Pentose phosphate pathway activity: effect on *in vitro* maturation and oxidative status of bovine oocyte

Running head: PPP activity in COCs.

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Abstract

The relationship between the pentose phosphate pathway (PPP) activity in COCs and oxidative and mitochondrial activity in bovine oocytes was evaluated, with the aim of analyzing the impact of two inhibitors (NADPH, 6-AN) and a stimulator (NADP) of the key enzymes of PPP on the maturation rate, the oxidative and mitochondrial activity, and the mitochondrial distribution in oocytes. The percentage of COCs with PPP activity, (assessed using BCB staining), glucose uptake, lactate production, and meiotic maturation rate diminished when 6-AN was added to the maturation media ($p<0.05$). The addition of NADPH did not modify glucose uptake and lactate production, while the COC PPP activity and the meiotic maturation rates decreased ($p<0.05$). The presence of NADP in the maturation medium had no effect on COC PPP activity rate, glucose uptake, lactate production and meiotic maturation rate. However, in the absence of gonadotrophin supplementation NADP stimulated both glucose uptake and lactate production at the highest concentration tested ($p<0.05$). NADP did not modify cleavage rate, but decreased blastocyst production ($p<0.05$). During IVM, oocyte oxidative and mitochondrial activity was observed to increase at 15 and 22 h of maturation, which was also related to a progressive mitochondrial migration. Inhibiting PPP with 6-AN or NADPH led to a reduced oxidative and mitochondrial activity compared with their respective control groups and an inhibition in mitochondrial migration was also observed ($p<0.05$). Stimulation of the pathway with NADP increased oxidative and mitochondrial activity at 9 h of maturation ($p<0.05$) and delayed mitochondrial migration. This study shows the significance of altering the PPP activity during bovine oocyte IVM, revealing there is a link between the activity of the pathway and the oxidative status of the oocyte.

Introduction

Cumulus-oocyte complexes (COCs) consume substrates from the ovarian follicular fluid during *in vivo* maturation and from culture media during *in vitro* maturation (IVM) (Alm *et al.* 2005) in order to derived these substrates towards diverse metabolic pathways involved in the maturation process (Sutton *et al.* 2003; Thompson 2006). As in other cell types, COCs metabolize glucose via glycolysis, the pentose phosphate pathway (PPP) and the hexosamine biosynthesis pathway (Downs and Utecht 1999; Gutnisky *et al.* 2007; Sutton *et al.* 2003), as well as the polyol pathway (Sutton-McDowall *et al.* 2010).

It has been observed that the uptake of glucose by bovine COCs is mainly directed towards the production of lactate due to a high glycolytic activity, however, it is known that glucose can also be oxidized in the PPP as well (reviewed in Sutton *et al.* 2003). In pigs, a close relationship between the PPP activity and the maturation process in the oocyte has been proposed (Herrick *et al.* 2006). It has been suggested that the PPP is a primary factor for the progression of nuclear maturation (Sato *et al.* 2007). Accordingly, it was demonstrated that the flux of glucose throughout PPP influences the resumption of oocyte nuclear maturation in mouse COC (Downs *et al.* 1998). 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 (Herrick *et al.* 2006; Sutton-McDowall *et al.* 2005).

In somatic cells, the major regulatory point of the PPP is at the glucose 6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49), with NADP/NADPH ratio having an important regulatory role *in vitro* but not *in vivo* (Stanton 2012). It was also proposed that G6PDH is

competitively inhibited by NADPH (Özer *et al*. 2002). 6-aminonicotinamide (6-AN) is a pharmacological inhibitor of the PPP that suppresses the two NADP-requiring enzymes of the pathway, G6PDH and 6-phosphogluconate dehydrogenase (Köhler *et al.* 1970). 6-AN can replace the nicotinamide moiety of pyridine nucleotides, the resulting metabolite inhibit the pyridine nucleotide linked reactions in a competitive manner (Köhler *et al.* 1970; Tyson *et al*. 2000). We previously reported activity of G6PDH in both oocytes and cumulus cells arising from immature and matured bovine COCs (Cetica *et al.* 2002).

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 important in preventing oxidative stress throughout the glutathione and the thioredoxin systems, and thus regulating the redox intracellular state (Tian *et al.* 1998). The redox state describes a complex relationship between oxidized and reduced forms of a large number of molecules that include NAD(P):NAD(P)H, FAD:$FADH_2$, GSH:GSSG (Harvey *et al.* 2002). Other sources of NADPH are the reactions catalyzed by the NADP-dependent isocitrate dehydrogenase and the malic enzyme, however it has been demonstrated that in cell lines deficient in G6PDH**,** the activity of these enzymes are not enough to replace the PPP production of NADPH (Pandolfi *et al.* 1995). On the other hand, in the mouse oocyte, the main source of NADPH seems to be the NADP-dependent isocitrate dehydrogenase (Dumollard et al. 2007),

In a previous report, we demonstrated that glycolytic activity in bovine COCs influences oxidative status and maturation of oocytes (Gutnisky *et al.* 2012). It has been suggested that a proportion of glucose consumed by bovine COCs is also directed to the PPP metabolic pathway, involved in maintaining intracellular redox state. Manipulating PPP activity by using physiological and pharmacological modulators of the key enzymes of

the pathway, should help us to elucidate the role of PPP on oocyte redox state and the relationship with meiotic competence *in vitro*. Thus, the main aim of this work was to study the PPP activity during cattle oocyte IVM, analyzing the effects of two inhibitors (NADPH, 6-AN) and a stimulator (NADP) of the key enzymes of the PPP on the maturation rate, the oxidative and mitochondrial activity, and the mitochondrial distribution in the oocytes.

Matherials and Methods

Matherials

Unless specified, all chemicals and reagents were purchased from Sigma Chemical (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.

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;

121 Lutropin-V; Bioniche) and 50 mg L^{-1} gentamicine sulfate under mineral oil at 39°C for 22 h
122 in an atmosphere of humidified 5% CO_2 in air.

123 To study the effect of the addition of PPP enzyme modulators on the pathway
124 activity, glucose uptake and lactate production in COCs and oocyte meiotic maturation,
125 COCs were matured in media supplemented with increasing concentrations of 6-AN (0.1;
126 1; 5 and 10 mM), NADPH (0.0125; 0.125; 1.25 and 12.5 mM) or NADP (0.0125; 0.125;
127 1.25 and 12.5 mM) under the conditions described above. The rationale for selecting the
128 concentrations of NADP and NADPH for the dose response curves is that they include the
129 concentration of NADP (1,5 mM) used to determine the activity of G6PDH in enzymatic
130 extracts arising from bovine COCs (Cetica *et al.* 2002). The concentrations of 6-AN were
131 selected as they were previously used to inhibit PPP activity in bovine COCs and somatic
132 cells (Commizzoli et al. 2003; Gupte *et al.* 2003). To study the effect of manipulating the
133 PPP activity in COCs on subsequent oxidative status, mitochondrial activity, mitochondrial
134 distribution and nuclear morphology in the oocytes, COCs were matured in media
135 supplemented with 5 mM 6-AN, 1.25 mM NADPH (determined as inhibitory
136 concentrations in the previous experiments) or 12.5 mM NADP (determined as stimulatory
137 concentration in the previous experiment) for 9, 15 and 22 h. These time points were
138 chosen as they are temporally associated with key events of the maturation process:
139 germinal vesicle breakdown (GVBD), metaphase I (MI) and extrusion of the first polar
140 body, respectively (Fleming and Saacke 1972; Gordon 1994; Kruip *et al.* 1983).
141
142 *Determination of COC PPP activity and evaluation of oocyte meiotic maturation*
143 The Brilliant Cresyl Blue (BCB) stain test can be used to evaluate the proportion of
144 COCs with measurable PPP actvity. 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.* 2007; Bhojwani *et al.* 2005).

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.* 2007; Bhojwani *et al.* 2005). 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.

After the determination of COC PPP activity rate, denuded oocytes were placed in a hypotonic medium of 2.9 mM sodium citrate at 37°C for 15 min, fixed on a slide with 3:1 ethanol: acetic acid (Tarkowski 1966), stained with 5% (v/v) Giemsa (Merck, Darmstadt, Germany) for 15 minutes and observed under a light microscope at magnifications of x100 and x400. Oocytes were considered mature when a metaphase II chromosome configuration was present.

Determination of glucose uptake and lactate production

In order to evaluate glucose uptake by bovine COCs under the presence of the PPP enzyme modulators, COCs were individually cultured in 20 µl drops of maturation media for 22 h as described above. Then, COCs were removed from each drop and the glucose content was determined from the spent maturation medium. Glucose concentration was

measured using a spectrophotometric assay based on the oxidation of glucose by glucose oxidase and subsequent production of hydrogen peroxide (Trinder 1969). Positive controls comprising 20 µl drops of maturation media and a standard of 100 mg/ml of glucose were included in each experiment.

In order to evaluate simultaneously the glycolytic activity in the same COCs under the presence of enzyme modulators, lactate production in the culture medium was conducted in the same droplets that glucose uptake was determined. Lactate production was measured using a spectrophotometric assay based on the oxidation of lactate and subsequent production of hydrogen peroxide (Barhan and Trinder 1972; Trinder 1969).

Evaluation of oocyte competence

Immature COCs were divided into four groups for IVM in the media described above (positive control), without supplementation of gonadotrophins (negative control) and supplemented with 12.5 mM NADP with or without gonadotrophins. After 21 h of maturation, in vitro fertilisation (IVF) was performed using frozen–thawed semen from a Holstein bull of proven fertility. Semen was thawed at 37°C in modified synthetic oviduct fluid (mSOF) (Takahashi and First 1992), filtered through a glass column, centrifuged twice at 500 x *g* for 5 min and then resuspended in fertilisation medium to a final concentration of 2×10^9 motile spermatozoa L^{-1} (Arzondo *et al.* 2012). Fertilisation was performed in IVF-mSOF, consisting of mSOF supplemented with 5 g bovine serum albumin (BSA) L^{-1} and 10000 U heparin L^{-1} under mineral oil at 39°C, in 5% CO_2 in air and 100% humidity for 20 h. Zygotes were denuded by repeated pipetting and placed in 500µL of *in vitro* culture (IVC)-mSOF, consisting of mSOF supplemented with 30 ml Minimum Essential Medium (MEM) amino acids (GIBCO) L^{-1}, 10 ml MEM non-essential

amino acid (GIBCO) L^{-1}, 2 mmoles L-glutamine L^{-1}, 6 g L^{-1} BSA and 5% (v/v) FBS (GIBCO), under mineral oil at 39ºC in 90% N_2 : 5% CO_2 : 5% O_2 and 100% humidity for 6 days. The IVF was evaluated by the ratio of cleaved embryos 48 h after fertilisation. An additional cohort of 10 oocytes from each replicate was maintained through the fertilisation procedure without exposure to sperm to test for parthenogenesis. Embryo development was evaluated by the ratio of blastocyst 16-18 h after fertilisation.

Evaluation of oocyte chromatin morphology

COCs were cultured in groups of 50 in 500 µl drops of the maturation media described above. One third of the oocytes were used to evaluate the chromatin morphology at 0, 9, 15 and 22 h of maturation and the remaining 2/3 of the oocytes were used to evaluate oxidative activity, mitochondrial activity and mitochondrial distribution. A total of 15-20 oocyte per treatment was used in this experiment. Denuded oocytes were fixed in a 40 mg L^{-1} paraformaldehyde solution for an hour and then incubated in a permeabilizing solution for an hour and a half. Finally the fixed oocytes were stained with 10 mg L^{-1} Hoechst 33342 solution for 15 min.

Oocyte nuclear status was observed at x400 under using 330- 380 nm (excitation) and 410 nm (emision) filters for a Jenamed II epifluorescence microscope (Carl Zeiss Jena).

Evaluation of oxidative activity, mitochondrial activity and mitochondrial distribution

COCs were cultured in groups of 50 in 500 µl drops of the maturation media described above. From the total number of oocytes used, 2/3 were used to determine the oxidative activity, mitochondrial activity and mitochondrial distribution at 0, 9, 15 and 22 h of maturation. Between 30 and 40 oocytes per treatment in 3 replicates were evaluated.

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

Fluorescent probes and confocal microscopy were used to analyze the parameters mentioned above. The dual stains of RedoxSensor red CC-1 (Molecular Probes, Eugene, OR, USA) and MitoTracker green FM (Molecular probes) were used in this experiment. Oocytes were coincubated with a final concentration of 1nM RedoxSensor red CC-1 and 0.5 nM MitoTracker green FM, for 30 min at 39˚C in the dark and then washed twice in PBS. Stained oocytes were then placed between slide and coverslip for the observation using a laser confocal microscope (Nikon C1 confocal scanning head, Nikon TE2000E; Nikon, Kanagawa, Japan). Images were taken only at the equator of the oocyte, then analyzed using the Adobe Photoshop CS2 (version 9, Adobe Systems Inc.).

Both red and green fluorescence emission intensities were determined in four different equatorial regions (squares) within three areas: cortical (1), middle (2) and central (3) of the oocyte, as depicted in Fig 1 a (Wakefield *et al.* 2008).

The oxidative activity was calculated as the sum of the average red fluorescence intensity in the three areas of the same oocyte. The mitochondrial activity was calculated as the sum of the average green fluorescence intensity in the three areas of the same oocyte. Ratios of green fluorescence intensity between the central area (3) and the cortical area (1) were then calculated to compare the distribution of active mitochondria.

Statistical analysis

To evaluate the relationship between COCs with functional PPP activity and oocyte meiotic maturation, a Chi-square test for independence was performed. The percentage of COCs with active PPP, oocyte meiotic maturation, cleavage and blastocyst obtained were

compared using a chi-square analysis for non-parametric data. The results of glucose uptake, lactate production, oxidative activity, mitochondrial activity and the ratio of green fluorescence intensity between the central area (3) and the cortical area (1) to evaluate mitochondrial distribution are expressed as the mean ± s.e.m. In the studies evaluating glucose uptake and lactate production the comparisons were made by analysis of variance (ANOVA) followed by Bonferroni post-test. The oxidative activity, the mitochondrial activity and the mitochondrial distribution were compared using a 2 x 4 factorial design. In all tests, a significant difference was taken at $p<0.05$.

Results

COC PPP activity and oocyte nuclear maturation

Firstly, in order to determine if there is association between COCs with measurable PPP activity and oocyte meiotic maturation, an independence test was conducted. This revealed that both parameters are not independent events, demonstrating a relationship between them ($n= 73$; $p<0.05$).

The effect of the addition of increasing concentrations of both inhibitors (6-AN and NADPH) and the stimulator (NADP) of the PPP on the oocyte meiotic maturation and activity of the pathway was studied. A dose dependent inhibition on meiotic maturation and COC PPP activity were observed when 6-AN or NADPH was added to the maturation media ($p<0.05$; Fig. 2 a and 2 b), while the addition of NADP did not modify these parameters (Fig. 2 c). Since the gonadotrophins might be masking the effect induced by NADP, the experiment was repeated without hormonal supplementation. However, meiotic maturation and COC PPP activity remained unaffected (Fig. 2 d).

Glucose uptake and lactate production

The addition of 6-AN to the maturation media induced a dose dependent inhibition on glucose uptake by COCs ($p<0.05$). Lactate production diminished around 50% in the presence of 6-AN at all concentrations assessed ($p<0.05$; Fig. 3 a).

The addition of NADPH or NADP did not modify the glucose uptake and the lactate production by COCs (Fig. 3 b and 3 c). Nevertheless, NADP at the highest concentration produced a rise in both glucose uptake and lactate production when it was added in the absence of gonadotrophins ($p<0.05$; Fig. 3 d).

Oocyte competence

Since the meiotic maturation was not affected by the presence of NADP during IVM, the IVF and embryo development was evaluated in order to determine possible subsequent effects. The dose of 12.5 mM NADP in maturation media in the presence or absence of gonadotrophins was used in these experiments because it was the concentration that stimulated glucose uptake. The supplementation with NADP did not modify the cleavage rates compared with their respective controls. Nevertheless, differences due to the hormonal supplementation were observed ($p<0.05$; fig. 4 a).

A significant decrease in blastocyst yield was observed in COCs matured with or without gonadotrophins in the presence of NADP ($p<0.05$). No differences were observed in the blastocyst rates obtained without hormonal supplementation (Fig. 4 b).

Oocyte chromatin morphology

To determine which stage of nuclear maturation was affected by the PPP inhibition in COCs, oocytes were also analyzed with the fluorochrome Hoechst 33342 at different

time points. In the control group 70.8% of oocyte had undergone GVBD after 9 h of maturation, while oocytes matured with 6-AN or NADPH had lower rates of GVBD ($p<0.05$). Furthermore, progression to MI was also significantly reduced in 6-AN or NADPH treatments at 15 h of maturation ($p<0.05$). The extrusion of the first polar body was observed in 80% of control oocytes at 22 h, but was significantly lower in 6-AN or NADPH treated oocytes ($p<0.05$), which remained blocked at the GV or MI stages (Table 1; Fig 1 c). In the presence of NADP, oocytes were equally as capable of reaching metaphase II at 22 h of maturation with respect to control, so we did not pursue an analysis of oocyte nuclear morphology with this treatment.

Oxidative activity of the oocyte

To determine the impact of the PPP activity in the COC on oxidative status within the oocyte, denuded oocytes were stained with Redox Sensor Red to quantify oxidative activity at different time points (0, 9, 15 and 22 h). In these experiments, 1.25 mM NADPH and 5 mM 6-AN were used as levels known to be 50% of the inhibitory concentrations observed for the PPP activity. Because NADP did not produce a significant increase in the PPP activity, the concentration of NADP (12.5 mM) that increased glucose uptake by COCs was used for this study.

Oocytes showed variation in the level of oxidative activity throughout maturation in the control group (Fig 1 b). Similar values were detected at 0 h and 9 h, but a remarkable increase was registered at 15 h ($p<0.05$); then a decrease to a lower value was observed at 22 h ($p<0.05$; Fig. 5 a-c). The addition of 6-AN to the maturation media induced a change in the pattern of oxidative activity with respect to the control; a significant decrease of the oxidative activity at 15 h ($p<0.05$; Fig. 5 a). In the presence of NADPH, oxidative activity

diminished significantly at 15 h and 22 h of maturation ($p<0.05$; Fig 5 b). On the other hand, the addition of NADP induced a significant increase in oxidative activity at 9 h of maturation ($p<0.05$) and a significant decrease at 15 h and 22 h with respect to the control ($p<0.05$; Fig 5 c).

Mitochondrial activity of the oocyte

To study the effect of manipulating PPP activity in COCs on mitochondrial activity within oocytes, fluorescence intensity of Mitotracker green was analyzed at the same time points and using the same 6-AN, NADPH and NADP concentrations described in the experiment above.

Mitochondrial activity changed in oocytes during maturation in the control groups, following a similar pattern to that described for oxidative activity ($p<0.05$; Fig 6 a-c). The addition of 6-AN or NADPH induced a significant decrease in mitochondrial activity at 15 h and 22 h of maturation with respect to control ($p<0.05$; Fig 6 a and b). The addition of NADP induced a significant increase in the mitochondrial activity at 9 h of maturation ($p<0.05$) and a significant decrease at 15 h and 22 h with respect to the control ($p<0.05$; Fig 6 c).

A high positive correlation between oxidative activity and mitochondrial activity of oocytes was observed for each treatment ($r>0.96$, $n=173$-212, $p<0.05$).

Mitochondrial distribution in the oocyte

Changes in mitochondrial distribution were observed during maturation in the control group. The distribution in immature oocytes was cortical, however a progressive mitochondria migration towards the central area was observed during maturation, which we

have observed in a previous study (Gutnisky *et al.* 2012). This observation was confirmed by analyzing the ratio between the intensity of green fluorescence between area 3 and area 1 of the oocyte ($p<0.05$; Fig 6 a-c). Mitochondria of oocytes treated with 6-AN or NADPH did not migrate ($p<0.05$; fig 7 a and 7 b). On the other hand, while mitochondria of oocytes from the control group completed the migration at 15 h, mitochondria of oocytes treated with NADP completed their migration at 22 h (Fig 7 c).

Discussion

The present study describes the effect of the addition of enzyme modulators of PPP during bovine oocyte IVM on the pathway activity in COCs, oocyte maturation rate, and the oxidative activity, mitochondrial activity and mitochondrial distribution of the oocytes.

The independence test between the proportion of COCs with measurable PPP activity and oocyte meiotic maturation revealed that both are associated events. Additionally, under increasing concentrations of the pharmacological (6-AN) or the physiological (NADPH) inhibitor of G6PDH, a dose dependent inhibition in both parameters was observed during in vitro maturation, confirming the close relationship between PPP activity and nuclear maturation in bovine COCs. This relationship is implicated from products of PPP activity for other metabolic pathways, like NADPH and ribose 5-phosphate (Nelson and Cox 2005).

Our findings showed that the supplementation of the maturation media with a pharmacological inhibitor of the PPP had an inhibitory dose-dependent effect on glucose uptake and a non-dose dependent inhibition on lactate production. Both results suggest a concomitant inhibitory effect on glycolytic activity. In agreement with these results, an indirect inhibition of the oocyte PPP with an inhibitor of the NADPH oxidase was

accompanied by an inhibition of the glycolytic pathway in pig oocytes (Herrick *et al.* 2006). Similar results were also obtained by inhibiting the oxidative arm of the PPP in mouse oocytes (Downs *et al.* 1998). It has been reported in somatic cells that inhibition of the 6-phosphogluconate dehydrogenase by 6-AN causes the accumulation of 6-phosphogluconate, a metabolite that might be responsible for the inhibition of the glycolytic enzyme phosphoglucose isomerase (Tyson *et al.* 2000). However, we cannot exclude a possible inhibition of glycolytic dehydrogenase activity by 6-AN, although the K*i* for the 6-phosphogluconate dehydrogenase was the lowest measured and several order of magnitude lower than the K*i* for other dehydrogenases studied (Köhler *et al.* 1970). In contrast, the addition of the physiological inhibitor of the pathway to the maturation media did not modify the lactate production, suggesting that the PPP inhibitory mechanism of NADPH does not involve co-inhibition of the glycolytic pathway as 6-AN.

The addition of the physiological stimulator of the PPP (NADP) to the maturation media with or without supplementation of gonatrophins failed to increase the percentage of COCs with active PPP, suggesting a high activity of the pathway. NADP supplementation seems to be unable to further stimulate the pathway during bovine oocyte IVM. On the other hand, an increase on glucose uptake and lactate production was observed when the highest concentration of NADP was added to maturation media without gonadotrophins, showing a higher glycolytic activity under this maturation condition. We previously reported that glycolysis can be stimulated by the addition of the physiological stimulator AMP in maturation media without gonadotrophic supplementation (Gutnisky *et al.* 2012). In the present study, the augmented glycolytic activity in the presence of NADP could be explained by both requirements of NADPH and ATP by the COC. In somatic cells, it has been described that the flux of glucose through the PPP has four different patterns

according to the NADPH, ribose 5-phosphate and ATP requirements. When the cell requires both NADPH and ATP, the end products of the PPP, glyceraldehyde 3-phosphate and fructose 6-phosphate, enter the glycolytic pathway in order to obtain ATP (Berg *et al.* 2002). Although the oocytes matured with NADP complete the meiotic and cytoplasmic maturation processes, the embryo development was impaired, suggesting that high concentrations of NADP may be altering developmental capacity of bovine oocyte.

As we reported in a previous study (Gutnisky *et al.* 2012), oocyte oxidative and mitochondrial activity are highly correlated and both fluctuate during IVM, with significant increases at 15 and 22 h compared with 0 h and 9 h of maturation. Modulating PPP activity in COCs with both enzymatic inhibitors and the putative stimulator of the pathway all modified oxidative activity within the oocyte, altering the characteristic pattern observed in untreated control oocytes. When NADPH was added to the maturation media, oxidative activity remained low throughout maturation, while 6-AN caused only a slight increase at 22 h, demonstrating the close relationship between PPP activity and oocyte redox state. In line with these results, inhibition of the PPP with both inhibitors had a negative effect on meiotic progression of the oocytes. These effects might be due to an alteration in the NADPH:NADP ratio caused by the inhibition of G6PDH. The activity of this enzyme is important in the regulation of the cell redox level (Tian *et al.* 1998) and in events related with the resumption of meiosis (Harvey *et al.* 2002; Herrick *et al.* 2006). In hamster oocytes, it have been suggested that PPP is not only important in preventing cell oxidative stress throughout the glutathione system, but also the maintaining of meiotic spindle morphology by protecting the spindle against oxidative damage (Zuelke *et al.* 1997).

In contrast, the addition of NADP to maturation media changed the pattern of oxidative activity with respect to control, accelerating the time of the oxidative burst to 9 h

409 of maturation. The addition of NADP to IVM medium may produce a precocious
410 activation of PPP and in this way alters the redox state towards a more oxidative state. In
411 agreement, it has been reported that fully grown oocytes at the GV stage have low activity
412 of the PPP pathway (Rodriguez-Gonzalez *et al.* 2002). This shift in the redox state within
413 the oocyte towards a more oxidized state, may contribute to the subsequent impaired
414 embryo developmental competence. We propose that a precocious oxidative burst causes
415 the oxidation of some target molecules implicated in events related to embryo development.
416 It has been observed that several transcription factors involved in diverse developmental
417 processes are regulated by the intracellular redox potential (Imai *et al*. 2000; Dickinson and
418 Forman 2002; Rahman *et al*. 2004; Liu *et al*. 2005; Funato *el at*. 2006). The recent
419 discovery that these factors can be sensitive to oxidation (e.g. reactive oxygen species, S-
420 glutathionylation, high NAD(P)/NAD(P)H ratio) is generating new insights into the
421 regulation of embryo development (Dumolard et *al*. 2007). In agreement, it has been
422 reported that low intracellular glutathione concentrations in oocytes might be responsible of
423 the lower embryo developmental capacity in cattle and pig oocytes (Brad *et al.* 2003; de
424 Matos and Furnus 2000).

425 The inhibition of the PPP activity in COCs with 6-AN or NADPH also modified the
426 mitochondrial activity during maturation, following the same pattern previously observed
427 for oxidative activity. These results, together with the high positive correlation observed
428 between both parameters, confirm that mitochondrial activity and oxidative activity are
429 closely related events. Furthermore, the presence of NADP increased mitochondrial activity
430 at 9 h of culture, as it was also observed for oxidative activity. Coincidentally, it has been
431 suggested that cytosolic redox state is linked to mitochondrial activity and mitochondrial
432 redox state in ovine embryos (Lieber 1991; Thompson *et al.* 1993).

During maturation, mitochondria migrated from the cortical area towards a more central distribution in untreated oocytes (control group), which we previously reported (Gutnisky *et al.* 2012). The inhibition of PPP activity with 6-AN or NADPH also inhibited the migration of the mitochondria, suggesting that mitochondrial activity is linked to mitochondrial redistribution. In agreement, it was proposed that ATP content and mitochondria redistribution are associated events in bovine oocytes (Stojkovic *et al.* 2001). Somewhat differently, the addition of NADP to maturation media delayed the mitochondrial migration which might be related to developmental competence as we have demonstrated that NADP affects the blatocyst rate. In line with these results, it has been proposed that the regulation of mitochondria distribution and segregation might be involved in the complexity of the segmentation axis during early cleavages (Tarazona *et al.* 2006).

In conclusion, we reported that the PPP activity in bovine COCs is necessary for successful meiotic and cytoplasmic maturation of the bovine oocyte. Fluctuations in the oocyte's oxidative and mitochondrial activities were detected during the progression of maturation, which if perturbed by the use of the PPP inhibitors had consequences for developmental competence.

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Caption of the figures:

Fig 1:

Schematic representation of the four different regions (squares) within three areas (1 – 3) of an oocyte used for determining mitochondrial fluorescence (1 a).

Oocytes stained with two fluorescent probes, MitoTracker Green (A) and RedoxSensor Red (B) and the co-localization at different time points (Original magnification x 400) (1 b).

Chromatin configuration at different hours of maturation. Oocytes satined with Hoeschst. (A) Geminal vesicle, (B) Germinal vesicle breakdown, (C) Metaphase I, (D) Anaphase, (E) Metaphase II and polar body. Original magnification (x 1000) (1 c).

Fig 2:

COC PPP activity rate and oocyte nuclear maturation with different concentrations of 6-AN (2 a), NADPH (2 b), NADP (2 c) and NADP without gonadotrophin supplementation (2 d).

[a, b, c] Bars of the same color with different super index differ significantly ($p<0,05$).

n=30-40 COCs for each treatment in 4 replicates.

Fig 3:

Glucose uptake and lactate production in COCs matured with different concentrations of 6-AN (3 a), NADPH (3 b), NADP (3 c) and NADP without gonadotrophin supplementation (3 d).

[a, b, c] Bars of the same color with different super index differ significantly (p<0,05).

n=30-40 COCs for each treatment in 4 replicates.

Table 1:

Effect of 6-AN 5 mM and NADPH 1.25 mM on chromatin morphology

Data show the percentage of oocytes at each stage of development (*n*=15–20 oocytes for each treatment in 3 replicates). Different superscript letters indicates significant differences in the percentage of oocytes at the same time point and same nuclear stage between treatments. GV, germinal vesicle; GVBD, GV breakdown; AN, anaphase; PBE, polar body extrusion.

Fig 4:

Cleavage rate following IVF (4 a) and blstocyst rate (4 b) from oocytes matured with NADP 12,5 mM with and without gonadotrophins.

[a, b] Bars with different super index differ significantly (p<0,05).

n=107-134 COCs for each treatment in 4 replicates.

Fig 5:

Oxidative activity within oocytes matured in the presence of 6-AN 5 mM (5 a), NADPH 1.25 mM (5 b) or NADP 12.5 mM (5 c).

[a, b, c] Bars of the same color with different super index differ significantly (p<0,05).

* Significant difference between treatments at the same time.

n=30-40 COCs for each treatment in 3 replicates.

Fig 6:

Mitochondrial activity within oocytes matured in the presence of 6-AN 5 mM (6 a), NADPH 1.25 mM (6 b) or NADP 12.5 mM (6 c).

[a, b, c] Bars of the same color and different super index differ significantly ($p<0,05$).

* Significant difference between treatments at the same time.

n=30-40 COCs for each treatment in 3 replicates.

Fig 7:

Active mitochondria distribution within oocytes matured in the presence of 6-AN 5 mM (7 a), NADPH 1.25 mM (7 b) or NADP 12.5 mM (7 c).

[a, b, c] Bars of the same color and different super index differ significantly ($p<0,05$).

* Significant difference between treatments at the same time.

n=30-40 COCs for each treatment in 3 replicates.

Table 1:

Effect of 6-AN 5 mM, NADPH 1.25 mM or NADP 12.5 mM on nuclear morphology

Data show the percentage of oocytes at each stage of development (*n*=15–20 oocytes for each treatment in 3 replicates). Different superscript letters indicates significant differences in the percentage of oocytes at the same time point and same nuclear stage between treatments. GV, germinal vesicle; GVBD, GV breakdown; AN, anaphase; PBE, polar body extrusion.