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Regulation of key enzymes of glucose metabolism in bovine COCs

Cynthia Gutnisky[1,2,3], Elizabeth Breininger[1,2], Gabriel Carlos Dalvit[1], Pablo Daniel Cetica[1,2]

[1]Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal (INITRA), Buenos Aires, Argentina.

[2]Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Investigaciones en Producción Animal (INPA), Facultad de Ciencias Veterinarias, Buenos Aires, Argentina.

Abstract

The aim of this work was to study the regulation of PFK 1 and G6PDH, two key enzymes involved in glucose metabolism in cumulus oocyte-complexes (COCs), and its relationship with the oocyte maturation process. It was observed that the activity of PFK 1 in the presence of ATP was inhibited whereas the addition of AMP increased the activity ($P < 0.05$). To verify the effect of the physiological modulators on the COC glycolytic pathway, the lactate production during IVM and the maturation rate were evaluated. In accordance with the enzymatic activity, the glycolytic activity evaluated by lactate production and the maturation rate diminished ($P < 0.05$) with the addition of ATP. While the AMP had a dose response effect on the lactate production, the maturation rate remained unaltered. It was observed that NADPH inhibited the activity of the G6PDH and the addition of NADP increased the activity of the enzyme ($P < 0.05$). To verify the effect of the physiological modulators on the COC pentose phosphate pathway (PPP), the proportion of colourless oocytes evaluated by brilliant cresyl blue (BCB) and the maturation rate were carried out. In presence of NADPH an inhibition ($P < 0.05$) on PPP and maturation rate was observed. On the other hand, NADP had no effect on PPP activity and maturation rate. The present study shows that the regulation of key enzymes of glucose metabolism in bovine COCs are regulated mainly by the energetic charge and the redox status. We also reported a tight relation between the activity of the PFK 1 and G6PDH enzymes, glycolytic and PPP activities and the oocyte maturation process.

Keywords: glucose 6-phosphate dehydrogenase, glycolysis, oocyte, pentose phosphate pathway, phosphofructokinase.

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 the germinal vesicle breakdown (GVBD), also involves cumulus expansion and cytoplasmic maturation. The glucose metabolism is crucial because progression through this dynamic process requires energy and substrates that arise from carbohydrates, aminoacids and lipids. In cattle oocytes, an adequate concentration of glucose in the maturation media is necessary for the maturation process and also for subsequent embryo development (Rose-Hellekant *et al*., 1998; Lim *et al*., 1999; Khurana and Niemann, 2000). Indeed, manipulation of glucose concentration during maturation can affect the kinetics of bovine oocyte meiosis (Sutton-McDowall *et al*., 2005).

Cumulus oocyte-complexes (COCs) metabolize glucose via glycolysis, pentose phosphate pathway (PPP) and the hexosamine biosynthesis pathway (Downs and Utecht, 1999; Sutton *et al*., 2003; Gutnisky *et al*., 2007), as well as the polyol pathway (Sutton-McDowall *et al*., 2010). Cumulus cells have a great glycolytic capacity, and this is why glycolysis represents the predominant pathway in COCs. It has been suggested that in 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 capacity to undertake glucose metabolism (Zuelke and Brackett, 1992; Sutton *et al*., 2003; Dumollard *et al*., 2007).

In somatic cells, the major regulatory point of the glycolytic pathway is the enzyme phosphofructokinase 1 (PFK1; E.C.2.7.1.11), with AMP and ATP having important positive and negative allosteric regulating roles, respectively (Schirmer and Evans, 1990; Clarenburg, 1992). The regulatory mechanism in COCs is, however, still lacking.

It has been proposed a close relationship between PPP activity and the maturation process (Herrick *et al*., 2006) and it has been suggested that this pathway is a key factor in the progression of nuclear maturation (Downs *et al*., 1998; Sato *et al*., 2007). Glucose flux throughout PPP influences the resumption of oocyte nuclear maturation in mouse COC (Downs *et al*., 1998; Sato *et al*., 2007). It has also been proposed that PPP is involved in the progression of all stages of meiosis, including the resumption of meiosis, MI-MII transition and the resumption of meiosis post-fertilization (Sutton-McDowall *et al*., 2005; Herrick *et al*., 2006). The PPP has two main metabolic goals: produce NADPH for reductive synthesis and/or yield ribose 5-phosphate as a nucleotide precursor. The NADPH produced by the PPP is also important in preventing oxidative stress throughout the glutathione and the thioredoxin systems, and thus regulating the redox intracellular state (Tian *et al*., 1998).

In somatic cells, the major regulatory point of the PPP is at the glucose 6-phosphate dehydrogenase

[3]Corresponding author: cgutnisky@fvet.uba.ar

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(G6PDH, E.C. 1.1.1.49), with NADP/NADPH ratio having an important regulatory role (Stanton, 2012), and it was also proposed that G6PDH is competitively inhibited by NADPH (Ozer *et al*., 2002). However, there is no information of the regulation of this enzyme in COCs.

In a previous work it was determined that the enzymatic activity of the two enzymes (PFK1 and G6PDH) in COCs did not vary during maturation (Cetica *et al*., 2002). PFK1 activity in cumulus cells was 102 times higher than in denuded oocytes, while the G6PDH activity in cumulus cell was 14 times higher than in denuded oocytes (Cetica *et al*., 2002). Thus, the aim of this work was to evaluate the regulation of these two key enzymes involved in glucose metabolism of COCs, and it relationship with the oocyte maturation process.

Materials and Methods

Materials

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

Recovery of cumulus-oocyte complexes

Bovine ovaries were collected at an abattoir within 30 min of slaughter and kept warm (30ºC) during the 2 h journey to the laboratory. Ovaries were washed with physiological saline containing 100000 IU L^{-1} penicillin and 100 mg L^{-1} streptomycin. 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.

Preparation of enzymatic extract

Pools containing 50 immature COCs were suspended in distilled water, frozen (-20°C) - thawed twice (room temperature), centrifuged at 10000 g for 20 min at 4°C. The enzyme activities were determined in the supernatants. Extracts were prepared so that the final amount of each enzyme ensured linear behavior during activity measurement, thus enabling absorbance per min variation to be calculated.

Determination of phosphofructokinase 1 activity

PFK 1 activity was determined in the supernatants of the enzymatic extracts made of pools containing 50 immature COCs (n = 5). Activity was measured in a spectrophotometer at 340 nm for 9 min at 37°C in 95 mmol Tris-HCl buffer, pH 8.2, 10 mM $MgCl_2$, 0.4 mM NADH, 10 mM NH_4Cl, 4 mM fructose 6-phosphate, in the presence of (1) 1.4 U/ml aldolase, (2) 40 U/ml phosphotriose isomerase or (3) 5 U/ml glycerol 3-phosphate dehydrogenase (Kotlarz and Buc, 1982). In order to evaluate the effect of the physiological modulators of the enzyme, at 3 and 6 min of each determination ATP (final concentration of 2 and 10 mM) or AMP (1 and 10 mM), were added. An enzymatic unit of PFK 1 was defined as the quantity of enzyme that catalyses the formation of 1 µmol fructose 1,6- biphosphate min^{-1} = the oxidation of 2 µmol NADH min^{-1}.

Determination of glucose 6-phosphate dehydrogenase activity

G6PDH activity was determined in the supernatants of the enzymatic extracts made of pools containing 50 immature COCs (n = 5). Activity was measured in a spectrophotometer at 340 nm for 9 min at 37°C in 22.5 mM glycine buffer, pH 7.5, 12.5 mM $MgCl_2$ and 1mM glucose 6-phosphate (Kornberg and Horecker, 1955). In order to evaluate the effect of the physiological modulators of the enzyme, at 3 and 6 min of each determination NADPH (final concentration of 1.25 and 12.5 mM) or NADP (0.15 and 12.5 mM) were added. An enzymatic unit of G6PDH was defined as the quantity of enzyme that catalyses the reduction of 1 µmol NADP min–1.

G6PDH: Glucose 6-phosphate + NADP→ 6 - phosphogluconolactone + NADPH

In vitro *maturation of cumulus-oocyte complexes*

COCs were cultured in Medium 199 (Earle´s salts, L-glutamine, 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 porcine L^{-1} follicle-stimulating hormone (FSH; Folltropin-V; Bioniche, Belleville, Ontario, Canada), 2 mg L^{-1} porcine luteinizing hormone (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 effect of the different enzyme modulators of glycolysis on IVM, COCs were individually cultured in 20 µl drops of maturation media supplemented with increasing concentrations of ATP (0, 2 and 10 mM) or increasing concentrations of AMP (0, 1 and 10 mM). These concentrations were chosen for being the concentrations described previously for the determination of the enzymatic activity (Kotlarz and Buc, 1982; Cetica *et al*., 2002).

To study the effect of the addition of the PPP enzyme modulators on IVM, COCs were matured in media supplemented with increasing concentrations of NADPH (1.25 and 12.5 mM) or NADP (0.15 and 12.5 mM) under the conditions described above. These concentrations were chosen for being the physiological concentrations described previously for the determination of the enzymatic activity (Kornberg and Horecker, 1955; Cetica *et al*., 2002).

To discard the stimulatory effect of gonadotropins an additional group of COCs were matured with the addition of increasing concentrations of AMP or NADP without the supplementation of these hormones.

Oocytes were denuded mechanically by repeated pipetting in PBS with 1 g L^{-1} hyaluronidase type I-S. Denuded oocytes were placed in a hypotonic

medium of 2.9 mmol L^{-1} sodium citrate at 37C for 15 min, fixed on a slide with 3:1 ethanol: acetic acid (Tarkowski, 1966), stained with 5% (v/v) Giemsa in distilled water (Merck, Darmstadt, Germany) for 15 min and observed under a light microscope at magnifications of 100X and 400X (Fig. 1). Oocytes were considered mature when a metaphase II chromosome configuration was present (n = 30-40 oocytes per group, 4 replicates).

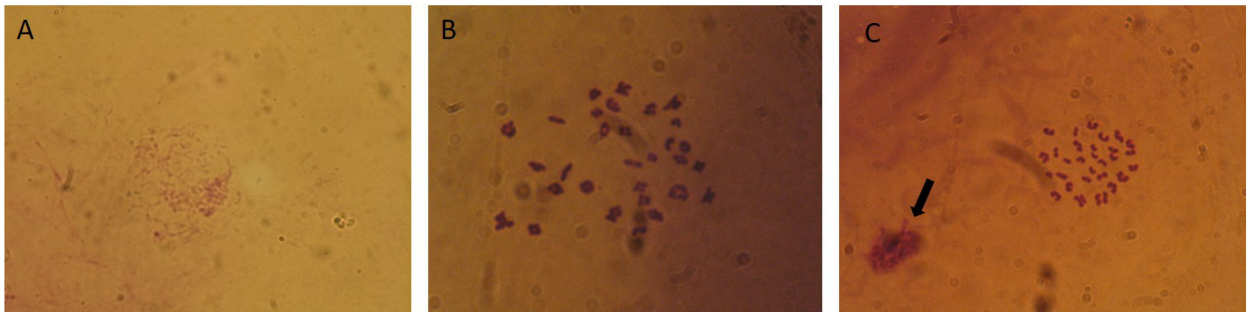

Figure 1. Different meiotic configurations with Giemsa stain, magnification 400X. A) Chromatin configuration of a germinal vesicle; B) Metaphase I and C) Metaphase II, the arrow shows the 1st polar body.

Determination of COC glycolytic activity by lactate production

After IVM period, COCs were removed from each 20 µl drop and lactate production was measured in the medium using a spectrophotometric assay based on the oxidation of lactate and subsequent revealed of hydrogen peroxide (Trinder, 1969; Barhan and Trinder, 1972).

Negative controls comprising 20 µl drops of maturation media were included in each experiment.

Determination of COC PPP activity by BCB stain

The Brilliant Cresyl Blue (BCB) stain test can be used to evaluate the proportion of COCs with measurable PPP activity. The BCB stain test is a non-destructive method that approximates G6PDH activity. COCs with high G6PDH activity convert BCB stain to a colorless compound. Conversely, COCs with low or no levels of G6PDH remain blue in color (Alm *et al*., 2005; Bhojwani *et al*., 2005, 2007).

For the determination of the proportion of COCs with active PPP, COCs were cultured in groups in the maturation media described above for 20.5 h and placed in the same media supplemented with 26 µm BCB for 1.5 h to complete maturation (Alm *et al*., 2005; Bhojwani *et al*., 2005, 2007). At the cessation of culture, COCs were washed twice in PBS and oocytes were denuded mechanically by repeated pipetting in PBS with 1 g hyaluronidase L^{-1} in order to evaluate the cytoplasm coloration. The evaluated oocytes were divided into two groups, coloured oocytes (low G6PDH activity) and colourless oocytes (high G6PDH activity). The proportion of COCs with active PPP was calculated as the relation between colourless oocytes and total oocytes per treatment.

Statistical analysis

The proportion of COCs with active PPP and oocyte meiotic maturation rates were compared by the homogeneity test using a chi-square analysis for non-parametric data. The results of lactate production and enzyme activity were expressed as the mean ± SEM. In the studies evaluating lactate production and enzyme activity the comparisons were made by analysis of variance (ANOVA) followed by Bonferroni post-test. In all tests, a significant difference was taken at $P < 0.05$.

Results

Effect of ATP and AMP on phosphofructokinase 1 activity and COC glycolytic activity

In order to confirm whether the physiological modulators act on the glycolytic enzyme PFK 1, the enzyme activity was determined in the presence of 2 concentrations of ATP or AMP. It was observed that the activity of PFK 1 in the presence of ATP 10 mM inhibited the activity of the enzyme a 55% comparing with the concentration of ATP 2 mM ($P < 0.05$; Fig. 2a) whereas the addition of AMP 10 mM increased the activity about three times respect to AMP 1 mM (Fig. 2b). In the absence of ATP or AMP the activity of the enzyme was not detected by the method used.

To verify the effect of the physiological modulators on the COC glycolytic pathway, the lactate production during IVM and the maturation rate were carried out. In accordance with the enzymatic activity, the glycolytic activity evaluated by lactate production and the maturation rate diminished with the addition of ATP 10 mM around 62 and 75%, respectively, with respect to ATP 2 mM (Fig. 2a), whereas the addition of AMP did not modify these variables. Because the gonadotropins may be masking the effects of AMP, the experiment was repeated without hormonal supplementation. In this group, AMP had a dose response effect on the lactate production without effect on the maturation rate (Fig. 2b).

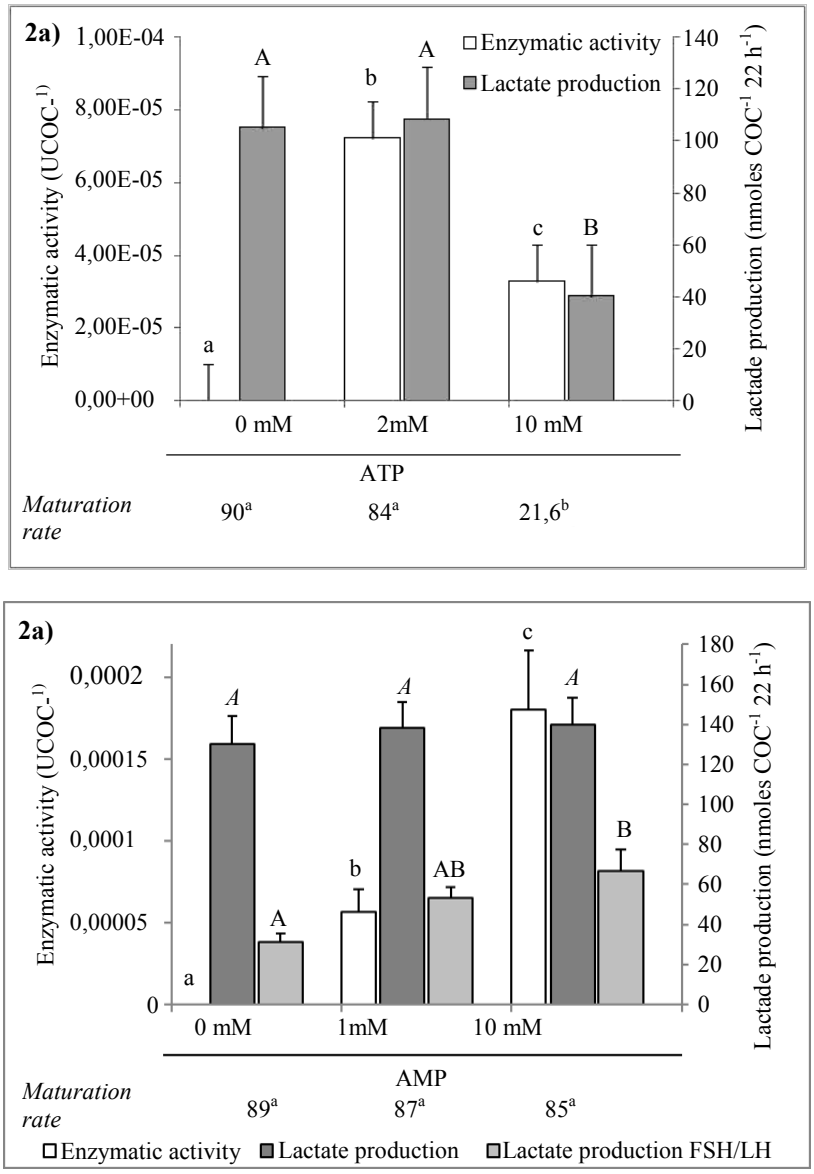

Figure 2. Activity of PFK1 in immature COCs, lactate production during maturation and oocyte nuclear maturation rate with different concentrations of ATP (2a) and AMP (2b). [a,b,c;A,B,C;*A,B,C*]Bars with different superscripts and same color are significantly different ($P < 0.05$). n =30-40 for each treatment of lactate production and maturation rate. n = 5 for the determination of enzymatic activity.

Effect of NADPH and NADP on glucose 6-phosphate dehydrogenase activity and COC PPP activity

In order to confirm whether the physiological modulators act on the PPP enzyme glucose 6-phosphate dehydrogenase, the enzyme activity was determined in the presence of 2 concentrations of NADPH or NADP. It was observed that NADPH 1.5 mM inhibited about 58% the activity of the G6PDH reaching a value of inhibition near 100% for NADPH 12.5 mM ($P < 0.05$; Fig. 3a).

The addition of NADP 12.5 mM increased the activity of the enzyme approximately 8.6 times respect to NADP 0.15 mM. In absence of NADP the activity of the enzyme was not detected by the method used ($P < 0.05$; Fig. 3b).

To verify the effect of the physiological modulators on the COC PPP pathway, the proportion of colourless oocytes evaluated by BCB and the maturation rate were carried out. In presence of NADPH 1.25 mM an inhibition on PPP was observed. The inhibition was approximately of 59%, being similar to the enzyme activity inhibition obtained with the same compound and concentration. A dose dependent inhibition on maturation rate was observed when NADPH was added on the culture media (Fig. 3a). On the other hand NADP had no effect on COC PPP activity evaluated by BCB and on the maturation rate (Fig. 3b). To discard a masking effect of gonadotropins the experiment was repeated without hormonal supplementation. However, meiotic maturation and PPP activity in COCs remained unaffected (Fig. 3b).

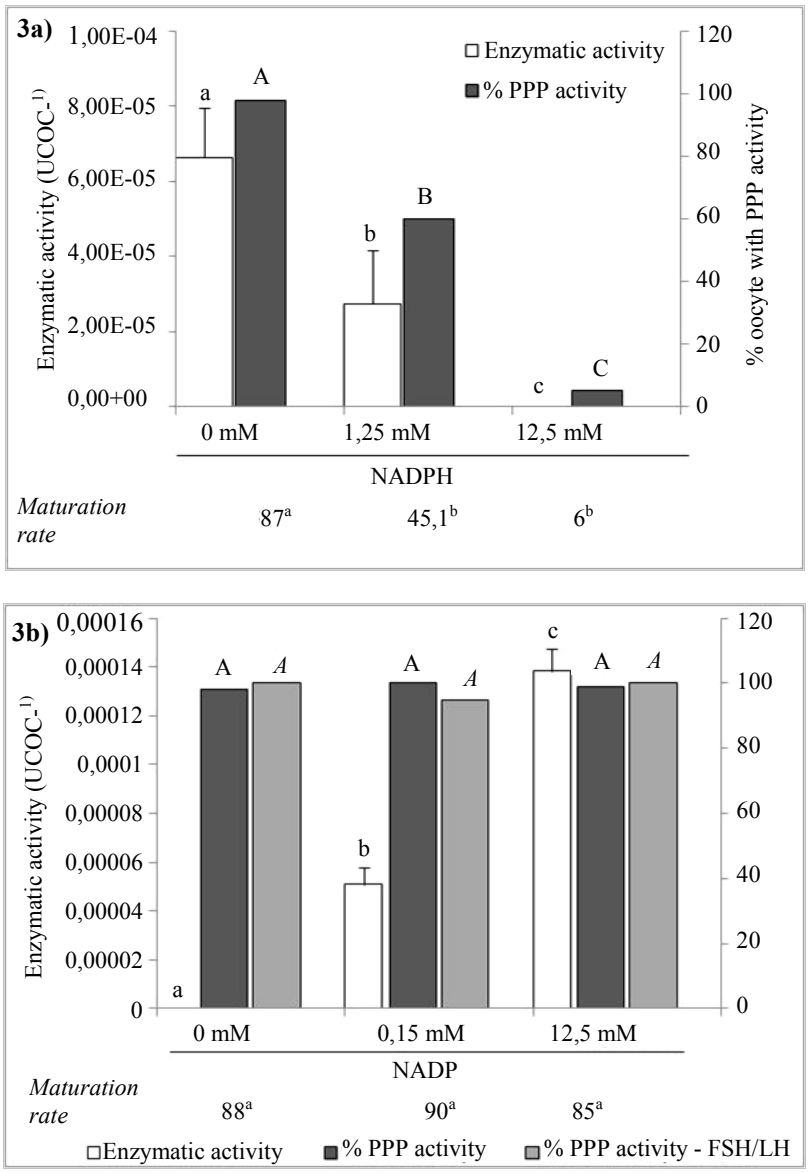

Figure 3. Activity of G6PDH in immature COCs, proportion of COCs with active PPP and oocyte nuclear maturation rate with different concentrations of NADPH (3a) and NADP (3b). [a,b,c;A,B,C;*A,B,C*] Bars with different superscripts and same color are significantly different ($P < 0.05$). n = 30-40 for each treatment of % PPP activity and maturation rate. n = 5 for the determination of enzymatic activity.

Discussion

The present study describes the effect of the proposed modulators on the activity of key enzymes involved in glucose metabolism, PFK1 and G6PDH. It was also evaluated the effect of the modulators on the glycolytic pathway activity and the PPP activity, evaluated by the lactate production and the proportion of colourless oocytes to BCB stain during maturation, respectively, and the nuclear maturation rate.

In a previous work, the activity of PFK1 and G6PDH has been detected in bovine COCs, the activity of these enzymes remained constant during maturation (Cetica *et al*., 2002). Our findings showed that PFK1 activity was modified in presence of the modulators ATP and AMP demonstrating that the PFK1 of bovine COCs was regulated by the energetic charge of the cell given by the relation between ATP/AMP, as it is done in somatic cells (Schirmer and Evans, 1990; Bruser *et al*., 2012). On the other hand, the absence of both modulators also diminished the activity of the enzyme since ATP would be acting, in addition to negative allosteric modulator, as substrate in low concentrations, and AMP would be required for the enzymatic activity. In concordance with these results, in somatic and tumoral cells, it has been demonstrated that ATP has a dual effect on PFK, where the enzyme is activated at concentrations of 1 mM of ATP and inhibited at higher concentration (Leite *et al*., 2007; Zancan *et al*., 2007; Sola-Penna *et al*., 2010). Thus, PFK has two binding sites for ATP, a catalytic binding site (with a binding constant of ~0.15 mM) and an allosteric inhibitor binding site (with a binding constant of ~2.5 mM; Leite *et al*., 2007; Marinho-Carvalho *et al*., 2009; Marcondes *et al*., 2010; Al Hasawi *et al*., 2014). Moreover, inhibition of COC glycolytic pathway evaluated by the

lactate production and oocyte nuclear maturation confirmed the inhibition of the enzyme during IVM and the relation between the pathway and the maturation process. The inhibition on the nuclear maturation might be due to a decrease in the flux of oxidative substrates from cumulus cells to the oocyte, like pyruvate and lactate. These substrates are essential for the bovine oocyte since it has been demonstrated the low capacity for glucose uptake that has the oocyte (Zuelke and Brackett, 1992; Cetica *et al*., 1999). In addition, the stimulation of the glycolytic key enzyme with AMP increase lactate production in the absence of gonadotropins. This behavior was not observed in the presence of gonadotropins probably because the glycolytic activity is maximum under the effect of these hormones.

The activity of G6PDH was modified in presence of NADP and NADPH. The addition of NADPH during the determination diminished the activity in a dose dependant manner while the addition of NADP stimulates it. These results demonstrate that the activity of G6PDH was regulated by the redox state of the bovine COC. In a previous work it has been observed that both the cumulus cells and the oocyte have G6PDH activity, being approximately 14 times higher the activity in the cumulus cells (Cetica *et al*., 2002). We also observed that the addition with NADPH during maturation induced a dose dependent inhibition on COC PPP activity and on oocyte meiotic maturation, confirming a tight relationship between the PPP and the nuclear maturation of the bovine COCs. This relationship could be because the products of the PPP might be targeted to metabolic pathways (Nelson and Cox, 2005) involved in the meiotic progress.

Despite the stimulatory effect of NADP on G6PDH activity, the addition of this physiological stimulator to the maturation media did not affect the rate of COCs with high PPP activity, suggesting that the pathway already had a high activity and NADP failed to stimulate even more the pathway independently the gonadotropin effect.

In conclusion, we observed that the regulation of key enzymes of glucose metabolism, PFK1 and G6PDH, in bovine COCs is similar to that reported for somatic cells, being regulated mainly by the energetic charge and the redox status, respectively. We also reported a tight relation between the activity of the PFK 1 and G6PDH enzymes, glycolytic and PPP activities and the oocyte maturation process in this species.

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