Reproduction in Domestic Animals

Reprod Dom Anim **48**, 545–553 (2013); doi: 10.1111/rda.12123
ISSN 0936–6768

Modulation of Glycolysis and the Pentose Phosphate Pathway Influences Porcine Oocyte *In Vitro* Maturation

GM Alvarez, EL Ferretti, C Gutnisky, GC Dalvit and PD Cetica

INITRA (Institute of Research and Technology in Animal Reproduction), Area of Biochemistry, School of Veterinary Sciences, University of Buenos Aires, Buenos Aires, Argentina

Contents
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 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 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.

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 glycolysis as well as of enzymatic inhibitors (6-AN, NADPH) and a stimulator (NADP) of PPP in IVM medium on the glycolytic activity (evaluated by lactate production) and PPP activity [evaluated by brilliant cresyl blue (BCB) test] in porcine COCs, glucose uptake per COC and oocyte maturation were analysed.

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 (28–33°C) for the 2- to 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. cumulus–oocyte complexes were aspirated from 3- to 8-mm antral follicles 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

Cumulus–oocyte complexes 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, ON, Canada) plus 0.5 mg/l porcine luteinising hormone (LH) (Lutropin-V, Bioniche) (control medium) under mineral oil at 39°C 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, 5, 7.5 and 10 mM NaF (glycolytic pharmacological inhibitor); 1, 10, 20 and 40 mM ATP (glycolytic physiological inhibitor); 1, 10, 20 and 40 mM AMP (glycolytic physiological stimulator); 0.01, 0.025, 0.05 and 0.1 mM 6-AN (PPP pharmacological inhibitor); 0.0125, 0.125, 1.25 and 12.5 mM NADPH (PPP physiological inhibitor); 0.0125, 0.125, 1.25 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 37°C, placed in a hypotonic medium of 10 g/l sodium citrate at 37°C 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 ×100 and ×400 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 37°C in PBS added with 2.5 μg/l fluorescein diacetate fluorochrome. Then, COCs were washed in PBS before being observed in an epifluorescence microscope using a 510 nm filter at × 100 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. Cumulus–oocyte complexes 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.

Cumulus–oocyte complexes 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 BCB test for immature oocytes was performed (Wongsrikeao et al. 2006b) with some modifications to be adapted to the porcine oocyte IVM. Cumulus–oocyte complexes were individually matured in 20-μl droplets of culture medium for 45 h and then transferred for the last 3 h 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.

Cumulus–oocyte complexes removed from the droplets were processed as previously described to evaluate oocyte meiotic maturation.

Statistical analysis and experimental design

Nonparametric 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 ($p < 0.05$, Fig. 1a). Glucose uptake per COC showed a similar behaviour in the presence of this compound in IVM medium ($p < 0.05$, Fig. 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 onwards ($p < 0.05$, Fig. 1c). However, cumulus cells and oocyte viability were not affected at any of the concentrations of NaF evaluated (Table 1).

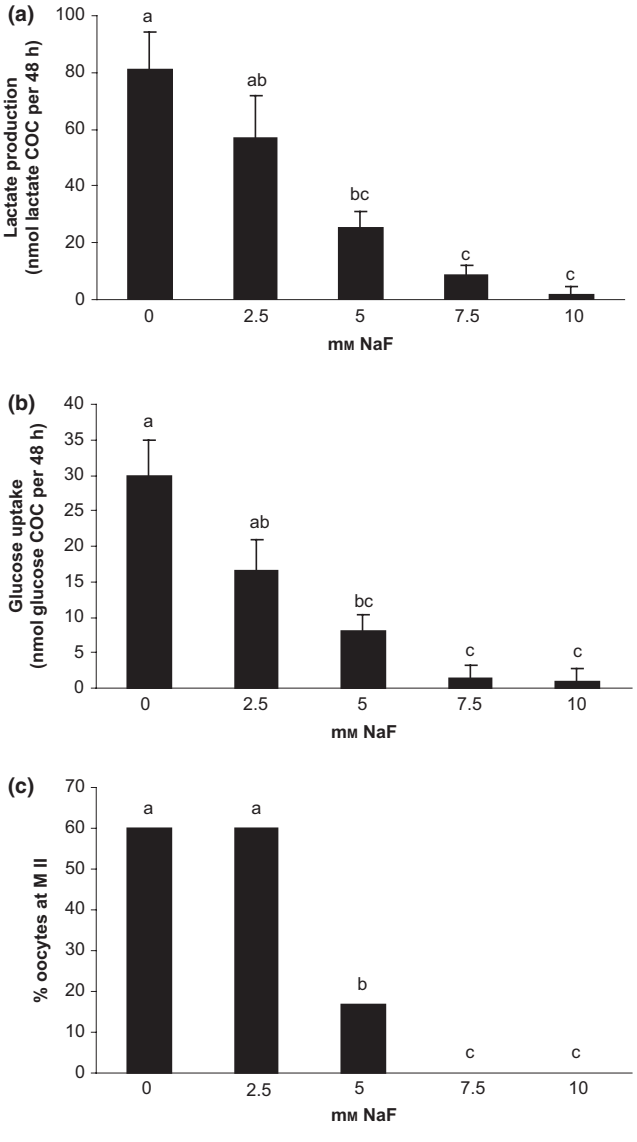

Fig. 1. (a) Lactate production by cumulus–oocyte complex (COC) during maturation with different concentrations of NaF. [(a,b,c)]Different superscripts over bars indicate significant differences ($p < 0.05$). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± 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

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 ($p < 0.05$, Fig. 2a,b). 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 (Fig. 2c). However, neither cumulus cells nor oocyte viability was affected at any of the concentrations of ATP used (Table 1).

Table 1. Percentage of live oocytes and live cumulus in cumulus–oocyte complex (COC) matured in the presence of different modulators

	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]	100[a]	100[a]	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]

n = 30 COCs for each value. Experiments were repeated three times.
[a]The same superscript indicates no significant difference within line.

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 (Fig. 3a,b). 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 (Fig. 3c). Neither cumulus cells nor oocyte viability was 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 ($p < 0.05$, Fig. 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 ($p < 0.05$; Fig. 4b,c). A good positive correlation between glucose

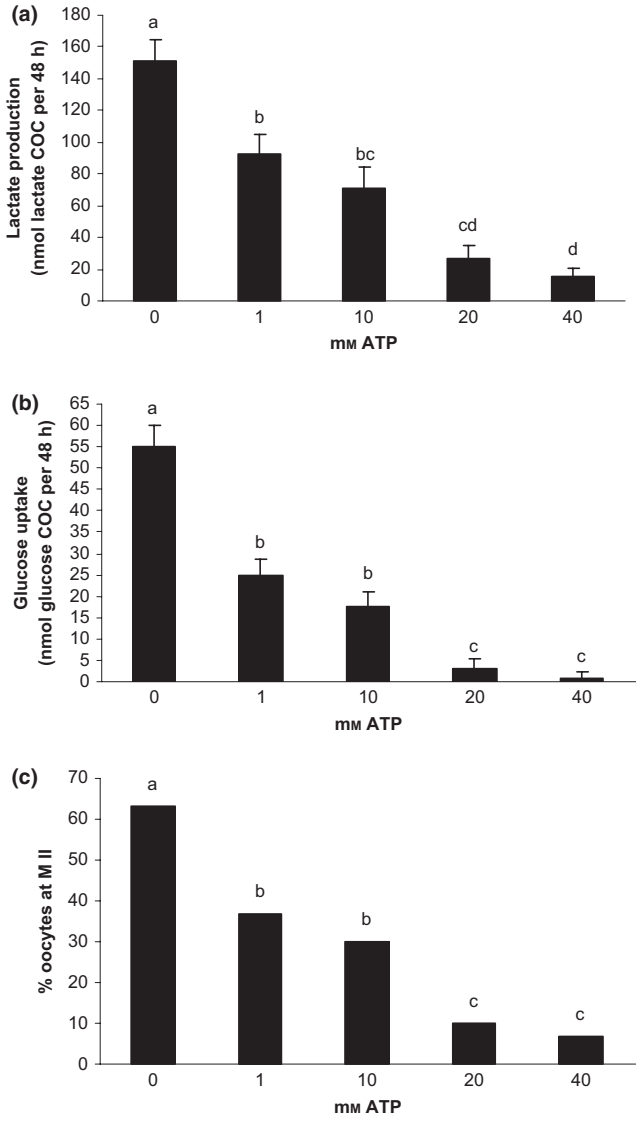

Fig. 2. (a) Lactate production by cumulus–oocyte complex (COC) during maturation with different concentrations of ATP. [a,b,c,d]Different superscripts over bars indicate significant differences ($p < 0.05$). n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± 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

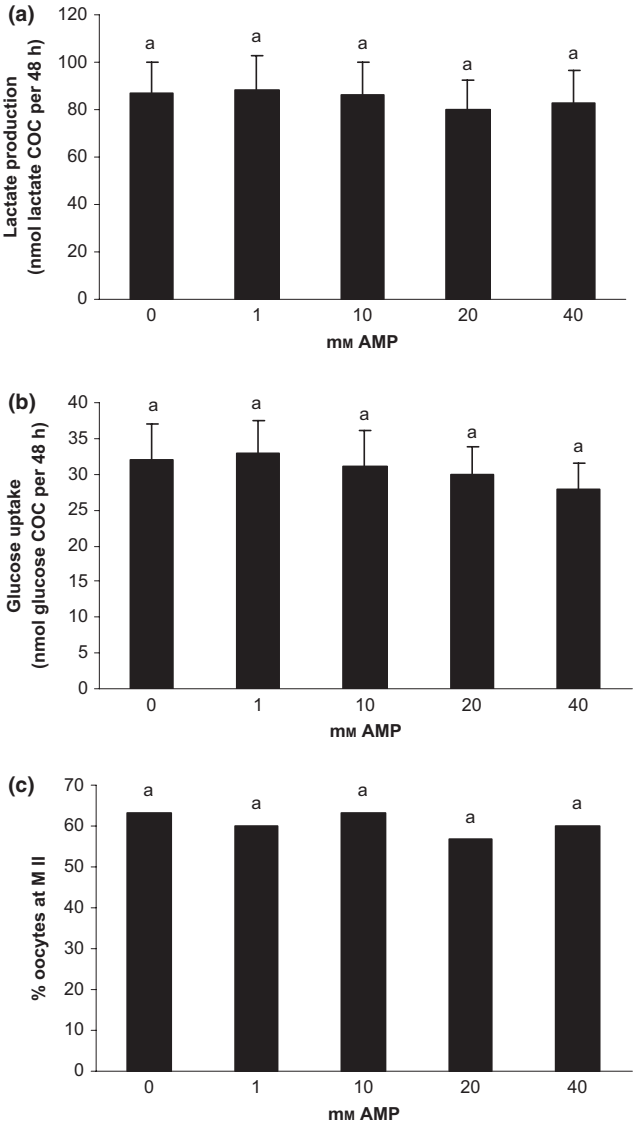

Fig. 3. (a) Lactate production by cumulus–oocyte complex (COC) during maturation with different concentrations of AMP. [a]The same superscript over bars indicates no significant difference. n = 30 COCs for each bar. Experiments were repeated three times. Data are presented as mean ± SEM. (b) Glucose 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

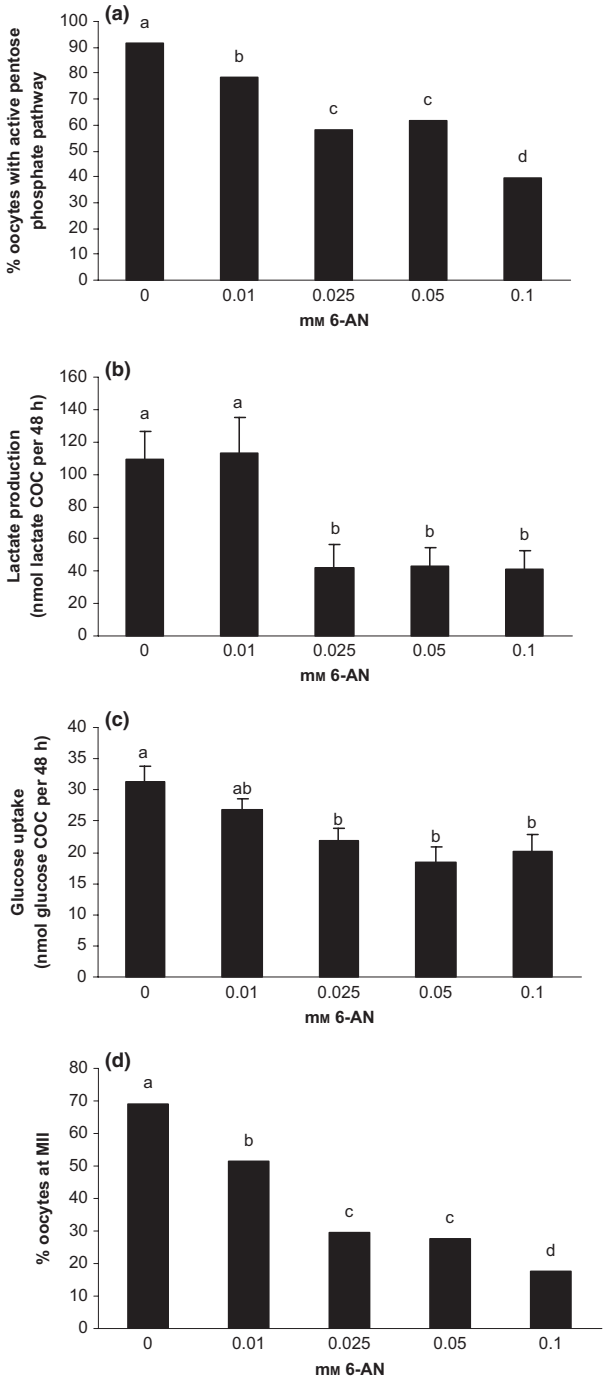

Fig. 4. (a) Percentage of oocytes with active pentose phosphate pathway, evaluated by brilliant cresyl blue test, after maturation with different concentrations of 6-AN. [(a,b,c,d)]Different superscripts over bars indicate significant differences ($p < 0.05$). n = 28–30 oocytes for each bar. Experiments 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

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 (Fig. 4d). However, neither cumulus cells nor oocyte viability was 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 and the meiotic maturation rate in the presence of the physiological inhibitor of the pathway ($p < 0.05$, Fig. 5a,d).Furthermore, lactate production and glucose uptake per COC remained constant in the presence of the different concentrations of NADPH (Fig. 5b,c). A high positive correlation between glucose uptake and lactate production was recorded ($r = 0.72$; $p = 0.0000$). Neither cumulus cells nor oocyte viability was 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 (Fig. 6a). Lactate production per COC decreased to a plateau from 0.125 to 12.5 mM NADP ($p < 0.05$, Fig. 6b), although glucose uptake was not modified with respect to control (Fig. 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 ($p < 0.05$, Fig. 6d). However, neither cumulus cells nor oocyte viability was 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

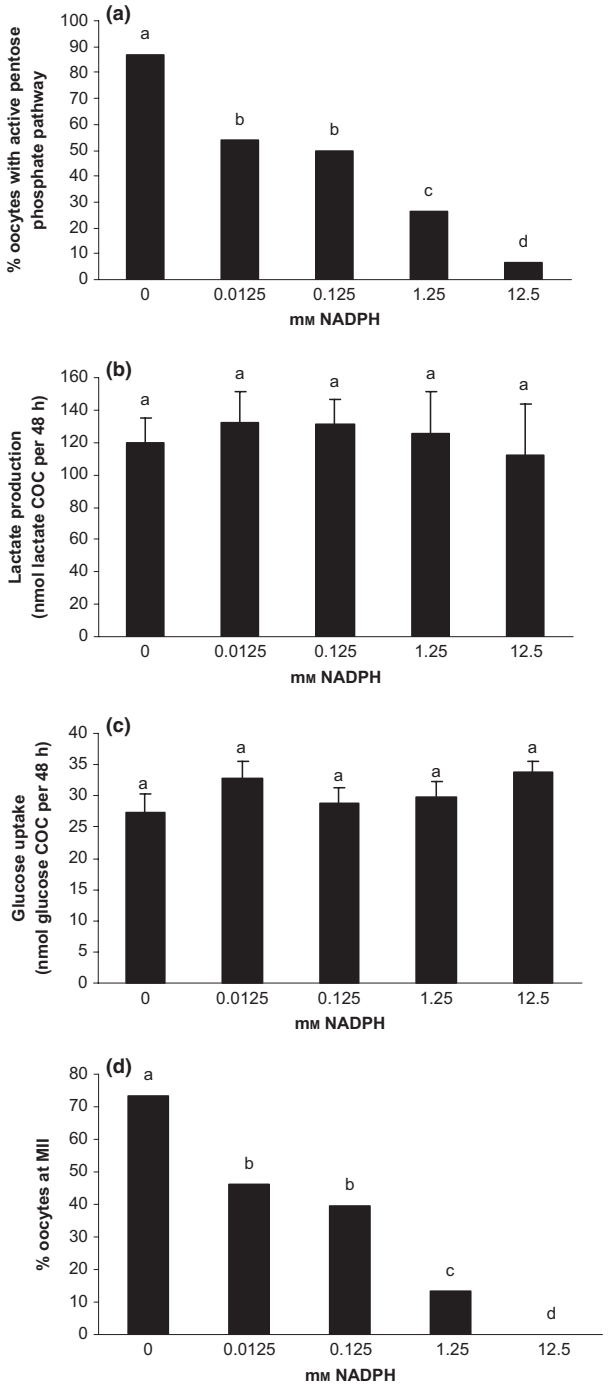

Fig. 5. (a) Percentage of oocytes with active pentose phosphate pathway, evaluated by brilliant cresyl blue test, after maturation with different concentrations of NADPH. [(a,b,c,d)]Different superscripts over bars indicate significant differences ($p < 0.05$). n = 29–30 oocytes for each bar. Experiments 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

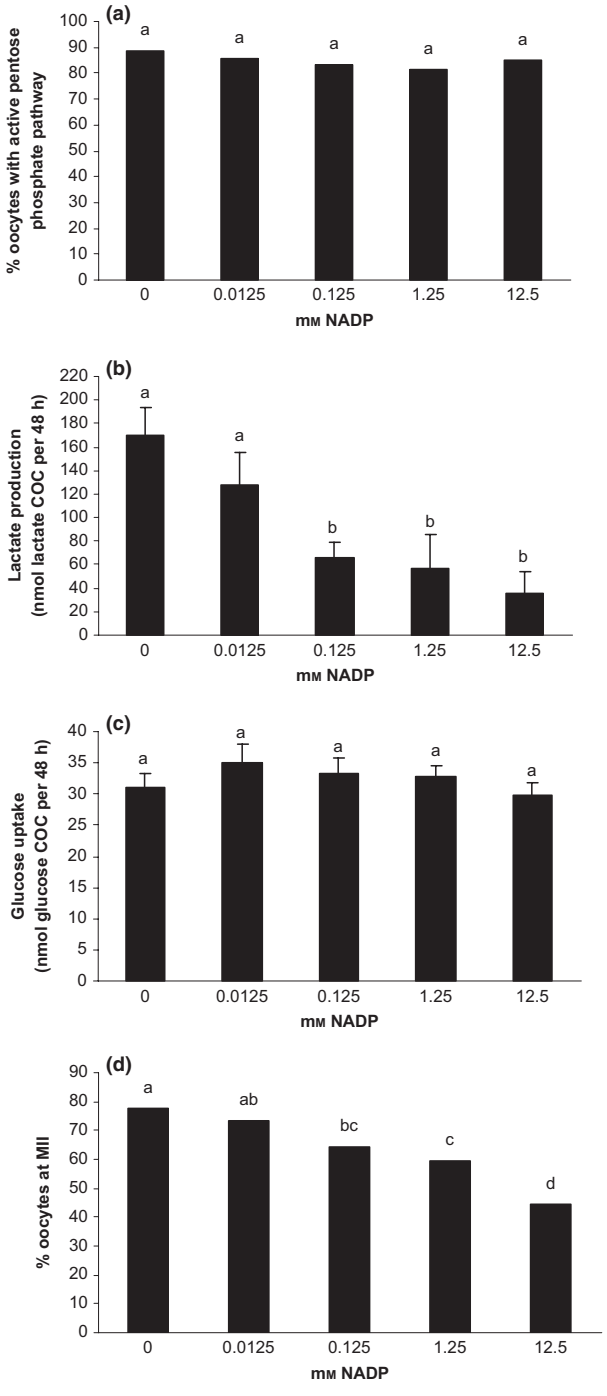

Fig. 6. (a) Percentage of oocytes with active pentose phosphate pathway, evaluated by brilliant cresyl blue test, after maturation with different concentrations of NADP. [(a)]The same superscript over bars indicates no significant difference. n = 28–30 oocytes for each bar. Experiments were repeated 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

concentrations of this inhibitor, demonstrating that glycolysis is the main fate of glucose consumed by 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. 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).

The effect of the modulation of intracellular metabolism in cumulus cells and oocytes by the addition of different nucleotides in the *in vitro* maturation media was previously reported in murine and bovine species (Chen et al. 1990; Cetica et al. 1999, 2003; Gutnisky et al. 2007). 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 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 of 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.

Pentose phosphate pathway 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 was 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 catabolized 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.

Acknowledgements

The authors thank the Japanese International Cooperation Agency (JICA) for technology transfer and equipment, the Porkind abattoir for ovaries, Astra Laboratories for ultra-pure water and ETC Internacional S.A. for donation of cell culture products and V.H. Chávez for his technical assistance.

Funding

This work was funded by the University of Buenos Aires, Argentina (grant number UBACyT V007).

Conflicts of interest

None of the authors have any conflict of interest to declare.

Author contributions

Gabriel Martín Alvarez performed the experiences to evaluate glycolysis and drafted the manuscript. Eugenia Lorena Ferretti performed the experiences to evaluate pentose phosphate pathway. Cynthia Gutnisky contributed to perform the experiences to evaluate pentose phosphate pathway and to the analysis and interpretation of data. Gabriel Carlos Dalvit contributed to the analysis and interpretation of data. Pablo Daniel Cetica designed the experiences and drafted the article.

References

Abeydeera LR, Wang WH, Prather RS, Day BN, 2001: Effect of incubation temperature on in vitro maturation of porcine oocytes: nuclear maturation, fertilisation and developmental competence. Zygote **9**, 331–337.

Alvarez GM, Dalvit GC, Achi MV, Miguez MS, Cetica PD, 2009: Immature oocyte quality and maturational competence of porcine cumulus-oocyte complexes subpopulations. Biocell **33**, 167–177.

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. Reprod Domest Anim **47**, 856–864.

Anderson DM, 1969: In vitro inhibition of glycolysis in blood and its effect on the haematocrit. J Comp Pathol **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 MA, Sirard MA, 1993: Effect of adenylate cyclase stimulation on meiotic resumption and cyclic AMP content of zona-free and cumulus-enclosed bovine oocytes in vitro. J Reprod Fertil **97**, 5–11.

Cetica PD, Pintos LN, Dalvit GC, Beconi MT, 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.

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 **124**, 675–681.

Cetica P, Pintos L, Dalvit G, Beconi M, 2003: Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation in vitro. Reproduction **126**, 753–763.

Chen L, Wert SE, Hendrix EM, Russell PT, Cannon M, Larsen WJ, 1990: Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. Mol Reprod Dev **26**, 236–247.

Clarenburg R, 1992: Carbohydrate metabolism. In: Reinhardt R (ed.), Physiological Chemistry of Domestic Animals. Mosby Year Book Inc, St Louis, MO, pp. 239–291.

Downs SM, 1995: The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte. Dev Biol **167**, 502–512.

Downs SM, Utecht AM, 1999: Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes. Biol Reprod **60**, 1446–1452.

Downs SM, Humpherson PG, Leese HJ, 1998: Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway. Biol Reprod **58**, 1084–1094.

Feig SA, Shohet SB, Nathan DG, 1971: Energy metabolism in human erythrocytes I. Effects of sodium fluoride. J Clin Investig **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 GC, Pintos LN, Thompson JG, Beconi MT, Cetica PD, 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 RA, 2002: Carbohydrate metabolism I: Major metabolic pathways and their control. In: Devlin TM (ed.), Textbook of Biochemistry. Wiley-Liss, New York, pp. 597–664.
Herrick JR, Brad AM, Krisher RL, 2006: Chemical manipulation of glucose metabolism in porcine oocytes: effects on nuclear and cytoplasmic maturation in vitro. Reproduction **131**, 289–298.
Hothersall JS, Zubairu S, McLean P, Greenbaum AL, 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. J Neurochem **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 **133**, 29–40.
Khurana NK, 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 RL, Brad AM, Herrick JR, Sparman ML, Swain JE, 2007: A comparative analysis of metabolism and viability in porcine oocytes during in vitro maturation. Anim Reprod Sci **98**, 72–96.
Lim JM, Lee BC, Lee ES, Chung HM, Ko JJ, Park SE, Cha KY, Hwang WS, 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 PA, 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, DF, pp. 153–161.
Nelson D, Cox M 2005: Glycolysis, gluconeogenesis, and the pentose phosphate pathway. In: Nelson D, Cox M (eds), Lehninger. Principles of Biochemistry. W. H. Freeman and Company, New York, pp. 521-559.
Preis KA, Seidel G Jr, Gardner DK, 2005: Metabolic markers of developmental competence for in vitro-matured mouse oocytes. Reproduction **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. Anim Reprod Sci **99**, 299–305.
Schirmer T, Evans PR, 1990: Structural basis of the allosteric behaviour of phosphofructokinase. Nature **343**, 140–145.
Shayiq RM, Kidwai AM, 1986: Effect of fluoride on uptake of D-glucose by isolated epithelial cells of rat intestine. Environ Res **41**, 388–399.
Simpfendorfer RW, Oelckers KB, Lopez DA, 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 MA, 1990: Temporary inhibition of meiosis resumption in vitro by adenylate cyclase stimulation in immature bovine oocytes. Theriogenology **33**, 757–767.
Sutton ML, Cetica PD, Beconi MT, Kind KL, Gilchrist RB, Thompson JG, 2003a: Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. Reproduction **126**, 27–34.
Sutton ML, Gilchrist RB, Thompson JG, 2003b: Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. Hum Reprod Update **9**, 35–48.
Thompson JG, 2006: The impact of nutrition of the cumulus oocyte complex and embryo on subsequent development in ruminants. J Reprod Dev **52**, 169–175.
Tyson RL, Perron J, Sutherland GR, 2000: 6-Aminonicotinamide inhibition of the pentose phosphate pathway in rat neocortex. NeuroReport **11**, 1845–1848.
Wongsrikeao P, Otoi T, Taniguchi M, Karja NW, 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.

Submitted: 8 May 2012; Accepted: 22 Oct 2012

Author's address (for correspondence): Gabriel Martín Alvarez, Chorroarín 280, C1427CWO, Buenos Aires, Argentina.
E-mail: galvarez@fvet.uba.ar