of maturation medium supplemented with 10 mM ATP or 3 mM NaF (determined as inhibitory concentrations in the previous experiments) for 9, 15 and 22 h, and 10 mM AMP (determined as a stimulatory concentration in the previous experiment) without gonadotrophin supplementation. These maturations time-points were chosen for being consecutives to key events of the maturation process, namely GVBD, MI and extrusion of the first polar body, respectively (Fleming and Saacke 1972; Kruip *et al.* 1983; Gordon 1994).

Determination of the glycolytic activity of COCs

The COCs were removed from each 20-μL drop and the glucose content was determined from the spent maturation medium. Glucose concentrations were determined using a spectrophotometric assay based on the oxidation of glucose by glucose oxidase and subsequent production of hydrogen peroxide (Trinder 1969). Positive controls comprising 20-μL drops of maturation medium were included in each experiment.

Lactate production in the culture medium was determined in the same droplets used to determine glucose uptake. Lactate production was measured using a spectrophotometric assay

based on the oxidation of lactate and the subsequent production of hydrogen peroxide (Trinder 1969; Barham and Trinder 1972).

Evaluation of oocyte meiotic maturation

The COCs removed from each 20-μL drop were used to evaluate meiotic maturation rates. Oocytes were denuded mechanically by repeated pipetting in phosphate-buffered saline (PBS) with 1 g L^{-1} hyaluronidase. Denuded oocytes were placed in a hypotonic medium of 2.9 mmol L^{-1} sodium citrate at 37°C for 15 min, fixed on a slide with 3 : 1 ethanol : acetic acid (Tarkowski 1966), stained with 5% (v/v) Giemsa (Merck, Darmstadt, Germany) for 15 min and observed under a light microscope at ×100 and ×400 magnification. Oocytes were considered mature when an MII chromosome configuration was evident.

Evaluation of oocyte cytoplasmic maturation

Immature COCs were divided into three groups for IVM in the medium described above (positive control), without supplementation of gonadotrophins (negative control) and only supplemented with 10 mM AMP. After 21 h maturation, IVF was performed using frozen–thawed semen from a Holstein bull of proven fertility. The semen was thawed at 37°C in modified synthetic oviducal fluid (mSOF; Takahashi and First 1992), centrifuged twice at 500*g* for 5 min and then resuspended in fertilisation medium to a final concentration of 2×10^9 motile spermatozoa L^{-1} after sperm selection by glass wool (Arzondo *et al.* 2012). Fertilisation was performed in IVF-mSOF, consisting of mSOF supplemented with 5 g bovine serum albumin (BSA) L^{-1} and 10 000 U heparin L^{-1}, under mineral oil at 39°C in 5% CO_2 in air and 100% humidity for 20 h. Zygotes were denuded by repeated pipetting and placed in 500 μL *in vitro* culture (IVC)-mSOF, consisting of mSOF supplemented with 30 mL amino acid MEM L^{-1} (GIBCO), 10 mL non-essential amino acid MEM L^{-1} (GIBCO), 2 mmol L-glutamine L^{-1}, 6 g L^{-1} BSA and 5% (v/v) FBS (GIBCO), under mineral oil at 39°C in 90% N_2 : 5% CO_2 : 5% O_2 and 100% humidity for 24 h. Cytoplasmic maturation was evaluated by the ratio of cleaved embryos. An additional cohort of 10 oocytes from each replicate was maintained through the fertilisation procedure without exposure to spermatozoa to test for parthenogenesis.

Evaluation of oxidative and mitochondrial activity, as well as mitochondrial distribution

From the total number of oocytes recovered, two-thirds were used to determine oxidative activity, mitochondrial activity and mitochondrial distribution after 0, 9, 15 and 22 h maturation. These time-points were chosen because they correspond to key events of the maturation process, namely GVBD (6–8 h), MI (12–14 h) and extrusion of the first polar body (18–21 h).

Cumulus cells were removed mechanically by repeated pipetting in PBS with 1 g L^{-1} hyaluronidase before the zona pellucida was dissolved with 5 g L^{-1} pronase for 1 min.

Fluorescent probes and confocal microscopy were used to analyse the parameters mentioned above. Dual staining with RedoxSensor red CC-1 (Molecular Probes, Eugene, OR, USA) and MitoTracker green FM (Molecular Probes) were used in this experiment. Oocytes were coincubated with final concentrations of 1 nM RedoxSensor red CC-1 and 0.5 nM MitoTracker green FM for 30 min at 37°C in the dark and then washed twice with PBS. Stained oocytes were then placed between a slide and coverslip for observation under a laser confocal microscope (Nikon C1 confocal scanning head, Nikon TE2000E; Nikon, Kanagawa, Japan). One optical section was examined for each oocyte. The images obtained were saved and then analysed using Adobe Photoshop CS2 (version 9; Adobe Systems Inc.)

Both red and green fluorescence emission intensities were determined in four different regions (squares) within three areas (i.e. Areas 1–3) of the oocyte, as shown in Fig. 1*a* (Wakefield *et al.* 2007).

Oxidative activity was calculated as the sum of the average red fluorescence intensity in the three areas of the same oocyte. Mitochondrial activity was calculated as the sum of the average green fluorescence intensity in the three areas of the same oocyte. Ratios of green fluorescence intensity between Areas 3 and 1 were then calculated to compare the distribution of active mitochondria.

Evaluation of oocyte nuclear morphology

The remaining one-third of oocytes were used to evaluate nuclear morphology after 0, 9, 15 and 22 h maturation. Denuded oocytes were fixed in a 40 mg L^{-1} paraformaldehyde solution for 1 h and then incubated in a permeabilising solution for 1.5 h. Finally, the fixed oocytes were stained with 10 mg L^{-1} Hoechst 33342 solution for 15 min.

Oocyte nuclear status was observed at a magnification of ×400 under a Jenamed II epifluorescence microscope (Carl Zeiss Jena, Buenos Aires, Argentina) with a 410-nm filter at each time-point.

Statistical analysis

The results of glucose uptake, lactate production, oxidative activity, mitochondrial activity and the ratio of green fluorescence intensity between Areas 3 and 1 to evaluate mitochondrial distribution are expressed as the mean ± s.e.m. In studies evaluating glucose uptake and lactate production, comparisons were made by ANOVA followed by Bonferroni's post test. Oxidative activity, mitochondrial activity and mitochondrial distribution were compared using a 2×4 or 3×4 factorial design. Meiotic and cytoplasmic maturation rates between treatments were compared using Chi-squared analysis for non-parametric data. In all tests, significance was set at $P < 0.05$.

Results

Glycolytic activity of COCs and oocyte maturation

To investigate the glycolytic pathway, COCs were incubated during maturation with increasing concentrations of pathway modulators. COCs matured in the presence of increasing concentrations of ATP exhibited dose-dependent inhibition of glucose uptake and lactate production ($P < 0.05$). A codependent inhibition on their progression to MII was also observed ($P < 0.05$; Fig. 2*a*).

The addition of increasing concentrations of NaF to the maturation medium produced a dose-dependent inhibition of

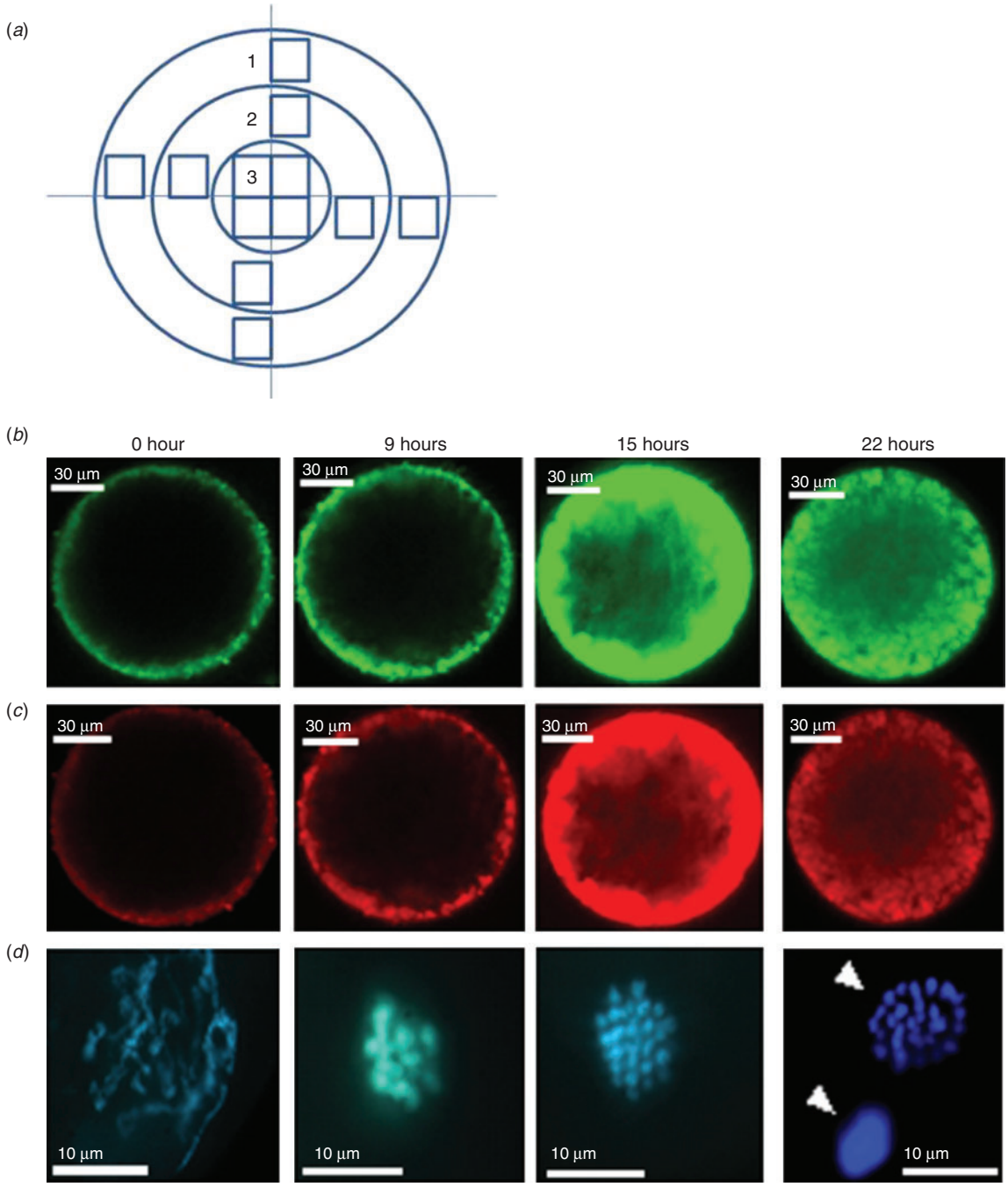

Fig. 1. (*a*) Schematic representation of the four different regions (squares) within three areas (Areas 1–3) of an oocyte used for determining mitochondrial fluorescence. (*b*, *c*) Oocytes stained with two fluorescent probes, namely MitoTracker green FM (Molecular Probes, Eugene, OR, USA) (*b*) RedoxSensor red CC-1 (Molecular Probes) (*c*), after different times of maturation. (*d*) Oocytes were stained with Hoechst DNA fluorochrome to determine nuclear maturation at different time points. Hour 0, germinal vesicle (GV) stage; Hour 9, GV breakdown; Hour 15, MI, Hour 22, extrusion of the first polar body. Arrows indicate the presence of an MII chromatin configuration and the first polar body.

both lactate production and nuclear maturation ($P < 0.05$). However, the inhibition of glucose uptake was not dose dependent ($P < 0.05$; Fig. 2*b*).

The addition of AMP to the maturation medium had no effect on glucose uptake and lactate production or on rates of nuclear maturation compared with the control group (Fig. 2*c*). To test whether the gonadotrophins (FSH, LH) were masking an effect of AMP, the experiment was repeated with maturation media devoid of gonadotrophins. In the absence of FSH and LH, AMP had stimulatory effects on both glucose uptake and lactate

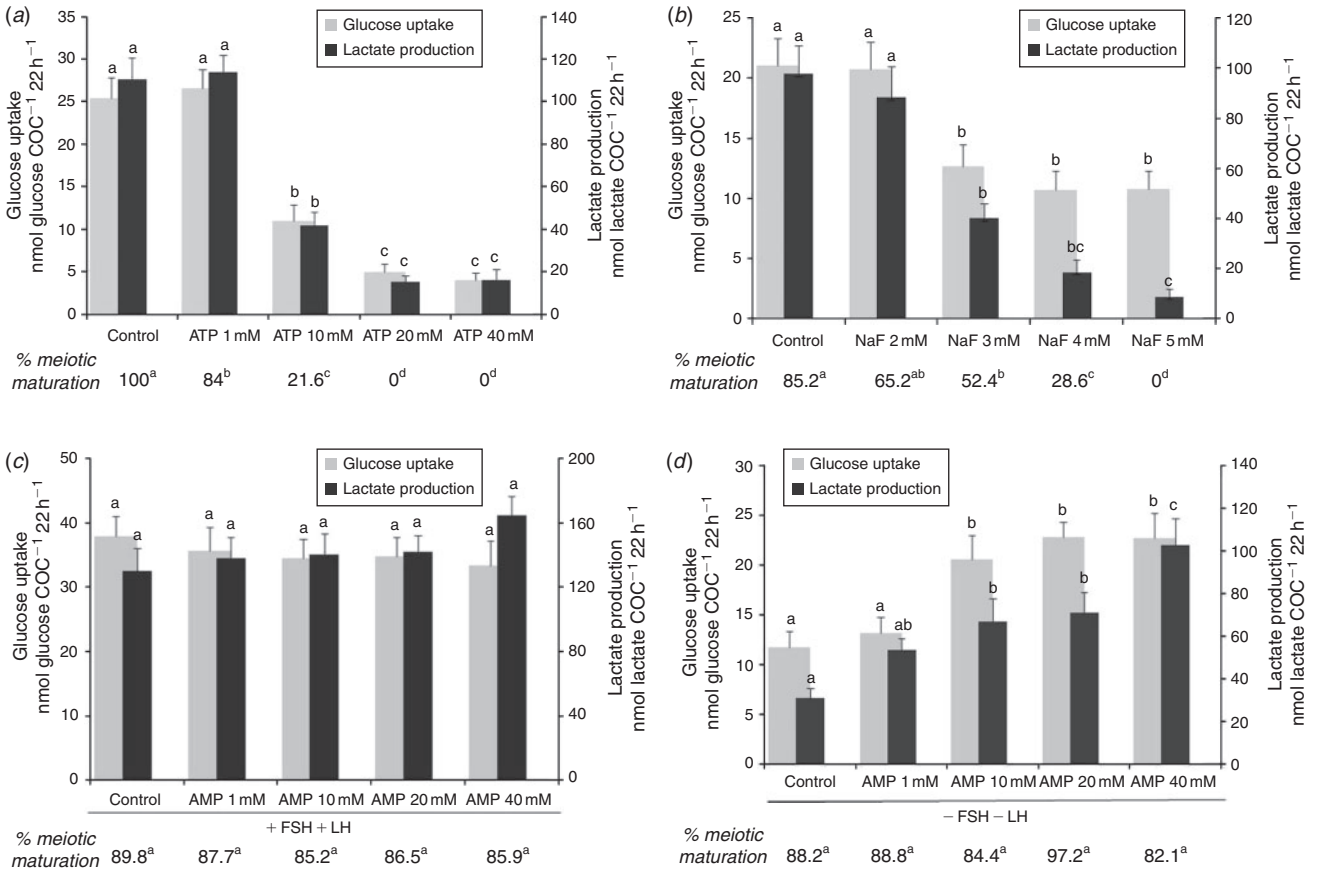

Fig. 2. Glucose uptake and lactate production in cumulus–oocyte complexes (COCs) matured for 22 h in the presence of different concentrations of (*a*) ATP, (*b*) NaF and (*c*, *d*) AMP with (*c*) or without (*d*) gonadotrophin supplementation. Data are the mean ± s.e.m. ($n = 30$–40 COCs for each treatment). Values for glucose uptake or lactate production with different superscript letters differ significantly ($P < 0.05$).

production ($P < 0.05$), with no effect on nuclear maturation (Fig. 2*d*).

Because the rate of nuclear maturation had not been altered with the addition of AMP, we evaluated the cytoplasmic maturation of oocytes cultured with 10 mM AMP without gonadotrophin supplementation. We chose this concentration because it was the lowest that stimulated the glycolytic pathway in COCs. A significant decrease in cleavage rates compared with both control groups (matured with and without gonadotrophins) was observed in the group of COCs matured in the presence of AMP ($P < 0.05$; Fig. 3).

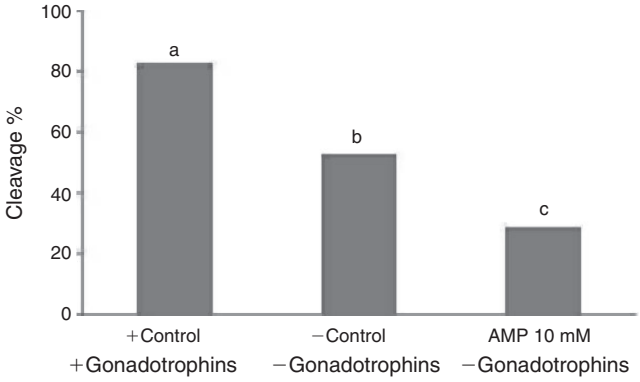

Fig. 3. Cleavage rate following IVF of oocytes matured for 22 h in the presence of 10 mM AMP without gonadotrophins. +Control, positive control; −Control, negative control. Data are the mean ± s.e.m. ($n = 101$–116 COCs for each treatment). Values with different superscript letters differ significantly ($P < 0.05$).

Oxidative activity of the oocyte

To confirm the participation of COC glycolysis in oxidative metabolism, oocytes were stained with RedoxSensor red CC-1 to quantify mitochondrial oxidative activity at different time-points (0, 9, 15 and 22 h). In these experiments, 10 mM ATP and 3 mM NaF were at the minimum inhibitory concentrations observed for glucose uptake and lactate production. For glycolytic pathway stimulation, we used 10 mM AMP to stimulate glucose uptake and lactate production.

Oocytes exhibited changes in their oxidative activity throughout maturation, with an increase in oxidative activity observed after 15 and 22 h maturation compared with Time 0 ($P < 0.05$). Although similar changes in oxidative activity were observed in oocytes matured in the presence of ATP or NaF, overall activity was significantly lower than the values obtained

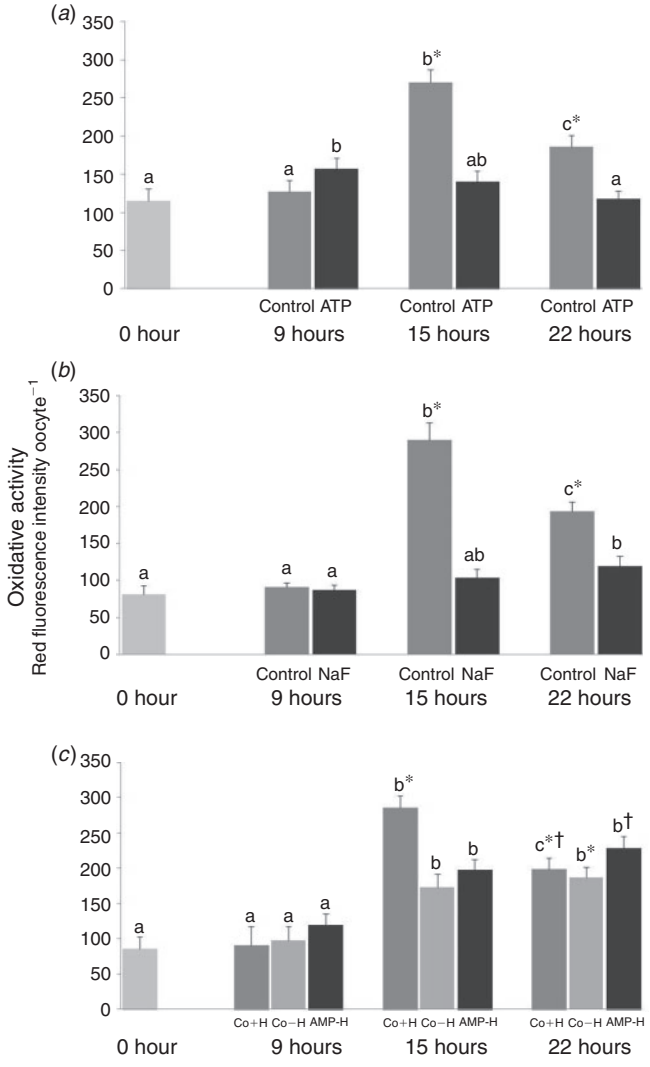

Fig. 4. Oxidative activity within oocytes matured for 0–22 h in the presence of (*a*) 10 mM ATP, (*b*) 3 mM NaF or (*c*) 10 mM AMP. Data are the mean ± s.e.m. ($n = 30$–40 COCs for each treatment). Within a graph and within each group (control or treated), values with different superscript letters differ significantly ($P < 0.05$). *†Columns with different symbols at the same time point differ significantly ($P < 0.05$).

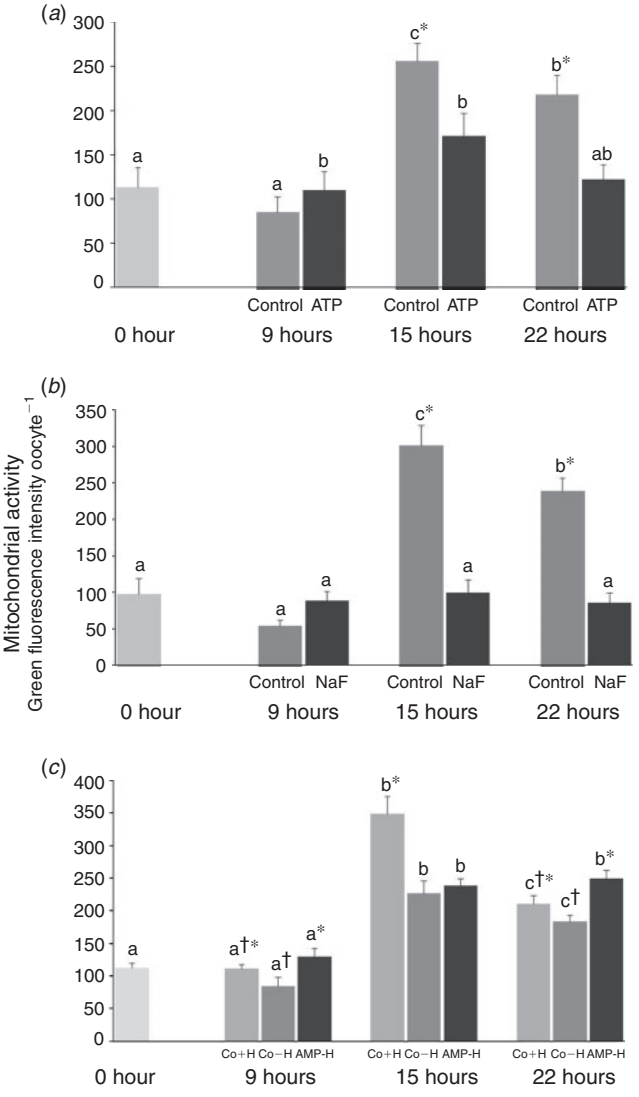

Fig. 5. Mitochondrial activity within oocytes matured for 0–22 h in the presence of (*a*) 10 mM ATP, (*b*) 3 mM NaF or (*c*) 10 mM AMP. Data are the mean ± s.e.m. ($n = 30$–40 COCs for each treatment). Within a graph and within each group (control or treated), values with different superscript letters differ significantly ($P < 0.05$). *†Columns with different symbols at the same time point differ significantly ($P < 0.05$).

for the control group ($P < 0.05$; Fig. 4*a*, *b*). In contrast, AMP (in the absence of gonadotrophins) stimulated oxidative activity after 15 and 22 h maturation ($P < 0.05$) compared with Time 0. Nevertheless, after 15 h maturation, the increase was significantly lower for oocytes matured with either AMP or the control without gonadotrophins compared with the FSH + LH control ($P < 0.05$). In addition, oocytes matured with AMP showed the highest oxidative activity after 22 h maturation ($P < 0.05$; Fig. 4*c*).

Mitochondrial activity of oocytes

To study the effects of manipulating glycolytic activity on mitochondrial activity within oocytes, the fluorescence intensity of MitoTracker green FM was analysed at the same time-points and using the same ATP, NaF and AMP concentrations described for the experiment above.

The intensity of green fluorescence varied throughout maturation. A significant increase in intensity was observed after 15 and 22 h maturation compared with Time 0 ($P < 0.05$). An increase was observed in the ATP group after 15 h maturation compared with Time 0 ($P < 0.05$) (Fig. 5*a*, *b*). Stimulation of glycolysis with AMP revealed an increase in mitochondrial activity after 15 and 22 h maturation compared with Time 0 ($P < 0.05$), as well as an increase in the same parameter compared with the respective controls after 9 and 22 h maturation ($P < 0.05$; Fig. 5*c*).

A significant positive correlation was observed between the oxidative activity and mitochondrial activity of oocytes for each treatment ($r > 0.82$, $P < 0.05$; $n = 199$–211).

Mitochondrial distribution in the oocyte

Changes in mitochondrial distribution were observed during maturation. The distribution in immature oocytes was cortical (Fig. 1); however, during maturation, progressive mitochondrial migration to the central area was observed (Fig. 1). This observation was confirmed analysing the ratio of the intensity of green fluorescence between Area 3 and Area 1 of the oocyte ($P < 0.05$). In contrast, oocytes treated with ATP, NaF or AMP showed partial migration compared with their respective controls ($P < 0.05$; Fig. 6*a*–*c*).

Oocyte nuclear morphology

To determine which stage of nuclear maturation is affected by inhibition of glycolysis, COCs were also analysed using the fluorochrome Hoechst 33342 at the time-points specified. In the control group, 70.8% of oocytes had passed GVBD after 9 h maturation, whereas the rates were significantly lower for oocytes matured with ATP or NaF ($P < 0.05$). The rates of oocytes in MI after 15 h maturation were also significantly lower for groups treated with ATP and NaF ($P < 0.05$). Extrusion of the first polar body was observed in 81.5% of control oocytes after 22 h maturation, but this rate decreased significantly ($P < 0.05$) in the treated groups, with most oocytes arrested at the GV stage (Table 1). Oocytes treated with AMP did not exhibit any differences in MII rates after 22 h maturation compared with controls, so we did not include the analysis of oocyte nuclear morphology in this experiment.

Discussion

The present study describes the effects of the addition of glycolytic modulators during bovine oocyte IVM on glucose uptake and lactate production of COCs, as well as on the maturation rate, oxidative activity, mitochondrial activity and mitochondrial distribution of oocytes.

When ATP or NaF were added to the maturation medium, glucose uptake and lactate production were reduced, as was the meiotic maturation rate, suggesting a relationship between COC glycolytic activity and the nuclear maturation capacity of the oocyte. Although dose-dependent inhibition of lactate production was seen with both modulators, only COCs matured with ATP exhibited dose-dependent inhibition of glucose uptake; this difference may be related to differences in the intracellular mechanisms of action of the two compounds. NaF is a specific pharmacological inhibitor of the glycolytic pathway that acts by inhibiting the enzyme enolase, whereas ATP is a physiological modulator of phosphofructokinase 1 and other glucose-dependent metabolic pathways. NaF and ATP exert their effects at different levels within the glycolytic pathway: ATP inhibits the pathway at the preparatory phase, whereas NaF acts at the pay-off phase (Nelson and Cox 2005). The later inhibition of glycolysis by NaF may allow a flux of intermediary metabolites to enter other pathways.

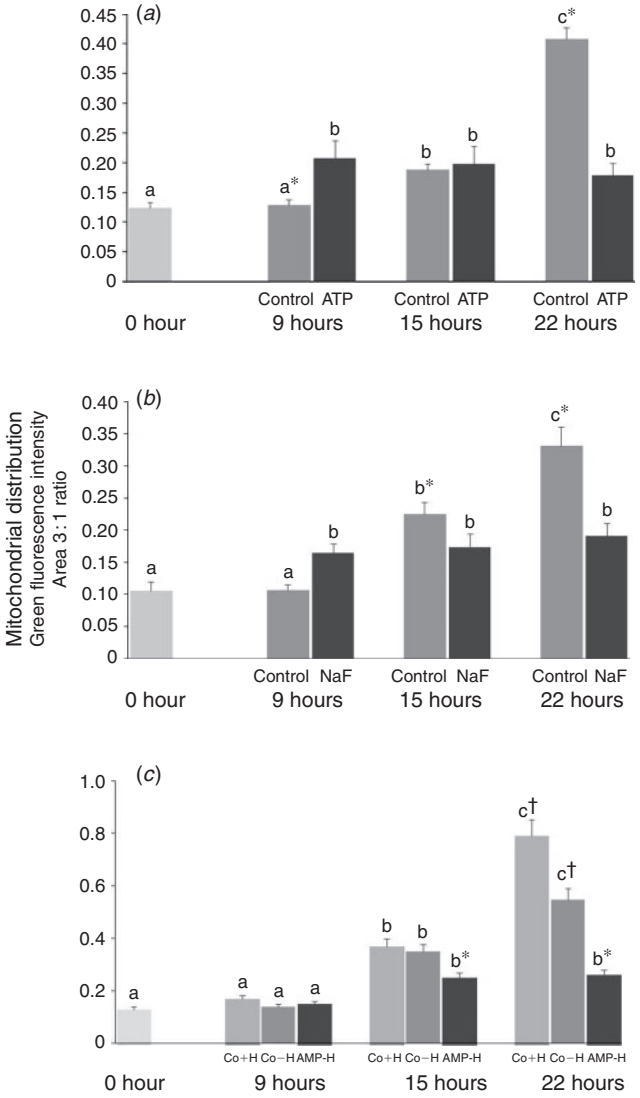

Fig. 6. Distribution of active mitochondria within oocytes matured for 0–22 h in the presence of (*a*) 10 mM ATP, (*b*) 3 mM NaF or (*c*) 10 mM AMP. Data are the mean ± s.e.m. ($n = 30$–40 COCs for each treatment). Within a graph and within each group (control or treated), values with different superscript letters differ significantly ($P < 0.05$). *Columns with different symbols at the same time point differ significantly ($P < 0.05$).

The dose-dependent inhibition of oocyte nuclear maturation in the presence of either ATP or NaF may be attributed a reduced contribution of oxidative substrates, such as pyruvate and lactate, by the cumulus cells. These substrates are essential for the bovine oocyte and the findings reflect the very low capacity for glucose metabolism by the oocyte (Cetica *et al.* 1999; Zuelke and Brackett 1992), as demonstrated by the considerably lower activity of phosphofructokinase 1 in denuded oocytes than in cumulus cells (Cetica *et al.* 2002).

In a previous study, we demonstrated that FSH + LH caused a significant (30.5%) increase in glucose uptake by COCs (Gutnisky *et al.* 2007). In the present study, we found that the

Table 1. Effects of 10 mM ATP and 3 mM NaF on nuclear morphology

Data show the percentage of oocytes at each stage of development ($n = 15$–20 oocytes for each treatment). Different superscript letters indicates significant differences in the percentage of oocytes at the same time point and same nuclear stage between treatments. GV, germinal vesicle; GVBD, GV breakdown; PBE, polar body extrusion

Duration of maturation (h)	Control				ATP				NaF			
	GV	GVBD	MI	PBE	GV	GVBD	MI	PBE	GV	GVBD	MI	PBE
0	100[a]				100[a]				100[a]			
9	29.2[a]	70.8[a]			100[b]	0[b]			88.9[b]	11.1[b]		
15	9.5[a]		90.5[a]		87.5[b]		12.5[b]		84.2[b]		15.8[b]	
22	11.1[a]		7.4[a]	81.5[a]	100[b]		0[a]	0[b]	58.8[c]		17.7[a]	23.5[c]

dose-dependent stimulation of glycolysis by AMP is not synergistic with FSH + LH stimulation. Furthermore, although the kinetics of meiotic maturation were not altered when AMP was added to the culture medium, the subsequent cleavage rates following IVF were significantly reduced, suggesting that stimulation of the glycolytic pathway by AMP may affect the cytoplasmic maturation of bovine oocytes. In line with these results, Preis *et al.* (2005) suggested that glucose uptake and lactate production by the COC are related to the capability of the oocyte to be fertilised. Moreover, AMP may be affecting the AMP : ATP ratio, resulting in the activation of AMP kinase. The activity of this enzyme is known to affect the nuclear maturation of bovine oocytes (Bilodeau-Goeseels *et al.* 2007).

In the present study, fluctuations in oxidative activity and mitochondrial activity of bovine oocytes were detected during IVM. In addition, both parameters were highly correlated and exhibited similar variations during maturation. Interestingly, the oxidative fluctuations observed here are not coincident with the changes in concentrations in reactive oxygen species detected during bovine oocyte maturation *in vitro* (Morado *et al.* 2009). Our results reveal that oxidative activity increases in bovine oocytes during maturation after 15 and 22 h culture. The increase in oxidative activity is coincident with key meiotic events of maturation, such as formation of the metaphase plate of the first meiotic division and extrusion of the first polar body (Gordon 1994), respectively. Protein synthesis increases threefold during MI compared with the GV stage (for a review, see Ferreira *et al.* 2009), suggesting that the increase in oxidative activity after 15 h maturation is related to new protein synthesis.

The reduced glycolytic activity induced by ATP and NaF is coincident with low oocyte oxidative activity after 15 and 22 h maturation, compared with untreated COCs. We also observed that inhibiting the glycolytic pathway arrested most of the oocytes at the GV stage. Although these results suggest that glycolytic activity of the COC may be decreased due to inhibition of GVBD and oocyte maturation, we have demonstrated previously that glycolytic activity in cumulus cells is not influenced by the presence of the oocyte (Sutton *et al.* 2003). Therefore, the pattern of oxidative activity would be dependent on changes in the glycolytic activity of the COCs and would therefore reflect ATP demand. Conversely, stimulation of the pathway by AMP affected the pattern of oocyte oxidative activity compared with the control group. The maintenance of a higher oxidative activity in the oocyte during IVM may, in some way, be affecting the cytoplasmic maturation of the female gamete.

Quantification of mitochondrial activity in untreated COCs increased significantly after 15 and 22 h maturation. Inhibition of COC glycolysis prevented this increase in mitochondrial activity, most likely as a consequence of the inhibition of glycolytic activity in COCs, resulting in a decreased supply of oxidative substrates to the oocyte. A similar observation was made for mitochondrial distribution: inhibition of glycolysis prevented mitochondrial migration during maturation. Stimulating the glycolytic pathway by AMP altered the pattern of mitochondrial activity compared with that in the control group. As discussed above with regard to oxidative activity, mitochondrial activity remained higher during IVM in the presence of AMP, suggesting that the increased glycolytic activity of cumulus cells may increase the contribution of reduced coenzymes and/or oxidative substrates to the oocyte, augmenting mitochondrial activity. Nevertheless, this does not appear to improve cytoplasmic maturation.

RedoxSensor red CC-1 (oxidative levels) and MitoTracker green FM (mitochondrial activity) were closely colocalised and within the cytosolic compartment of the oocyte, this being congruent with the characteristic pattern of metabolically active cells (Chen and Gee 2000). This relationship may be the result of an increase in cytosolic reductive agents, such as lactate and malate, within the oocyte. These metabolites can act as reductive compounds when they are substrates of lactate dehydrogenase and malate dehydrogenase, respectively (Cetica *et al.* 1999, 2003). It has been reported that mitochondrial reorganisation and a burst of ATP production during oocyte maturation are completely inhibited if cell cycle progression is inhibited (Yu *et al.* 2010). In agreement with these findings, we determined that changes in oxidative and mitochondrial activity are related to modifications in normal mitochondrial migration, suggesting that the same is true in bovine oocytes.

In conclusion, we report herein that glycolytic pathway activity in COCs is necessary for successful IVM of the bovine oocyte. Modifications in the oxidative and mitochondrial activities in the oocyte are associated with increases at 15 and 22 h incubation during maturation. Inhibiting glycolysis reduced these parameters in the oocyte at these time-points, with an inhibition of mitochondrial migration in oocytes also detected. Stimulation of the glycolytic pathway by AMP in the absence of

gonadotrophins also changed oxidative behaviour, which reduced oocyte cytoplasmic competence.

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