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MODULATION OF GLYCOLYSIS AND THE PENTOSE PHOSPHATE PATHWAY INFLUENCES PORCINE OOCYTE IN VITRO MATURATION

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Running title: Glycolysis and PPP in porcine IVM

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Abstract

Glycolytic and pentose phosphate pathway (PPP) activities were modulated in porcine cumulus-oocyte complexes (COCs) during in vitro maturation (IVM) by the addition of inhibitors or stimulators of key enzymes of the pathways in order to elucidate their relative participation in oocyte maturation. The activities of glycolysis and PPP were evaluated by lactate production per COC and by the Brilliant Cresyl Blue (BCB) test, respectively. Glucose uptake per COC and the oocyte maturation rate were also evaluated. Lactate production, glucose uptake and the percentage of oocytes reaching metaphase II decreased in a dose-dependent manner in the presence of the pharmacological (NaF) or the physiological (ATP) inhibitors of glycolysis ($P<0.05$). The addition of the physiological stimulator of glycolysis (AMP) caused no effect on lactate production, glucose uptake or the meiotic maturation rate. The pharmacological (6-AN) and the physiological (NADPH) inhibitors of PPP induced a dose-dependent decrease in the percentage of oocytes with high PPP activity and in the nuclear maturation rate ($P<0.05$). The physiological stimulator of PPP (NADP) caused no effect on the percentage of oocytes with high PPP activity. The glycolytic and PPP activities of porcine COCs and maturational competence of oocytes seem to be closely related events. This study shows for the first time the regulatory effect of ATP and NADPH as physiological inhibitors of glycolysis and PPP in porcine COCs, respectively. Besides, these pathways seem to reach their maximum activities in porcine COCs during IVM because no further increases were achieved by the presence of AMP or NADP.

Key words: Glycolysis, pentose phosphate pathway, COCs, oocyte, porcine

Introduction

The oocyte and the surrounding cumulus cells are structurally and physiologically coupled. Cumulus-oocyte complexes (COCs) can consume different substrates from the ovarian follicular fluid during in vivo maturation and from culture media during in vitro maturation (IVM), to be fated towards diverse metabolic pathways involved in the maturation process (Sutton et al. 2003b; Thompson 2006).

A close relationship between the presence of glucose in the maturation medium and the progression of meiosis has been observed in mouse oocytes (Downs 1995). Similarly, an adequate glucose concentration in the maturation medium improves the bovine oocyte IVM and the subsequent embryo development (Lim et al. 1999; Khurana and Niemann 2000). In the porcine species, the addition of glucose to the maturation medium accelerates the meiotic progression of oocytes (Sato et al. 2007) and increases the percentage of oocytes reaching the metaphase II nuclear stage (Wongsrikeao et al. 2006a; Funahashi et al. 2008). Additionally, glucose metabolism is important in oocyte cytoplasmic maturation, which in turn is necessary for embryo development (Krisher et al. 2007).

The glycolytic pathway has been proposed as the main fate for the glucose consumed by murine, bovine and porcine COCs (Downs and Utecht 1999; Cetica et al. 2002; Preis et al. 2005; Krisher et al. 2007; Alvarez et al. 2012). Evidence suggests that cumulus cells metabolize glucose, producing glycolytic metabolites, mainly lactate, used by the oocyte during maturation (Cetica et al. 1999; Sutton et al. 2003a; Alvarez et al. 2012). In somatic cells, the modulation of the glycolytic pathway is thought to take place in the enzyme phosphofructokinase 1, being AMP and ATP the allosteric stimulator and inhibitor, respectively (Schirmer and Evans 1990; Nelson and Cox

2005). Additionally, this pathway is inhibited by several pharmacological compounds, such as sodium fluoride (NaF), which is widely used to inhibit glycolytic activity (Mayes and Bender 2004).

Glucose can be alternatively oxidized through the pentose phosphate pathway (PPP), which appears to be linked to the regulation of oocyte maturation (Downs and Utecht 1999; Funahashi et al. 2008). The PPP has several working alternatives according to specific cell requirements: the metabolites obtained can be either used in other pathways (e.g. synthesis of nucleotides, glycolysis) or recycled in the PPP. The PPP activity is dependent on the intracellular concentrations of NADP and NADPH, which modulate the pathway positively and negatively, respectively, acting mainly on the enzyme glucose 6-phosphate dehydrogenase (Nelson and Cox 2005). Additionally, this enzyme can be inhibited pharmacologically by 6-aminonicotinamide (6-AN) (Hothersall et al. 1981).

The glucose consumed by porcine COCs seems to be oxidized mainly through the glycolytic pathway and the PPP. The modulation of these pathways through the regulation of the activity of key enzymes by different compounds may thus allow us to establish their relative participation in the porcine oocyte in vitro maturation process. The effects of the addition of enzymatic inhibitors (NaF, ATP) and a stimulator (AMP) of ~~phosphofructokinase 1~~ glycolysis as well as of enzymatic inhibitors (6-AN, NADPH) and a stimulator (NADP) of ~~glucose 6-phosphate dehydrogenase~~ PPP in IVM medium on the ~~activities of glycolysis~~ glycolytic activity (evaluated by lactate production) and PPP activity (evaluated by BCB test) in porcine COCs, glucose uptake per COC and oocyte maturation were analyzed.

Materials and Methods

Materials

Unless otherwise specified, all chemicals used were from Sigma Chemical Company (St. Louis, MO, USA).

Recovery and classification of cumulus-oocyte complexes

Ovaries from slaughtered gilts were transported in a warm environment (28-33ºC) for the 2-3 h journey to the laboratory. Ovaries were washed in 0.9% (w/v) NaCl containing 100 000 IU/L penicillin and 100 mg/L streptomycin. COCs were aspirated from 3-8 mm antral follicles by using a 10 mL syringe and an 18-gauge needle, and oocytes surrounded by a dense cumulus were selected for in vitro culture.

Oocyte in vitro maturation

COCs were individually cultured in medium 199 (Earle's salts, L-glutamine, 2.2 mg/L sodium bicarbonate; GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) foetal bovine serum (GIBCO), 0.57 mM cysteine, 50 mg/L gentamicin sulphate, and 0.5 mg/L porcine follicle-stimulating hormone (FSH) (Folltropin-V, Bioniche, Belleville, Ontario, Canada) plus 0.5 mg/L porcine luteinizing hormone (LH) (Lutropin-V, Bioniche) (control medium) under mineral oil at 39ºC for 48 h in a 5% CO_2 atmosphere (Abeydeera et al. 2001).

Different regulators of glycolysis and PPP were added to the control medium: 2.5 mM, 5 mM, 7.5 mM and 10 mM NaF (glycolytic pharmacological inhibitor); 1 mM,

10 mM, 20 mM and 40 mM ATP (glycolytic physiological inhibitor); 1 mM, 10 mM, 20 mM and 40 mM AMP (glycolytic physiological stimulator); 0.01 mM, 0.025 mM, 0.05 mM and 0.1 mM 6-AN (PPP pharmacological inhibitor); 0.0125 mM, 0.125 mM, 1.25 mM and 12.5 mM NADPH (PPP physiological inhibitor); 0.0125 mM, 0.125 mM, 1.25 mM and 12.5 mM NADP (PPP physiological stimulator).

Evaluation of oocyte maturation

In vitro matured oocytes were denuded by gentle pipetting after incubation in 1 g/l hyaluronidase in phosphate-buffered saline (PBS) for 5 min at 37ºC, placed in a hypotonic medium of 10 g/L sodium citrate at 37ºC for 15 min, fixed on a slide with Carnoy fixing solution (3:1 ethanol:acetic acid), and stained with 5% (v/v) Giemsa (Merck, Darmstadt, Germany) for 15 min. They were then observed under the light microscope at x100 and x400 magnification. Only oocytes with condensed and well-defined metaphase II chromosome configurations were considered meiotically mature (Alvarez et al. 2009).

Evaluation of the viability of cumulus-oocyte complexes

To evaluate viability of cumulus cells and oocytes, an aliquot of COCs from each treatment group was incubated for 10 min at 37ºC in PBS added with 2.5 µg/l fluorescein diacetate fluorochrome. Then, COCs were washed in PBS before being observed in an epifluorescence microscope (Zeiss, Germany) using a 510 nm filter at x100 magnification. Live cells were distinguished from dead ones based on their green fluorescence (Alvarez et al. 2009).

Evaluation of glycolytic activity

To evaluate glycolytic activity in COCs during IVM, lactate production per COC was determined. COCs were individually matured in 20-µl droplets of culture medium, then removed from the droplets and the lactate content of the spent maturation medium was assessed. Lactate concentration was measured using a spectrophotometric assay based on the oxidation of this compound by lactate oxidase and the subsequent determination of the hydrogen peroxide formed (Barham and Trinder 1972).

Additionally, glucose uptake per COC was measured in a similar manner by determining the glucose content of the spent maturation medium but using glucose oxidase (Barham and Trinder 1972; Gutnisky et al. 2007).

Twenty-microlitre droplets of maturation medium without cells were included in each experiment to provide glucose and lactate reference concentrations.

COCs removed from the droplets were processed as previously described to evaluate oocyte meiotic maturation.

Evaluation of pentose phosphate pathway activity

To evaluate PPP activity during IVM in COCs, the Brilliant Cresyl Blue (BCB) test for immature oocytes was performed (Wongsrikeao et al. 2006b) with some modifications to be adapted to the porcine oocyte IVM. COCs were individually matured in 20-µl droplets of culture medium for 45 hours and then transferred for the last 3 hours of IVM to the same culture medium which had been added with 4.8 µM of BCB. Oocytes were denuded as previously described and finally separated into two different groups according to their cytoplasmic colouration: BCB-positive oocytes (with

blue cytoplasmic colouration) indicate a low activity of PPP, whereas BCB-negative oocytes (with no blue cytoplasmic colouration) indicate a high activity of PPP.

Additionally, lactate production and glucose uptake per COC were determined by assessing lactate and glucose contents of the spent maturation medium, as described above.

COCs removed from the droplets were processed as previously described to evaluate oocyte meiotic maturation.

Statistical analysis and experimental design

Non-parametric values were recorded as percentages and analysed using a Chi-squared test. Parametric values were reported as means ± SEM and comparisons were made by ANOVA. The Pearson test was used to determine the correlation between glucose uptake and lactate production per COC. Significance was set at $P<0.05$.

Results

Effect of NaF on glycolytic activity

Lactate, an end product of glycolysis, was measured in IVM medium to assess the activity of glycolysis in porcine COCs in the presence of different concentrations of the pharmacological inhibitor of the pathway. Lactate production per COC disclosed a dose-dependent decrease in the presence of NaF ($P<0.05$, Figure 1a). Glucose uptake per COC showed a similar behaviour in the presence of this compound in IVM medium ($P<0.05$, Figure 1b). A very high positive correlation between glucose uptake and

lactate production was observed (r=0.86; P=0.0000). The oocyte meiotic maturation rate decreased from 5 mM NaF onward (P<0.05, Figure 1c). However, cumulus cells and oocyte viability were not affected at any of the concentrations of NaF evaluated (Table 1).

Effect of ATP on glycolytic activity

As observed with NaF, both lactate production and glucose uptake per COC decreased in a dose-dependent manner in the presence of the physiological inhibitor of the glycolytic pathway (P<0.05, Figure 2a and 2b). Again, a very high positive correlation between glucose uptake and lactate production was observed (r=0.86; P=0.0000). The oocyte meiotic maturation rate also showed a dose-dependent decrease in the presence of ATP (Figure 2c). However, neither cumulus cells nor oocyte viability were affected at any of the concentrations of ATP used (Table 1).

Effect of AMP on glycolytic activity

There was no difference in lactate production or glucose uptake per COC in the presence of increasing concentrations of the physiological stimulator of glycolysis (Figure 3a and 3b). A very strong positive correlation between glucose uptake and lactate production was observed (r=0.87; P=0.0000). The oocyte meiotic maturation rate did not show variation in the presence of AMP (Figure 3c). Neither cumulus cells nor oocyte viability were affected at any of the concentrations of AMP assessed (Table 1).

Effect of 6-AN on pentose phosphate pathway activity

The BCB test was used to evaluate the activity of PPP in porcine COCs in the presence of different concentrations of the pharmacological inhibitor of the metabolic pathway. The results showed a dose-dependent decrease in the percentage of oocytes with high PPP activity of with the addition of 6-AN in IVM medium ($P<0.05$, Figure 4a). Both lactate production and glucose uptake per COC decreased to a plateau from 0.025 to 0.1 mM 6-AN, not showing a dose-dependent effect ($P<0.05$; Figure 4b and 4c). A good positive correlation between glucose uptake and lactate production was observed ($r=0.63$; $P=0.0000$). The oocyte meiotic maturation rate decreased in a dose-dependent manner in the presence of the pharmacological inhibitor of PPP (Figure 4d). However, neither cumulus cells nor oocyte viability were affected at any of the concentrations of 6-AN studied (Table 1).

Effect of NADPH on pentose phosphate pathway activity

As observed with 6-AN, there was a dose-dependent decrease in both the percentage of oocytes with high PPP activity of and the meiotic maturation rate in the presence of the physiological inhibitor of the pathway ($P<0.05$, Figure 5a and 5d). However, lactate production and glucose uptake per COC remained constant in the presence of the different concentrations of NADPH (Figure 5b and 5c). A high positive correlation between glucose uptake and lactate production was recorded ($r=0.72$; $P=0.0000$). Neither cumulus cells nor oocyte viability were affected at any of the concentrations of NADPH evaluated (Table 1).

Effect of NADP on pentose phosphate pathway activity

The addition of increasing concentrations of the physiological stimulator of PPP showed no effect on the activity of the pathway (Figure 6a). Lactate production per COC decreased to a plateau from 0.125 to 12.5 mM NADP ($P<0.05$, Figure 6b), although glucose uptake was not modified respect to control (Figure 6c). A weak positive correlation between glucose uptake and lactate production was determined ($r=0.31$; $P=0.0019$). A slightly but significant decrease in the oocyte meiotic maturation rate was observed in the presence of NADP ($P<0.05$, Figure 6d). However, neither cumulus cells nor oocyte viability were affected at any of the concentrations of NADP assessed (Table 1).

Discussion

The glucose consumption by COCs during in vitro culture is necessary for proper oocyte maturation (Thompson 2006). The fate of glucose towards glycolysis and PPP could be directly implicated in the acquisition of maturational competence. Here, the modulation of the glycolytic and PPP activities by means of enzymatic effectors demonstrated the impact of these metabolic pathways on the progression of oocyte maturation, increasing the understanding of the participation of each pathway in the maturation process.

The glucose consumed by porcine COCs would mainly be converted to lactate, suggesting significant glycolytic activity by cumulus cells (Alvarez et al. 2012). NaF is a well-characterized pharmacological inhibitor of glycolysis in somatic cells, and its action has been described on the enzyme enolase (Harris 2002). Lactate production and glucose uptake per COC decreased in a dose-dependent manner when porcine oocyte

IVM took place in the presence of NaF. Noteworthy, the very strong positive correlation between both parameters remained despite the addition of different concentrations of this inhibitor, demonstrating that glycolysis is the main fate of glucose consumed by porcine COCs. ~~The concentrations of NaF found to have inhibitory effect in the present study were higher than those reported to diminish glucose consumption in several types of eukaryotic cells (Anderson 1969; Feig et al. 1971; Shayiq and Kidwai 1986), confirming the high glycolytic activity in porcine COCs.~~ Interestingly, when maturation medium was added with 5 mM NaF, the percentage of oocytes reaching metaphase II, as well as lactate production and glucose uptake per COC, were 75% lower than those of the control group, suggesting that oocyte nuclear maturation and glycolytic activity in COCs are very closely related events in porcine species. ~~Interestingly, when maturation medium was added with 5 mM NaF, the percentage of oocytes reaching metaphase II was 75% lower than that of the control group was a quarter than control group, and lactate production and glucose uptake per COC also showed a quarter of the activity than control groups, suggesting that oocyte nuclear maturation and glycolytic activity in COCs are very close related events in porcine species.~~ In porcine COCs matured in medium added with other glycolytic pharmacological inhibitors, cumulus cells were removed at the conclusion of IVM and no effect on glycolytic activity was determined in denuded porcine oocytes (Herrick et al. 2006). This result is in agreement with the suggestion that glycolysis is a predominant pathway in porcine cumulus cells (Alvarez et al. 2012). The concentrations of NaF found to have inhibitory effect in the present study were higher than those reported to diminish glucose consumption in several types of eukaryotic cells (Anderson 1969; Feig et al. 1971; Shayiq and Kidwai 1986), confirming the high glycolytic activity in porcine COCs. The inhibition of oocyte maturation due to stimulation of adenylate cyclase by NaF has been reported in bovine

COCs. However, the concentration used to obtain this effect was higher than in the present study (Sirard 1990) or was utilized in combination with 3-isobutyl-1-methylxanthine to achieve the inhibition of nuclear maturation (Bilodeau et al. 1993).

ATP has been pointed out as a physiological regulator of glycolysis, being a negative allosteric effector of the main key enzyme of the pathway (Harris 2002; Kamp et al. 2007). Lactate production and glucose uptake per COC also showed a dose-dependent decrease when porcine oocyte IVM was carried out with the addition of ATP to the maturation medium, and the very high positive correlation between both parameters remained despite the diverse concentrations of the inhibitor assessed. These results showed for the first time the regulatory effect of ATP on the glycolytic activity of porcine COCs and reinforce the statement that glycolysis is the principal metabolic route in these complexes. It is interesting to note that the inhibitory concentrations of ATP determined in this work were about the ones reported to be effective on enzymatic extracts of phosphofructokinase 1 (Harris 2002; Kamp et al. 2007). In the present work, when porcine COCs were cultured in the presence of 1 mM ATP, the oocyte nuclear nuclear maturation rate, as well as the lactate production and glucose uptake per COC, was 50% lower than that observed in the control group, confirming the close relationship between oocyte meiotic maturation and glycolytic activity in porcine COCs during IVM.

The AMP has been identified as a positive allosteric effector of the main key enzyme of glycolysis (Harris 2002; Simpfendorfer et al. 2006; Kamp et al. 2007). In the experiment performed in the presence AMP in culture medium, no effect was observed on lactate production and glucose uptake per COC, suggesting no stimulating effect by this compound on glycolysis in porcine COCs. In a previous work, we demonstrated the stimulation of glycolysis in COCs by the supplementation of IVM

medium with gonadotropins in this species (Alvarez et al. 2012). We can propose that AMP has no effect on glycolytic activity of porcine COCs or that the stimulatory effect of gonadotropins on glycolysis could overlap the effect of AMP. It is important to remark that the concentrations of AMP evaluated in this study were either the same as or higher than the ones reported previously to be effective for stimulating phosphofructokinase 1 (Simpfendorfer et al. 2006; Kamp et al. 2007).

6-AN is an effective pharmacological inhibitor of the PPP, acting as a competitive inhibitor of the enzyme glucose 6-phosphate dehydrogenase (Tyson et al. 2000). The addition of this compound in the IVM medium of porcine oocytes induced a dose-dependent decrease in both the percentage of oocytes with high PPP activity and the nuclear maturation rate, indicating an association between both events. The interference of 6-AN on the meiotic progression of murine and porcine oocytes has been previously reported (Downs et al. 1998; Sato et al. 2007; Funahashi et al. 2008). Glucose uptake and lactate production per COC were inhibited at higher concentrations of 6-AN, but not in a dose-dependent manner, showing a good correlation between them. The specific inhibition of the PPP by 6-AN seems to reduce the amount of glucose used by COCs through this pathway, but the concurrent decrease in lactate production suggests a reduction in glycolytic activity as well. This effect could be explained by some kind of enzymatic inhibition of glycolysis or by the decrease in PPP end products fated to the glycolytic pathway. In coincidence, PPP inhibition also induces a decrease in glucose uptake and lactate production in murine COCs (Downs et al. 1998). This effect on glycolysis due to the inhibition of the PPP with diphenyleneiodonium has also been reported in mature porcine oocytes isolated from cumulus cells (Herrick et al. 2006). The accumulation of 6-phosphogluconate due to the inhibition of the PPP enzyme 6-phosphogluconate dehydrogenase with 6-AN has been

previously observed in somatic cells; this compound seems to inhibit the glycolytic enzyme phosphoglucose isomerase (Tyson et al. 2000). Thus, the inhibition of PPP with 6-AN would lead to a secondary inhibition of glycolysis in COCs, both affecting the maturational capability of porcine oocytes.

PPP activity is physiologically regulated by the intracellular NADP/NADPH ratio: a high ratio increases glucose consumption through the pathway, whereas a low ratio induces the inhibition of the pathway (Clarenburg 1992; Nelson and Cox 2005). In the present work, the addition of NADPH in the IVM medium caused a dose-dependent decrease in both the percentage of oocytes with high PPP activity and the nuclear maturation rate, reinforcing the evidences that both events are related. These results also show for the first time the regulatory effect of NADPH on PPP activity of porcine COCs. In contrast to that observed with 6-AN, neither glucose uptake nor lactate production per COC were altered by the addition of NADPH to the maturation medium, and a high positive correlation between both parameters was maintained. Therefore, we can propose that the decrease in PPP activity by increasing levels of its physiological inhibitor would not impair the glycolytic activity in porcine COCs during IVM, in contrast to that observed with the pharmacological inhibitors of the pathway.

The addition of NADP, a physiological stimulator of PPP, in the IVM medium caused no effects on the percentage of oocytes with high activity of this metabolic route. PPP activity seems to be high during porcine oocyte maturation and NADP supplementation seems to be unable to further stimulate this pathway. NADP did not modify glucose uptake per COC, although at higher concentrations it induced a reduction in lactate production by porcine COCs during IVM. The decrease observed in lactate production suggests some variation in glucose fate when NADP was present. A possible explanation is that NADP induces most of the consumed glucose to be destined

to PPP, and thus fewer molecules would be catabolised in glycolysis. This reduced glycolytic activity in COCs could justify the decrease in the number of porcine oocytes that reached metaphase II stage. Important interrelationships between different carbohydrate pathways in COCs during IVM have been described, and the over or under activation of one of them can impact on the activity of the others and subsequently affect oocyte maturational and/or developmental competence (Thompson 2006).

Finally, the viability of cumulus cells and oocytes was not affected at any of the concentrations of the different modulators used to control glycolytic and PPP activities, denoting that no toxic effect could be attributed to the results obtained.

In conclusion, the inhibition of glycolysis or PPP during IVM of porcine COCs leads to a decrease in nuclear oocyte in vitro maturation, demonstrating the importance of glucose utilization through these pathways for the progression of meiosis in the porcine gamete. This study shows for the first time that ATP and NADPH would act as physiological negative regulators of glycolytic and PPP activities in porcine COCs, respectively. Besides, glycolysis and PPP seem to reach their maximum activities in porcine COCs under the IVM conditions used in the present study because no further increase was achieved by AMP or NADP. The modulation of alternative pathways involved in glucose metabolism and their relationship with oocyte maturation will further contribute to the elucidation of the role of this hexose in the IVM process.

Conflicts of interest

The authors declare they have no conflicts of interest that might impede their impartiality with respect to the work performed.

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Figure legends

Figure 1.

a. Lactate production by cumulus-oocyte complex (COC) during maturation with different concentrations of NaF. [a, b, c] Different superscripts over bars indicate significant differences ($P<0.05$). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

b. Glucose uptake by COC during maturation with different concentrations of NaF. [a, b, c] Different superscripts over bars indicate significant differences ($P<0.05$). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

c. Percentage of oocytes reaching metaphase II (M II) after maturation with different concentrations of NaF. [a, b, c] Different superscripts over bars indicate significant differences ($P<0.05$). n = 27-30 oocytes for each bar. Experiments were repeated three times.

Figure 2.

a. Lactate production by cumulus-oocyte complex (COC) during maturation with different concentrations of ATP. [a, b, c, d] Different superscripts over bars indicate significant differences ($P<0.05$). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

b. Glucose uptake by COC during maturation with different concentrations of ATP. [a, b, c] Different superscripts over bars indicate significant differences ($P<0.05$). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

c. Percentage of oocytes reaching metaphase II (M II) after maturation with different concentrations of ATP. [a, b, c] Different superscripts over bars indicate significant differences ($P<0.05$). n = 29-30 oocytes for each bar. Experiments were repeated three times.

Figure 3.

a. Lactate production by cumulus-oocyte complex (COC) during maturation with different concentrations of AMP. [a] The same superscript over bars indicates no significant difference. n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

b. Glucose uptake by COC during maturation with different concentrations of AMP. [a] The same superscript over bars indicates no significant difference. n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

c. Percentage of oocytes reaching metaphase II (M II) after maturation with different concentrations of AMP. [a] The same superscript over bars indicates no significant difference. n = 29-30 oocytes for each bar. Experiments were repeated three times.

Figure 4.

a. Percentage of oocytes with active pentose phosphate pathway, evaluated by BCB test, after maturation with different concentrations of 6-AN. [a, b, c, d] Different superscripts over bars indicate significant differences ($P<0.05$). n = 28-30 oocytes for each bar. Experiments were repeated three times.

b. Lactate production by cumulus-oocyte complex (COC) during maturation with different concentrations of 6-AN. [a, b] Different superscripts over bars indicate significant differences ($P<0.05$). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

c. Glucose uptake by COC during maturation with different concentrations of 6-AN. [a, b] Different superscripts over bars indicate significant differences ($P<0.05$). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

d. Percentage of oocytes reaching metaphase II (M II) after maturation with different concentrations of 6-AN. [a, b, c, d] Different superscripts over bars indicate significant differences ($P<0.05$). n = 28-30 oocytes for each bar. Experiments were repeated three times.

Figure 5.

a. Percentage of oocytes with active pentose phosphate pathway, evaluated by BCB test, after maturation with different concentrations of NADPH. [a, b, c, d] Different superscripts over bars indicate significant differences (P<0.05). n = 29-30 oocytes for each bar. Experiments were repeated three times.

b. Lactate production by cumulus-oocyte complex (COC) during maturation with different concentrations of NADPH. [a] The same superscript over bars indicates no significant difference. n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

c. Glucose uptake by COC during maturation with different concentrations of NADPH. [a] The same superscript over bars indicates no significant difference. n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

d. Percentage of oocytes reaching metaphase II (M II) after maturation with different concentrations of NADPH. [a, b, c, d] Different superscripts over bars indicate significant differences (P<0.05). n = 29-30 oocytes for each bar. Experiments were repeated three times.

Figure 6.

a. Percentage of oocytes with active pentose phosphate pathway, evaluated by BCB test, after maturation with different concentrations of NADP. [a] The same superscript over bars indicates no significant difference. n = 28-30 oocytes for each bar. Experiments were repeated three times.

b. Lactate production by cumulus-oocyte complex (COC) during maturation with different concentrations of NADPH. [a, b] Different superscripts over bars indicate

significant differences (P<0.05). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

c. Glucose uptake by COC during maturation with different concentrations of NADP. [a] The same superscript over bars indicates no significant difference. n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM.

d. Percentage of oocytes reaching metaphase II (M II) after maturation with different concentrations of NADP. [a, b, c, d] Different superscripts over bars indicate significant differences (P<0.05). n = 28-30 oocytes for each bar. Experiments were repeated three times.

Table 1. Percentage of live oocytes and live cumulus in cumulus-oocyte complex (COC) matured in the presence of different modulators. [a] The same superscript indicates no significant difference within line. n = 30 COCs for each value. Experiments were repeated three times.

REFERENCES

Abeydeera L R, Wang W H, Prather R S, Day B N, 2001: Effect of incubation temperature on in vitro maturation of porcine oocytes: nuclear maturation, fertilisation and developmental competence. Zygote **9**, 331-337.

Alvarez G, Dalvit G, Cetica P, 2012: Influence of the Cumulus and Gonadotropins on the Metabolic Profile of Porcine Cumulus–Oocyte Complexes During In Vitro Maturation. Reproduction in domestic animals = Zuchthygiene, In Press.

Alvarez G M, Dalvit G C, Achi M V, Miguez M S, Cetica P D, 2009: Immature oocyte quality and maturational competence of porcine cumulus-oocyte complexes subpopulations. Biocell **33**, 167-177.

Anderson D M, 1969: In vitro inhibition of glycolysis in blood and its effect on the haematocrit. Journal of comparative pathology **79**, 525-535.

Barham D, Trinder P, 1972: An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst **97**, 142-145.

Bilodeau S, Fortier M A, Sirard M A, 1993: Effect of adenylate cyclase stimulation on meiotic resumption and cyclic AMP content of zona-free and cumulus-enclosed bovine oocytes in vitro. Journal of reproduction and fertility **97**, 5-11.

Cetica P, Pintos L, Dalvit G, Beconi M, 2002: Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. Reproduction (Cambridge, England) **124**, 675-681.

Cetica P D, Pintos L N, Dalvit G C, Beconi M T, 1999: Effect of lactate dehydrogenase activity and isoenzyme localization in bovine oocytes and utilization of oxidative substrates on in vitro maturation. Theriogenology **51**, 541-550.

Clarenburg R, 1992: Carbohydrate metabolism, In: Reinhardt, R. (Ed.), Physiological chemistry of domestic animals, Mosby Year Book Inc, Saint Louis, pp. 239-291.

Downs S M, 1995: The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte. Developmental biology **167**, 502-512.

Downs S M, Humpherson P G, Leese H J, 1998: Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway. Biology of reproduction **58**, 1084-1094.

Downs S M, Utecht A M, 1999: Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes. Biology of reproduction **60**, 1446-1452.

Feig S A, Shohet S B, Nathan D G, 1971: Energy metabolism in human erythrocytes. I. Effects of sodium fluoride. The Journal of clinical investigation **50**, 1731-1737.

Funahashi H, Koike T, Sakai R, 2008: Effect of glucose and pyruvate on nuclear and cytoplasmic maturation of porcine oocytes in a chemically defined medium. Theriogenology **70**, 1041-1047.

Gutnisky C, Dalvit G C, Pintos L N, Thompson J G, Beconi M T, Cetica P D, 2007: Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte in vitro maturation, fertilisation and embryo development. Reprod Fertil Dev **19**, 488-497.

Harris R A, 2002: Carbohydrate metabolism I: Major metabolic pathways and their control, In: Devlin, T.M. (Ed.), Textbook of Biochemistry, Wiley-Liss, New York, pp. 597-664.

Herrick J R, Brad A M, Krisher R L, 2006: Chemical manipulation of glucose metabolism in porcine oocytes: effects on nuclear and cytoplasmic maturation in vitro. Reproduction (Cambridge, England) **131**, 289-298.

Hothersall J S, Zubairu S, McLean P, Greenbaum A L, 1981: Alternative pathways of glucose utilization in brain; changes in the pattern of glucose utilization in brain

resulting from treatment of rats with 6-aminonicotinamide. Journal of neurochemistry **37**, 1484-1496.

Kamp G, Schmidt H, Stypa H, Feiden S, Mahling C, Wegener G, 2007: Regulatory properties of 6-phosphofructokinase and control of glycolysis in boar spermatozoa. Reproduction (Cambridge, England) **133**, 29-40.

Khurana N K, Niemann H, 2000: Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. Theriogenology **54**, 741-756.

Krisher R L, Brad A M, Herrick J R, Sparman M L, Swain J E, 2007: A comparative analysis of metabolism and viability in porcine oocytes during in vitro maturation. Animal reproduction science **98**, 72-96.

Lim J M, Lee B C, Lee E S, Chung H M, Ko J J, Park S E, Cha K Y, Hwang W S, 1999: In vitro maturation and in vitro fertilization of bovine oocytes cultured in a chemically defined, protein-free medium: effects of carbohydrates and amino acids. Reprod Fertil Dev **11**, 127-132.

Mayes P A, Bender D, 2004: Glucólisis y la oxidación del piruvato, In: Murray, R. (Ed.), Harper. Bioquímica Ilustrada, Editorial El Manual Moderno, S.A. de C.V.. , México, D.F., pp. 153-161.

Nelson D, Cox M, 2005: Glycolysis, gluconeogenesis, and the pehtose phosphate pathway, In: Nelson D, C.M. (Ed.), Lehninger. Principles of biochemistry, W. H. Freeman and Company, New York, pp. 521-559.

Preis K A, Seidel G, Jr., Gardner D K, 2005: Metabolic markers of developmental competence for in vitro-matured mouse oocytes. Reproduction (Cambridge, England) **130**, 475-483.

Sato H, Iwata H, Hayashi T, Kimura K, Kuwayama T, Monji Y, 2007: The effect of glucose on the progression of the nuclear maturation of pig oocytes. Animal reproduction science **99**, 299-305.

Schirmer T, Evans P R, 1990: Structural basis of the allosteric behaviour of phosphofructokinase. Nature **343**, 140-145.

Shayiq R M, Kidwai A M, 1986: Effect of fluoride on uptake of D-glucose by isolated epithelial cells of rat intestine. Environmental research **41**, 388-399.

Simpfendorfer R W, Oelckers K B, Lopez D A, 2006: Phosphofructokinase from muscle of the marine giant barnacle Austromegabalanus psittacus: kinetic characterization and effect of in vitro phosphorylation. Comp Biochem Physiol C Toxicol Pharmacol **142**, 382-389.

Sirard M A, 1990: Temporary inhibition of meiosis resumption in vitro by adenylate cyclase stimulation in immature bovine oocytes. Theriogenology **33**, 757-767.

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

Sutton M L, Gilchrist R B, Thompson J G, 2003b: Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. Human reproduction update **9**, 35-48.

Thompson J G, 2006: The impact of nutrition of the cumulus oocyte complex and embryo on subsequent development in ruminants. The Journal of reproduction and development **52**, 169-175.

Tyson R L, Perron J, Sutherland G R, 2000: 6-Aminonicotinamide inhibition of the pentose phosphate pathway in rat neocortex. Neuroreport **11**, 1845-1848.

Wongsrikeao P, Otoi T, Taniguchi M, Karja N W, Agung B, Nii M, Nagai T, 2006a: Effects of hexoses on in vitro oocyte maturation and embryo development in pigs. Theriogenology **65**, 332-343.

Wongsrikeao P, Otoi T, Yamasaki H, Agung B, Taniguchi M, Naoi H, Shimizu R, Nagai T, 2006b: Effects of single and double exposure to brilliant cresyl blue on the selection of porcine oocytes for in vitro production of embryos. Theriogenology **66**, 366-372.

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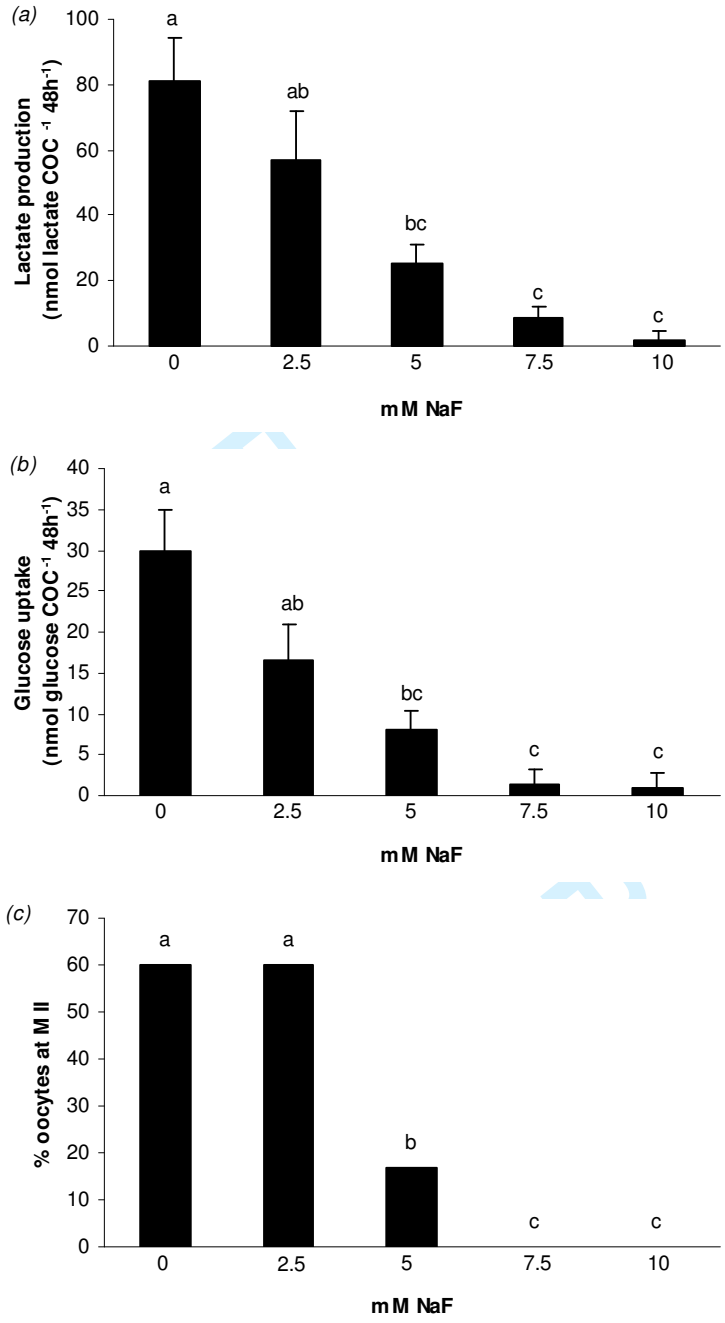

(a)
Lactate production (nmol lactate COC -1 48h-1)
100
80
60
40
20
0
a
ab
bc
c
c
0
2.5
5
7.5
10
mM NaF
(b)
Glucose uptake (nmol glucose COC-1 48h-1)
40
35
30
25
20
15
10
5
0
a
ab
bc
c
c
0
2.5
5
7.5
10
mM NaF
(c)
% oocytes at M II
70
60
50
40
30
20
10
0
a
a
b
c
c
0
2.5
5
7.5
10
mM NaF

Figure 2

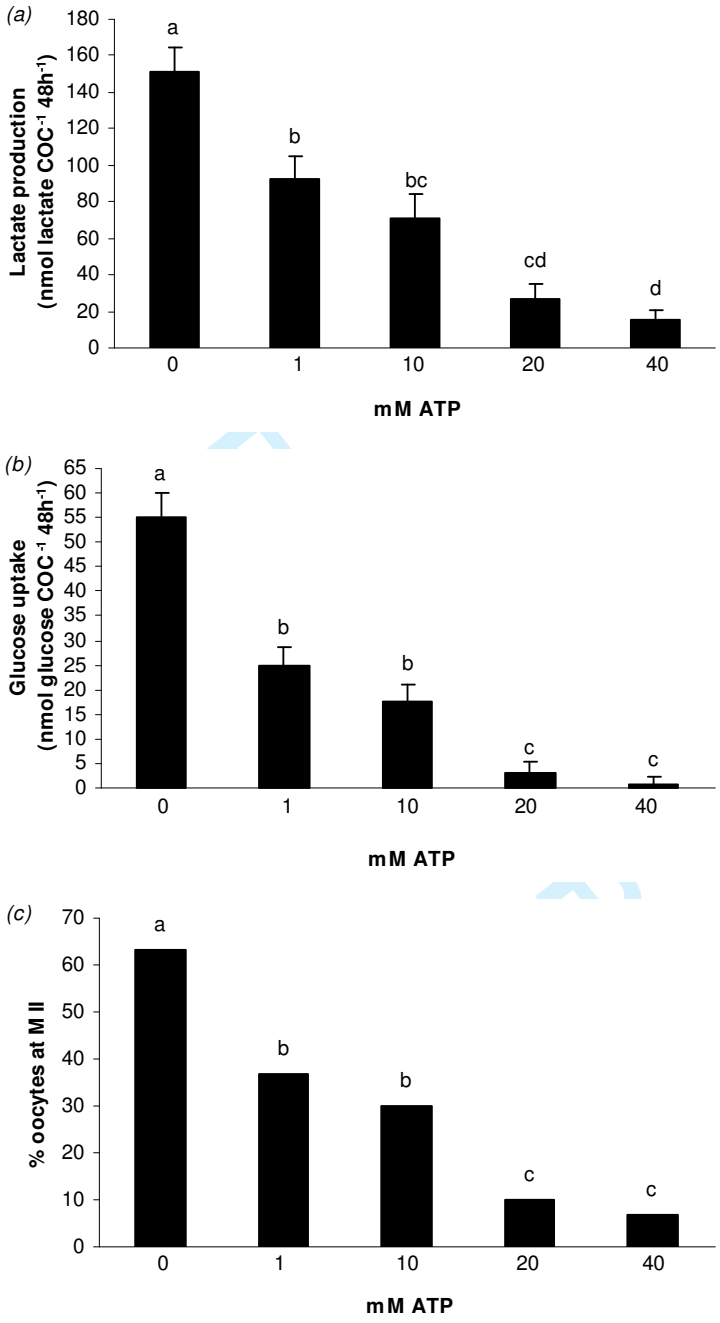

(a)
Lactate production (nmol lactate COC-1 48h-1)
180
160
140
120
100
80
60
40
20
0
a
b
bc
cd
d
0
1
10
20
40
mM ATP
(b)
Glucose uptake (nmol glucose COC-1 48h-1)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
a
b
b
c
c
0
1
10
20
40
mM ATP
(c)
% oocytes at M II
70
60
50
40
30
20
10
0
a
b
b
c
c
0
1
10
20
40
mM ATP

Figure 3

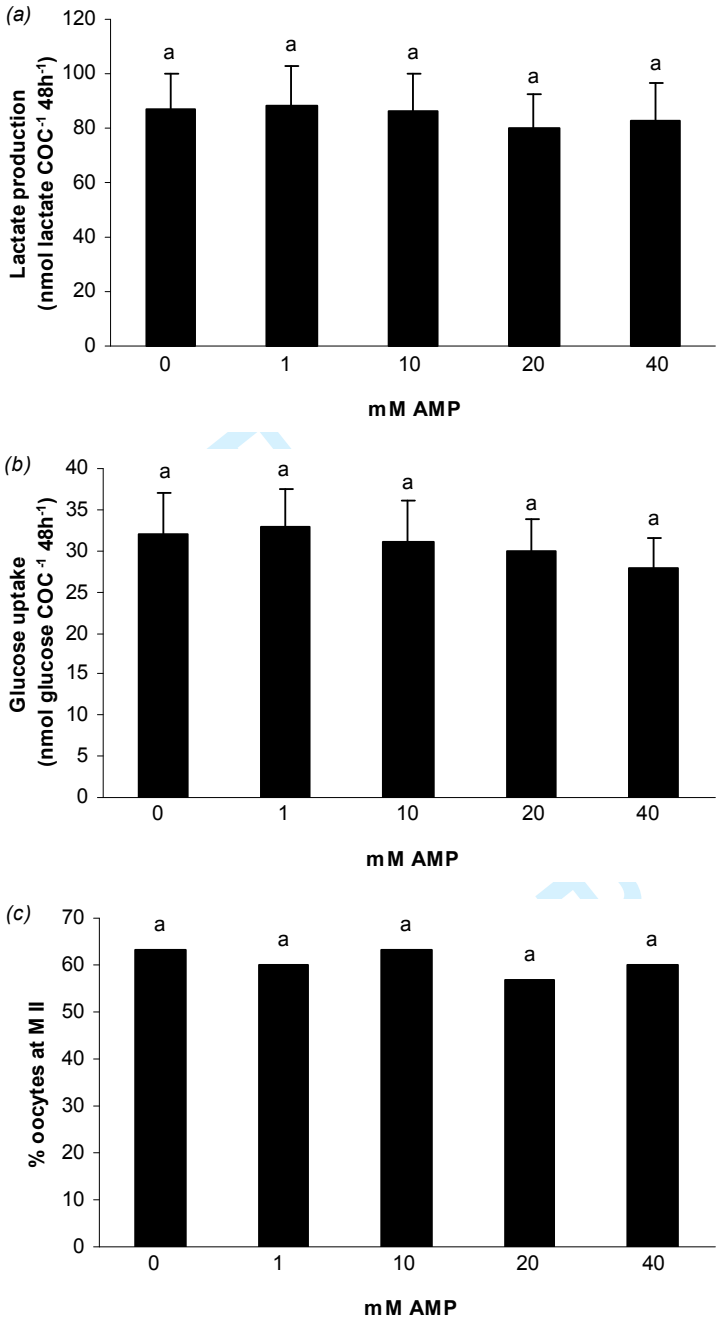
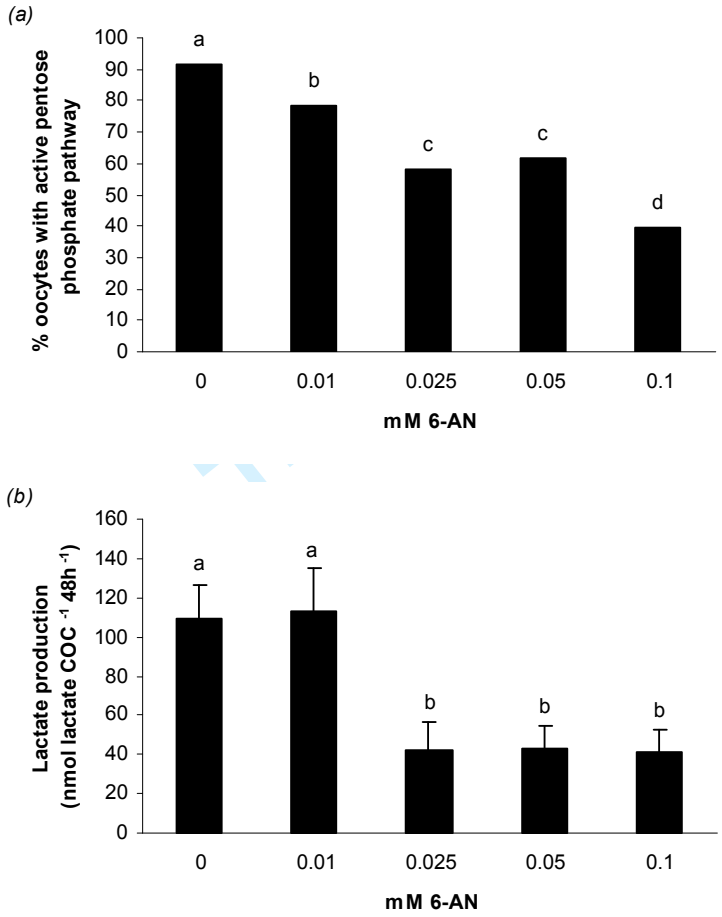
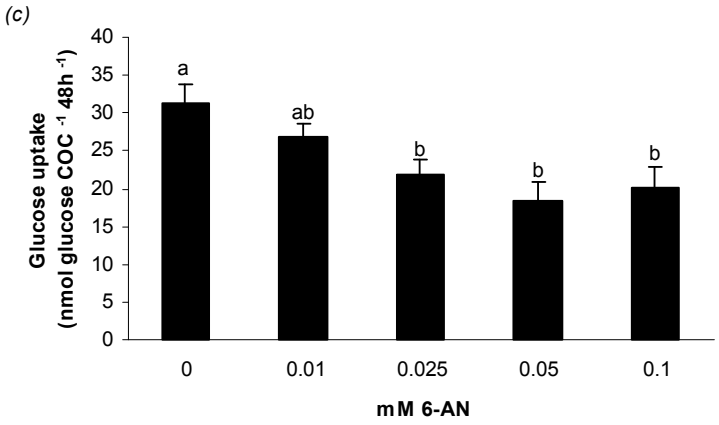

(a)
Lactate production (nmol lactate COC-1 48h-1)
120
100
80
60
40
20
0
a
a
a
a
a
0
1
10
20
40
mM AMP
(b)
Glucose uptake (nmol glucose COC-1 48h-1)
40
35
30
25
20
15
10
5
0
a
a
a
a
a
0
1
10
20
40
mM AMP
(c)
% oocytes at M II
70
60
50
40
30
20
10
0
a
a
a
a
a
0
1
10
20
40
mM AMP

Figure 4


(a)
% oocytes with active pentose phosphate pathway
100
90
80
70
60
50
40
30
20
10
0
a
b
c
c
d
0
0.01
0.025
0.05
0.1
mM 6-AN
(b)
Lactate production (nmol lactate COC -1 48h -1)
160
140
120
100
80
60
40
20
0
a
a
b
b
b
0
0.01
0.025
0.05
0.1
mM 6-AN


(c)
Glucose uptake (nmol glucose COC -1 48h -1)
40
35
30
25
20
15
10
5
0
a
ab
b
b
b
0
0.01
0.025
0.05
0.1
mM 6-AN

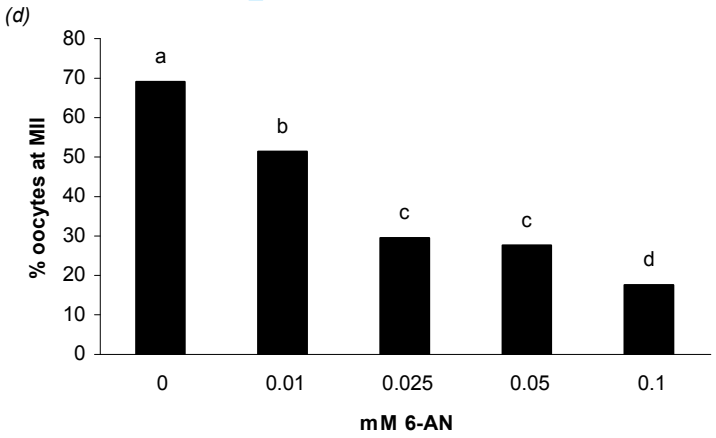
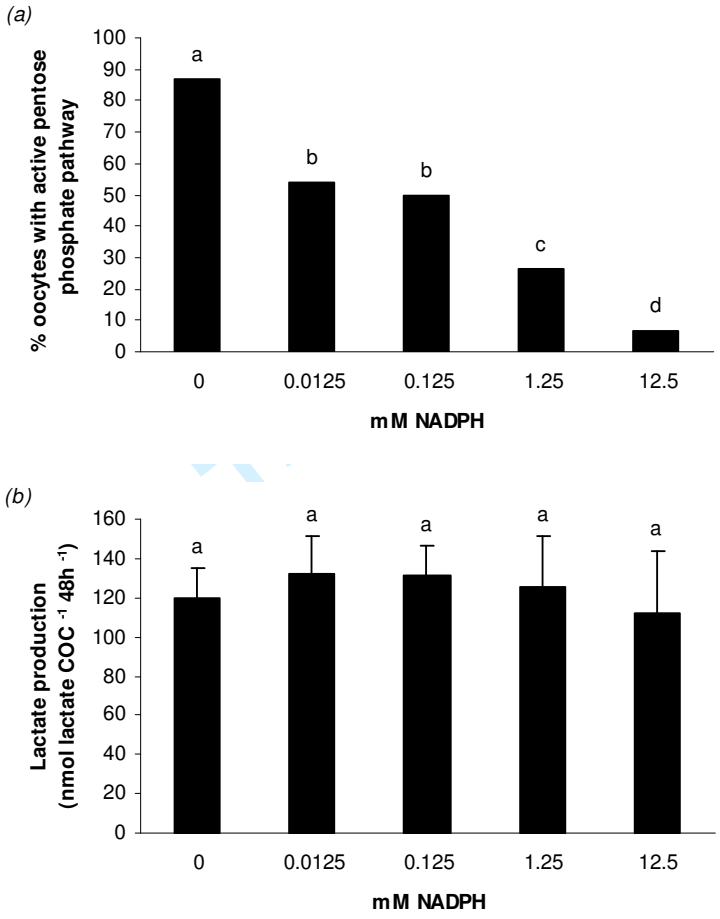
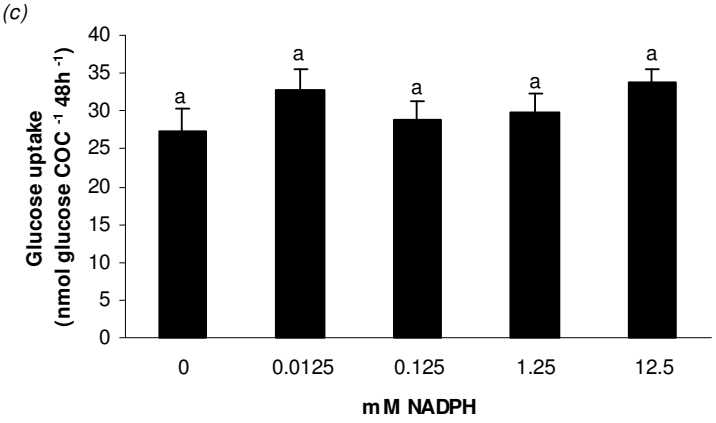

(d)
% oocytes at MII
80
70
60
50
40
30
20
10
0
a
b
c
c
d
0
0.01
0.025
0.05
0.1
mM 6-AN

Figure 5


(a)
% oocytes with active pentose phosphate pathway
100
90
80
70
60
50
40
30
20
10
0
a
b
b
c
d
0
0.0125
0.125
1.25
12.5
mM NADPH
(b)
Lactate production (nmol lactate COC -1 48h -1)
160
140
120
100
80
60
40
20
0
a
a
a
a
a
0
0.0125
0.125
1.25
12.5
mM NADPH

(c)


Glucose uptake
(nmol glucose COC -1 48h -1)
40
35
30
25
20
15
10
5
0
a
a
a
a
a
0
0.0125
0.125
1.25
12.5
mM NADPH

(d)

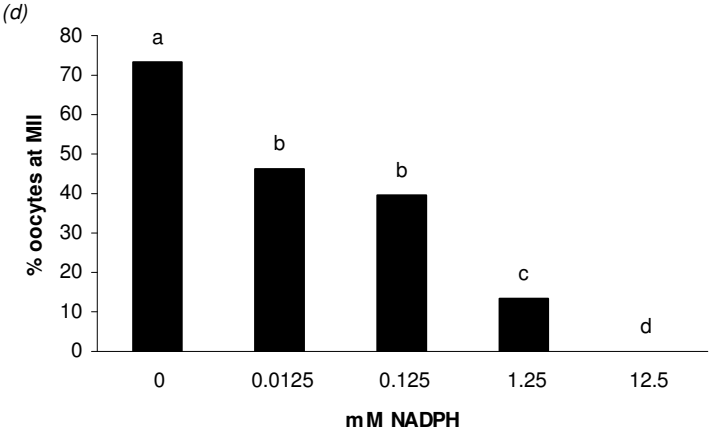
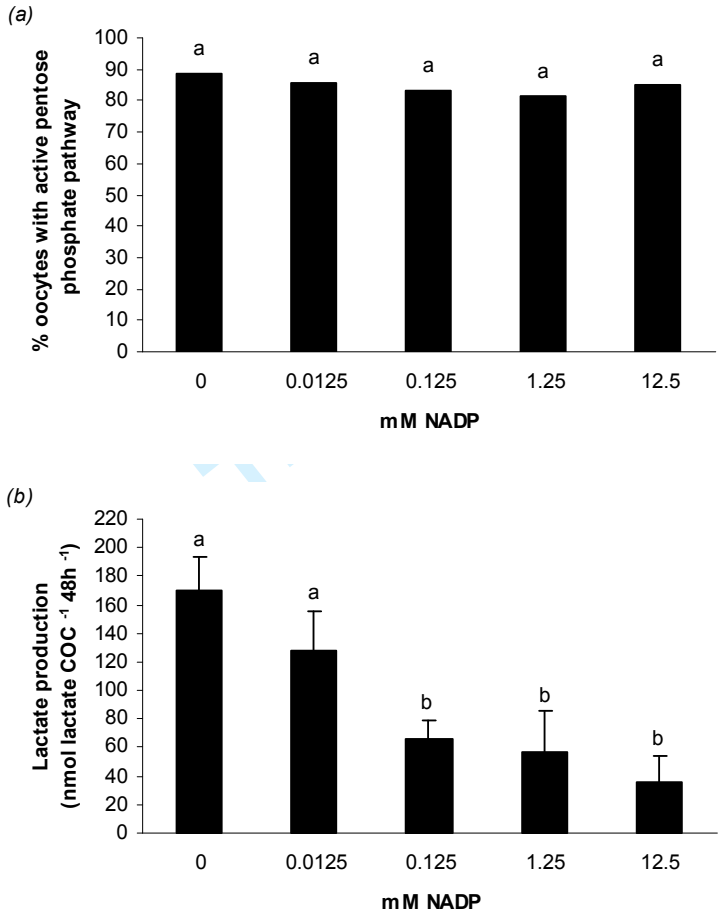
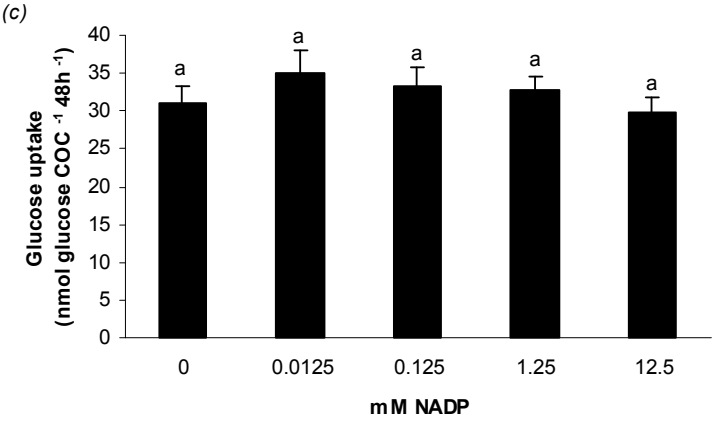

% oocytes at MII
80
70
60
50
40
30
20
10
0
a
b
b
c
d
0
0.0125
0.125
1.25
12.5
mM NADPH

Figure 6


(a)
% oocytes with active pentose phosphate pathway
100
90
80
70
60
50
40
30
20
10
0
a
a
a
a
a
0
0.0125
0.125
1.25
12.5
mM NADP
(b)
Lactate production (nmol lactate COC -1 48h -1)
220
200
180
160
140
120
100
80
60
40
20
0
a
a
b
b
b
0
0.0125
0.125
1.25
12.5
mM NADP

(c)


Glucose uptake
(nmol glucose COC -1 48h -1)
40
35
30
25
20
15
10
5
0
a
a
a
a
a
0
0.0125
0.125
1.25
12.5
mM NADP

(d)

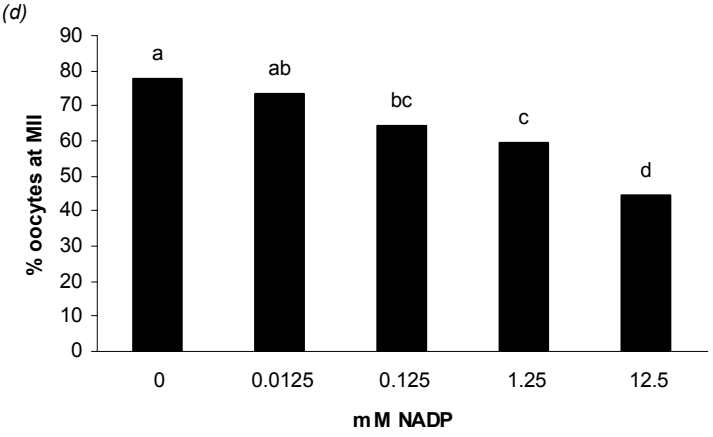

% oocytes at MII
90
80
70
60
50
40
30
20
10
0
a
ab
bc
c
d
0
0.0125
0.125
1.25
12.5
mM NADP

Table 1. Effect of modulators on COC vitality

	NaF				
	0 mM	**2.5 mM**	**5 mM**	**7.5 mM**	**10 mM**
% live oocytes	100 [a]	93.3 [a]	96.7 [a]	100 [a]	100 [a]
% live cumulus	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]
	ATP				
	0 mM	**1 mM**	**10 mM**	**20 mM**	**40 mM**
% live oocytes	96.7 [a]	100 [a]	93.3 [a]	100 [a]	96.7 [a]
% live cumulus	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]
	AMP				
	0 mM	**1 mM**	**10 mM**	**20 mM**	**40 mM**
% live oocytes	96.7 [a]	93.3 [a]	100 [a]	96.7 [a]	100 [a]
% live cumulus	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]
	6-AN				
	0 mM	**0.01 mM**	**0.025 mM**	**0.05 mM**	**0.1 mM**
% live oocytes	96.7 [a] [a]	100 [a]	100	100 [a]	93.3 [a]
% live cumulus	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]
	NADPH				
	0 mM	**0.0125 mM**	**0.125 mM**	**1.25 mM**	**12.5 mM**
% live oocytes	100 [a]	100 [a]	100 [a]	96.7 [a]	96.7 [a]
% live cumulus	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]
	NADP				
	0 mM	**0.0125 mM**	**0.125 mM**	**1.25 mM**	**12.5 mM**
% live oocytes	93.3 [a]	93.3 [a]	100 [a]	100 [a]	100 [a]
% live cumulus	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]

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