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

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Abstract. The relationship between pentose phosphate pathway (PPP) activity in cumulus–oocyte complexes (COCs) and oxidative and mitochondrial activity in bovine oocytes was evaluated with the aim of analysing the impact of two inhibitors (NADPH and 6-aminonicotinamide (6-AN)) and a stimulator (NADP) of the key enzymes of the PPP on the maturation rate, oxidative and mitochondrial activity and the mitochondrial distribution in oocytes. The proportion of COCs with measurable PPP activity (assessed using brilliant cresyl blue staining), glucose uptake, lactate production and meiotic maturation rate diminished when 6-AN (0.1, 1, 5 and 10 mM for 22 h) was added to the maturation medium ($P < 0.05$). The addition of NADPH did not modify glucose uptake or lactate production, but reduced PPP activity in COCs and meiotic maturation rates ($P < 0.05$). The presence of NADP (0.0125, 0.125, 1.25 and 12.5 mM for 22 h of culture) in the maturation medium had no effect on PPP activity in COCs, glucose uptake, lactate production and meiotic maturation rate. However, in the absence of gonadotropin supplementation, NADP stimulated both glucose uptake and lactate production at 12.5 mM (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 maturation, which was also related to progressive mitochondrial migration. Inhibiting the PPP with 6-AN or NADPH led to reduced oxidative and mitochondrial activity compared with the respective control groups and inhibition of mitochondrial migration ($P < 0.05$). Stimulation of the PPP with NADP increased oxidative and mitochondrial activity at 9 h maturation ($P < 0.05$) and delayed mitochondrial migration. The present study shows the significance of altering PPP activity during bovine oocyte IVM, revealing that there is a link between the activity of the PPP and the oxidative status of the oocyte.

Additional keywords: glucose metabolism, Mitotracker green, Redox Sensor red.

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Introduction

Cumulus–oocyte complexes (COCs) consume substrates from the ovarian follicular fluid during *in vivo* maturation and from culture media during IVM (Alm *et al.* 2005) and divert these substrates towards diverse metabolic pathways involved in the maturation process (Sutton *et al.* 2003; Thompson 2006). As in other cell types, COCs metabolise glucose via glycolysis, the 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).

It has been observed that the uptake of glucose by bovine COCs is directed mainly towards the production of lactate due to high glycolytic activity; however, it is known that glucose can also be oxidised in the PPP (for a review, see Sutton *et al.* 2003). In pigs, a close relationship between 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 the PPP influences the resumption of oocyte nuclear maturation in mouse COC (Downs *et al.* 1998). It has also been proposed that the PPP is involved in the progression of all stages of meiosis, including the resumption of meiosis, MI–MII transition and the resumption of meiosis after fertilisation (Sutton-McDowall *et al.* 2005; Herrick *et al.* 2006).

In somatic cells, the major regulatory point of the PPP is at glucose 6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49), with the 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, namely G6PDH and 6-phosphogluconate dehydrogenase (Köhler *et al.* 1970). 6-AN can replace the nicotinamide moiety of pyridine nucleotides, with the resulting metabolite inhibiting the pyridine nucleotide-linked reactions in a competitive manner (Köhler *et al.* 1970; Tyson *et al.* 2000). Previously, we reported G6PDH activity in both oocytes and cumulus cells arising from immature and matured bovine COCs (Cetica *et al.* 2002).

The PPP has two main metabolic goals: (1) to produce NADPH for reductive synthesis; and/or (2) to yield ribose 5-phosphate as a nucleotide precursor. The NADPH produced by the PPP is important in preventing oxidative stress throughout the glutathione and thioredoxin systems, and thus regulating the redox intracellular state (Tian *et al.* 1998). The redox state describes a complex relationship between oxidised and reduced forms of a large number of molecules, including NAD(P) : NAD(P)H, flavin adenine dinucleotide (FAD) : flavin adenine dinucleotide reduced form (FADH2), reduced glutathione (GSH) : glutathione disulfide (GSSG; Harvey *et al.* 2002). Other sources of NADPH are the reactions catalysed by the NADP-dependent isocitrate dehydrogenase (NADP-IDH) and malic enzyme; however, it has been demonstrated that in G6PDH-deficient cell lines the activity of these enzymes is not enough to replace the PPP production of NADPH (Pandolfi *et al.* 1995). Conversely, in mouse oocytes, the main source of NADPH seems to be the NADP-IDH (Dumollard *et al.* 2007).

In a previous study we demonstrated that glycolytic activity in bovine COCs influences oxidative status and the 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 elucidate the role of the PPP in oocyte redox state and the relationship with meiotic competence *in vitro*. Thus, the main aim of the present study was to investigate PPP activity during cattle oocyte IVM, analysing the effects of two inhibitors (NADPH and 6-AN) and a stimulator (NADP) of the key enzymes of the PPP on maturation rate, oxidative and mitochondrial activity and mitochondrial distribution in oocytes.

Materials and methods

Materials

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

Recovery of COCs

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

IVM of COCs

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

To investigate the effects of the addition of PPP enzyme modulators on pathway activity, glucose uptake and lactate production in COCs and oocyte meiotic maturation, the COCs were matured in media supplemented with increasing concentrations of 6-AN (0.1, 1, 5 and 10 mM), NADPH (0.0125, 0.125, 1.25 and 12.5 mM) or NADP (0.0125, 0.125, 1.25 and 12.5 mM) under the conditions described above. The rationale for selecting these concentrations of NADP and NADPH for the dose–response curves is that they include the concentration of NADP (1.5 mM) used to determine the activity of G6PDH in enzymatic extracts arising from bovine COCs (Cetica *et al.* 2002). The concentrations of 6-AN were selected on the basis of previous reports in which they were used to inhibit PPP activity in bovine COCs and somatic cells (Comizzoli *et al.* 2003; Gupte *et al.* 2003). To investigate the effects of manipulating PPP activity in COCs on subsequent oxidative status, mitochondrial activity, mitochondrial distribution and nuclear morphology in oocytes, the COCs were matured in media supplemented with 5 mM 6-AN, 1.25 mM NADPH (determined as inhibitory concentrations in previous experiments) or 12.5 mM NADP (determined as stimulatory concentrations in the previous experiment) for 9, 15 and 22 h. These time points were chosen because they are temporally associated with key events of the maturation process, namely germinal vesicle breakdown (GVBD), MI and extrusion of the first polar body, respectively (Fleming and Saacke 1972; Kruip *et al.* 1983; Gordon 1994).

Determination of PPP activity in COCs and evaluation of oocyte meiotic maturation

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

To determine the proportion of COCs with an 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 end of culture, the COCs were washed twice in phosphate-buffered

saline (PBS) and oocytes were denuded mechanically by repeated pipetting in PBS with 1 g L^{-1} hyaluronidase to evaluate the cytoplasm coloration. The oocytes evaluated were divided into two groups: coloured oocytes (low G6PDH activity) and colourless oocytes (high G6PDH activity). The proportion of COCs with an active PPP was calculated as the number of colourless oocytes as a percentage of total oocytes per treatment.

After the determination of PPP activity in COCs, 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 min and observed under a light microscope at a magnification of ×100 and ×400. Oocytes were considered mature when an MII chromosome configuration was present.

Determination of glucose uptake and lactate production

To evaluate glucose uptake by bovine COCs in the presence of the PPP enzyme modulators, COCs were cultured individually in 20-μL drops of maturation medium 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 concentrations were 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 medium and a standard of 1 g L^{-1} glucose were included in each experiment.

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

Evaluation of oocyte competence

Immature COCs were divided into four groups for IVM in the media described above (positive control), without supplementation of gonadotropins (negative control) and supplemented with 12.5 mM NADP with or without gonadotropins. After 21 h maturation, 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*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 L^{-1} bovine serum albumin (BSA) and 10 000 U L^{-1} heparin under mineral oil at 39°C, in 5% CO_2 in air and 100% humidity for 20 h. Zygotes were denuded by repeated pipetting and placed in 500 μL *in vitro* culture (IVC)-mSOF, consisting of mSOF supplemented with 30 mL L^{-1} minimum essential medium (MEM) amino acids (GIBCO), 10 mL L^{-1} MEM non-essential amino acid (GIBCO), 2 mmol L^{-1} L-glutamine, 6 g L^{-1} BSA and 5% (v/v) FBS (GIBCO), under mineral oil at 39°C in 90% N_2 : 5% CO_2 : 5% O_2 and 100% humidity for 6 days. IVF was evaluated as the ratio of cleaved embryos 48 h after fertilisation. An additional cohort of 10 oocytes from each replicate was maintained throughout the fertilisation procedure without exposure to spermatozoa to test for parthenogenesis. Embryo development was evaluated as the ratio of blastocysts 168 h after fertilisation.

Evaluation of oocyte chromatin morphology

The COCs were cultured in groups of 50 in 500-μL drops of the maturation media described above. One-third of oocytes were used to evaluate chromatin morphology at 0, 9, 15 and 22 h maturation, whereas the remaining two-thirds of oocytes were used to evaluate oxidative activity, mitochondrial activity and mitochondrial distribution. A total of 15–20 oocytes per treatment was used in this experiment. Denuded oocytes were fixed in 40 mg L^{-1} paraformaldehyde solution for 1 h and then incubated in a permeabilising solution for 1.5 h. Finally, the fixed oocytes were stained with 10 mg L^{-1} Hoechst 33342 solution for 15 min.

Oocyte nuclear status was observed at a magnification of ×400 under using 330–380 nm (excitation) and 410 nm (emission) filters for a Jenamed II epifluorescence microscope (Carl Zeiss Jena, Buenos Aires, Argentina).

Evaluation of oxidative activity, mitochondrial activity and mitochondrial distribution

In these experiments, the COCs were cultured in groups of 50 in 500-μL drops of maturation media described above. Of the total number of oocytes, two-thirds were used to determine oxidative activity, mitochondrial activity and mitochondrial distribution at 0, 9, 15 and 22 h maturation. Between 30 and 40 oocytes were evaluated per treatment in three replicates.

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

Fluorescent probes and confocal microscopy were used to analyse oxidative activity, mitochondrial activity and mitochondrial distribution. Dual staining with RedoxSensor red CC-1 (Molecular Probes, Eugene, OR, USA) and MitoTracker green FM (Molecular Probes) was used in this experiment. Oocytes were coincubated with a final concentration of 1 nM 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 placed between a slide and coverslip for observation under a laser confocal microscope (Nikon C1 confocal scanning head, Nikon TE2000E; Nikon, Kanagawa, Japan). Images were taken only at the equator of the oocyte and were analysed using Adobe Photoshop CS2 version 9 (Adobe Systems Inc., San Jose, CA, USA).

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

Oxidative activity was calculated as the sum of the average red fluorescence intensity in the three areas of the same oocyte. Mitochondrial activity was calculated as the sum of the average green fluorescence intensity in the three areas of the same oocyte. Ratios of green fluorescence intensity between

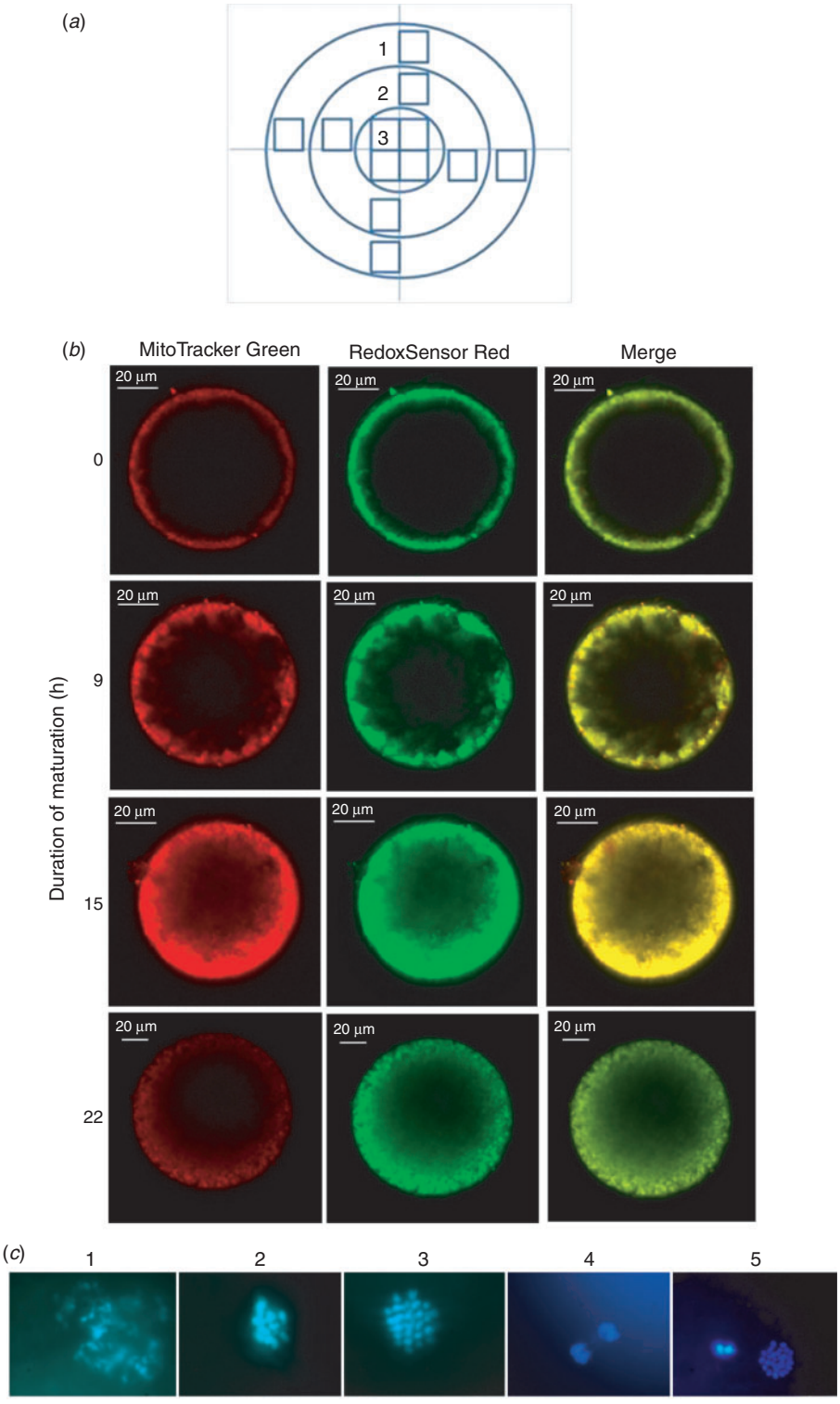

Fig. 1. (*a*) Schematic representation of the four different regions (squares) within three areas (i.e. cortical (1), middle (2) and central (3)) of an oocyte used for determining mitochondrial fluorescence. (*b*) Oocytes were stained with two fluorescent probes, namely MitoTracker Green and RedoxSensor Red, as indicated and colocalised at different time points (Merge). Chromatin configuration was evaluated after different periods of maturation, as indicated. (*c*) Hoechst staining of oocytes showing oocytes at the (1) germinal vesicle, (2) germinal vesicle breakdown, (3) MI, (4) anaphase and (5) MII and polar body stages. Original magnification ×1000.

the central and cortical areas 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-squared test for independence was performed. The proportion of COCs with an active PPP, oocyte meiotic maturation, cleavage and blastocyst rates were compared using a Chi-squared test for non-parametric data. Data for glucose uptake, lactate production, oxidative activity, mitochondrial activity and the ratio of green fluorescence intensity between the central and cortical areas (i.e. mitochondrial distribution) are expressed as the mean ± s.e.m. In studies evaluating glucose uptake and lactate production, comparisons were made by analysis of variance (ANOVA) followed by the Bonferroni post-test. Oxidative activity, mitochondrial activity and mitochondrial distribution were compared using a 2×4 factorial design. In all tests, $P < 0.05$ was considered significant.

Results

PPP activity in COCs and oocyte nuclear maturation

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

The effects of the addition of increasing concentrations of both inhibitors (6-AN and NADPH) and the stimulator (NADP) of the PPP on oocyte meiotic maturation and activity of the pathway were evaluated. A dose-dependent inhibition of meiotic maturation and PPP activity in COCs was observed when 6-AN or NADPH was added to the maturation medium ($P < 0.05$; Fig. 2*a*, *b*), whereas the addition of NADP did not modify these parameters (Fig. 2*c*). Because the gonadotropins may be masking the effects of NADP, the experiment was repeated without hormonal supplementation. However, meiotic maturation and PPP activity in COCs remained unaffected (Fig. 2*d*).

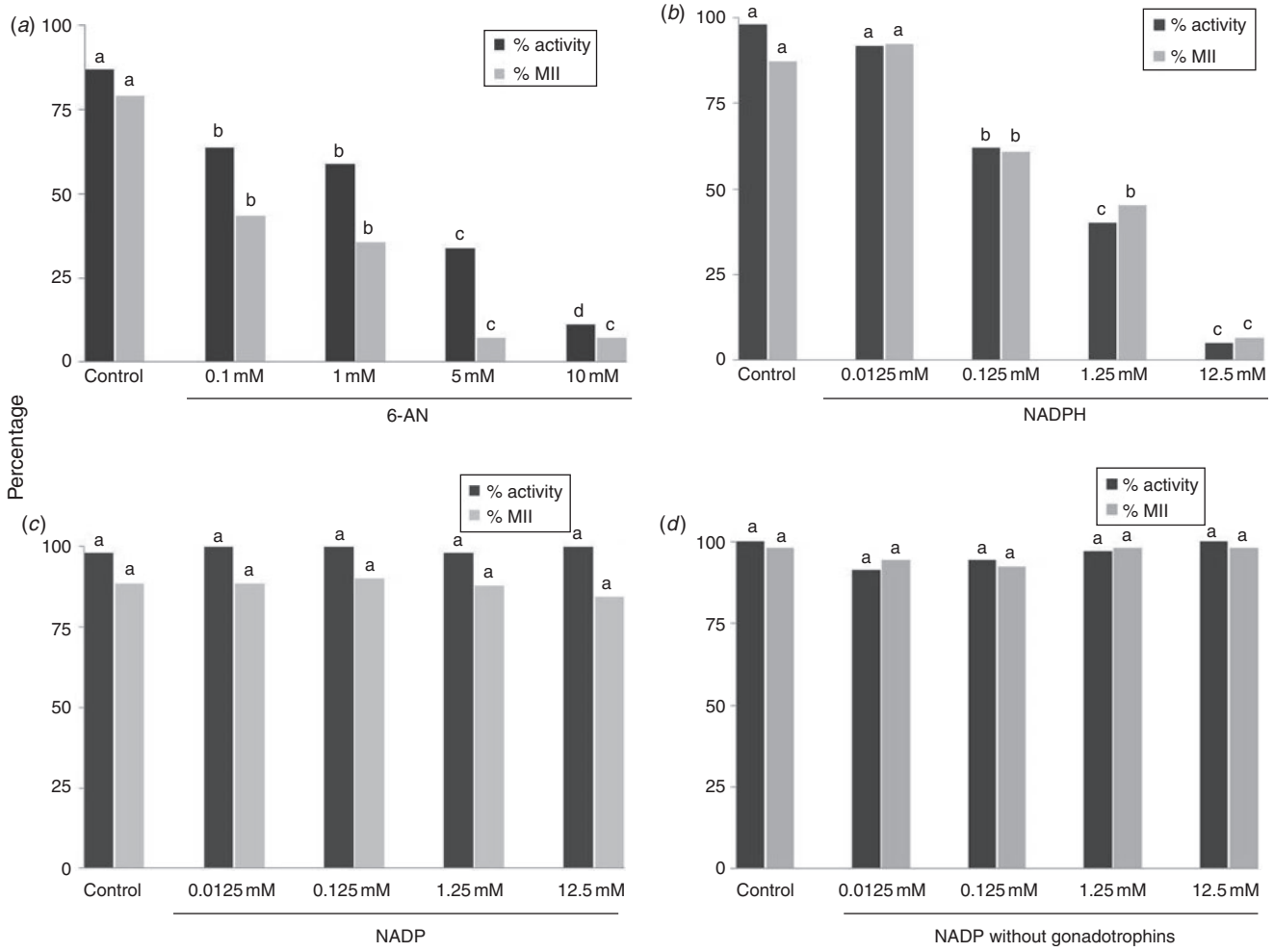

Fig. 2. Proportion of cumulus–oocyte complexes (COCs) with an active pentose phosphate pathway (PPP) and oocyte nuclear maturation rate following incubation with different concentrations of (*a*) 6-aminonicotinamide (6-AN), (*b*) NADPH, (*c*) NADP and (*d*) NADP without gonadotropin supplementation. Data show mean values ($n = 30$–40 COCs for each treatment in four replicates). Bars of the same colour with different letters differ significantly ($P < 0.05$).

Glucose uptake and lactate production

The addition of 6-AN to the maturation medium induced a dose-dependent inhibition of glucose uptake by COCs ($P < 0.05$). Lactate production diminished approximately 50% in the presence of 6-AN at all concentrations tested ($P < 0.05$; Fig. 3*a*).

The addition of NADPH or NADP did not modify glucose uptake and lactate production by COCs (Fig. 3*b*, *c*). Nevertheless, NADP at 12.5 mM NADP (the highest concentration) produced an increase in both glucose uptake and lactate production when it was added in the absence of gonadotropins ($P < 0.05$; Fig. 3*d*).

Oocyte competence

Because meiotic maturation was not affected by the presence of NADP during IVM, IVF and embryo development were evaluated to determine possible subsequent effects. A concentration of 12.5 mM NADP in the maturation medium (in the presence or absence of gonadotropins) was used in these experiments because this was the concentration that stimulated glucose uptake. Supplementation of the maturation medium with NADP did not modify cleavage rates compared with respective controls. Nevertheless, differences due to hormonal supplementation were observed ($P < 0.05$; Fig. 4*a*).

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

Oocyte chromatin morphology

To determine which stage of nuclear maturation was affected by inhibition of the PPP in COCs, oocytes were also analysed with the fluorochrome Hoechst 33342 at different time points. In the control group, 70.8% of oocytes had undergone GVBD after 9 h maturation, whereas oocytes matured in the presence of 6-AN or NADPH exhibited lower rates of GVBD ($P < 0.05$). Furthermore, progression to MI was significantly reduced by 6-AN or NADPH treatment at 15 h maturation ($P < 0.05$). 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 germinal vesicle (GV) or MI stages (Table 1; Fig. 1*c*). In the presence of NADP, oocytes were equally capable of reaching MII at 22 h maturation compared with control, so we did not pursue analysis of oocyte nuclear morphology with this treatment.

Oxidative activity of the oocyte

To determine the impact of PPP activity in COCs 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

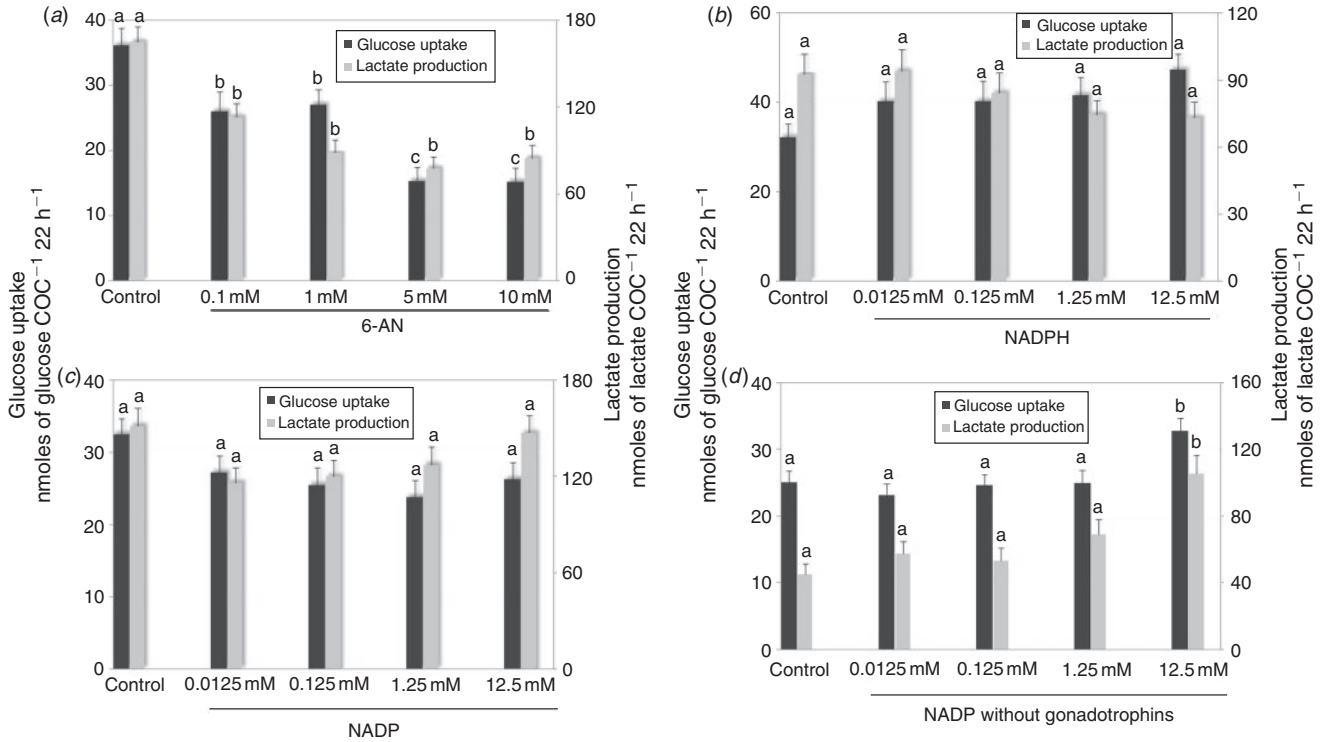

Fig. 3. Glucose uptake and lactate production in cumulus–oocyte complexes (COCs) matured with different concentrations of (*a*) 6-aminonicotinamide (6-AN), (*b*) NADPH, (*c*) NADP and (*d*) NADP without gonadotropin supplementation. Data are the mean ± s.e.m. ($n = 30$–40 COCs for each treatment in four replicates). Bars of the same colour with different letters differ significantly ($P < 0.05$).

NADPH and 5 mM 6-AN were used as 50% inhibitory concentrations of PPP activity. Because NADP did not produce a significant increase in the PPP activity, the concentration of NADP that increased glucose uptake by COCs was used in these experiments (i.e. 12.5 mM).

Oocytes exhibited variations in oxidative activity throughout maturation in the control group (Fig. 1*b*). Similar values were detected at 0 and 9 h, but a marked increase was seen at 15 h ($P < 0.05$), followed by a decrease to lower values at 22 h ($P < 0.05$; Fig. 5*a–c*). The addition of 6-AN to the maturation medium changed the pattern of oxidative activity compared with the control group; specifically, in the presence of 6-AN, a significant decrease of the oxidative activity was seen at 15 h ($P < 0.05$; Fig. 5*a*). In the presence of NADPH, oxidative activity diminished significantly at 15 and 22 h maturation ($P < 0.05$; Fig. 5*b*). Conversely, the addition of NADP induced

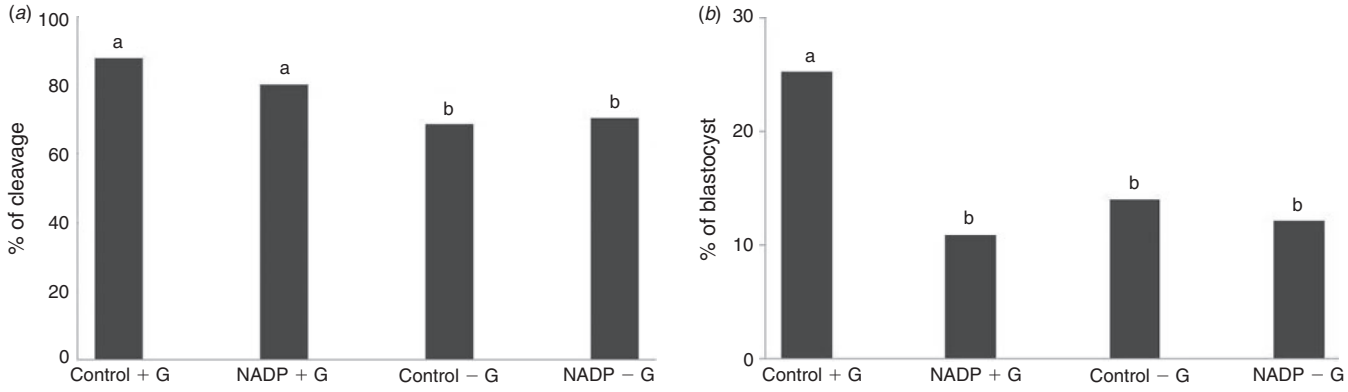

Fig. 4. (*a*) Cleavage rate following IVF and (*b*) blastocyst rate from oocytes matured with 12.5 mM NADP with and without gonadotropins (G). Data show mean values ($n = 107–134$ cumulus–oocyte complexes for each treatment in four replicates). Bars with different letters differ significantly ($P < 0.05$).

Table 1. Effects of 5 mM 6-aminonicotinamide (6-AN), 1.25 mM NADPH and 12.5 mM NADP on nuclear morphology

Data show the percentage of oocytes at each stage of development ($n = 15–20$ oocytes for each treatment in three replicates). Different superscript letters indicate 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

Duration of maturation (h)	Control					NADPH					6-AN				
	GV	GVBD	MI	AN	PBE	GV	GVBD	MI	AN	PBE	GV	GVBD	MI	AN	PBE
0	100^{a}					100^{a}					100^{a}				
9	29.2^{a}	70.8^{a}				56.7^{a}	43.3^{b}				92.59^{b}	7.41^{c}			
15	5.13^{a}	0^{a}	94.87^{a}			27.4^{b}	29.1^{b}	43.5^{b}			46.43^{c}	0^{a}	53.57^{b}		
22	2.5^{a}		12.5^{a}	5^{ab}	80^{a}	18.3^{b}		36.6^{b}	0^{a}	45.1^{b}	6.89^{a}		62.08^{c}	17.24^{b}	13.79^{c}

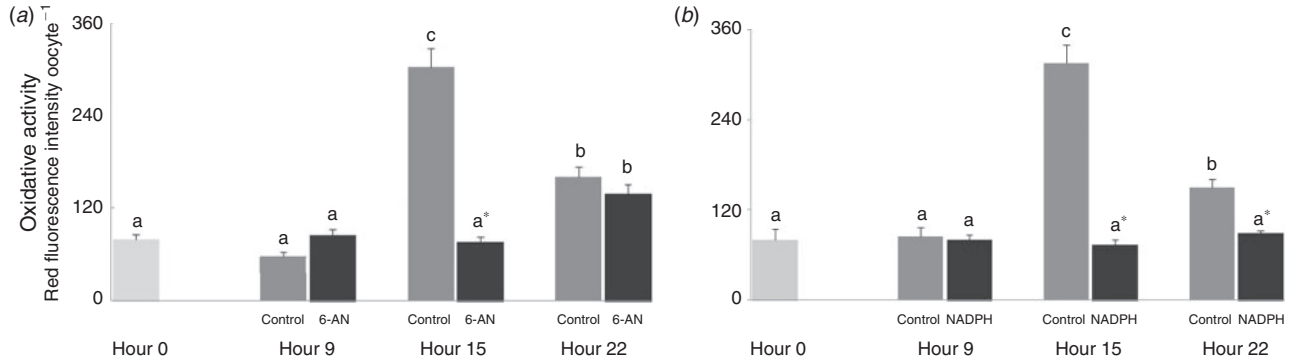

Fig. 5. Oxidative activity within oocytes matured in the presence of (*a*) 5 mM 6-aminonicotinamide (6-AN), (*b*) 1.25 mM NADPH or (*c*) 12.5 mM NADP. Data are the mean ± s.e.m. ($n = 30–40$ COCs for each treatment in three replicates). Bars of the same colour with different letters differ significantly ($P < 0.05$).

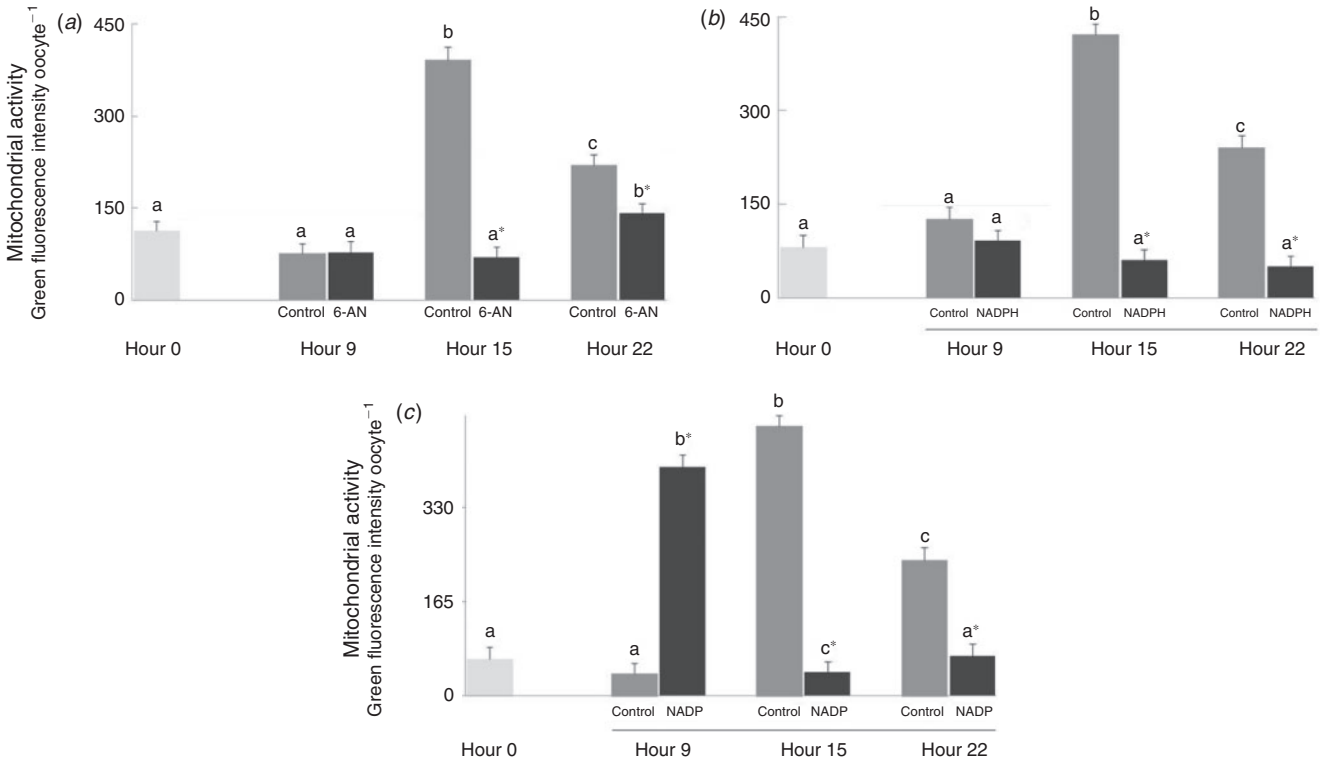

Fig. 6. Mitochondrial activity within oocytes matured in the presence of (*a*) 5 mM 6-aminonicotinamide (6-AN), (*b*) 1.25 mM NADPH or (*c*) 12.5 mM NADP. Data are the mean ± s.e.m. ($n = 30$–40 COCs for each treatment in three replicates). Bars of the same colour with different letters differ significantly ($P < 0.05$). The asterisks indicate significant differences between treatments at the same time point.

a significant increase in oxidative activity at 9 h maturation ($P < 0.05$) and a significant decrease at 15 and 22 h compared with control ($P < 0.05$; Fig. 5*c*).

Mitochondrial activity in oocytes

To investigate the effects of manipulating PPP activity in COCs on mitochondrial activity within oocytes, the fluorescence intensity of MitoTracker green was analysed at the same time points and using the same concentrations of 6-AN, NADPH and NADP 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 significantly decreased mitochondrial activity at 15 and 22 h maturation compared with control ($P < 0.05$; Fig. 6*a*, *b*). The addition of NADP significantly increased mitochondrial activity at 9 h maturation ($P < 0.05$), but significantly decreased activity at 15 and 22 h maturation compared with control ($P < 0.05$; Fig. 6*c*).

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

Mitochondrial distribution in oocytes

Changes in mitochondrial distribution were observed during maturation in the control group. The distribution of mitochondria in immature oocytes was cortical; however, progressive mitochondrial 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 analysing the ratio between the intensity of green fluorescence the cortical and central areas of the oocyte ($P < 0.05$; Fig. 6*a–c*). The mitochondria in oocytes treated with 6-AN or NADPH did not migrate ($P < 0.05$; Fig. 7*a*, *b*). Furthermore, although mitochondria in oocytes in the control group completed their migration at 15 h, the mitochondria of oocytes treated with NADP completed their migration at 22 h (Fig. 7*c*).

Discussion

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

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

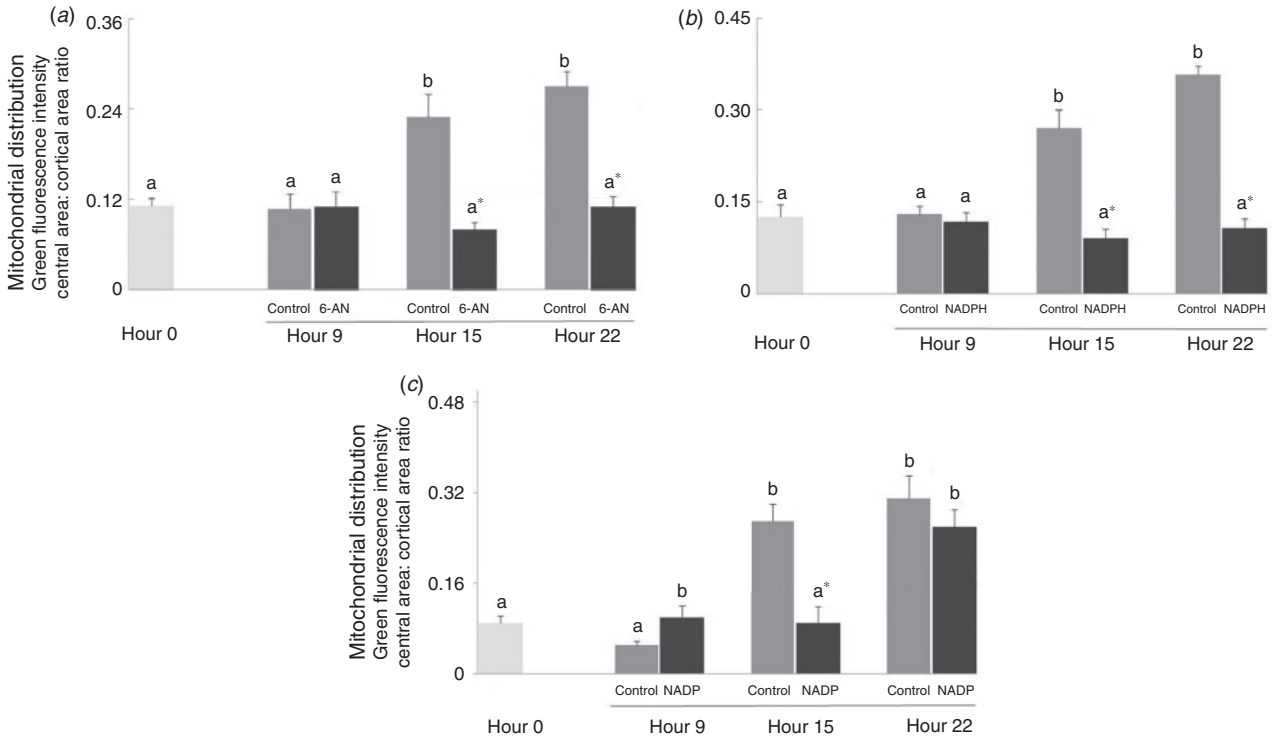

Fig. 7. Active mitochondria distribution within oocytes matured in the presence of (*a*) 5 mM 6-aminonicotinamide (6-AN), (*b*) 1.25 mM NADPH or (*c*) 12.5 mM NADP. Data are the mean ± s.e.m. ($n = 30$–40 COCs for each treatment in three replicates). Bars of the same colour with different letters differ significantly ($P < 0.05$). The asterisks indicate significant differences between treatments at the same time point.

Our findings showed that supplementation of the maturation media with a pharmacological inhibitor of the PPP had an inhibitory dose-dependent effect on glucose uptake and dose-independent inhibitory effect on lactate production. Both results suggest a concomitant inhibitory effect on glycolytic activity. In agreement with these results, indirect inhibition of the oocyte PPP with an inhibitor of NADPH oxidase was accompanied by inhibition of the glycolytic pathway in pig oocytes (Herrick *et al.* 2006). Similar results have been reported following inhibition of the oxidative arm of the PPP in mouse oocytes (Downs *et al.* 1998). It has been reported in somatic cells that inhibition of 6-phosphogluconate dehydrogenase by 6-AN causes the accumulation of 6-phosphogluconate, a metabolite that may be responsible for the inhibition of the glycolytic enzyme phosphoglucose isomerase (Tyson *et al.* 2000). However, we cannot exclude the possible inhibition of glycolytic dehydrogenase activity by 6-AN, although the K_i for 6-phosphogluconate dehydrogenase was the lowest measured and several orders 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 medium did not modify lactate production, suggesting that the mechanism underlying PPP inhibition by NADPH does not involve coinhibition of the glycolytic pathway, as is the case for 6-AN.

The addition of the physiological stimulator of the PPP (NADP) to maturation media with or without gonadotropin supplementation failed to increase the percentage of COCs with an active PPP, suggesting a high activity of the pathway. NADP supplementation seems to be unable to further stimulate the pathway during bovine oocyte IVM. Conversely, an increase in glucose uptake and lactate production was observed when the highest concentration of NADP (12.5 mM) was added to maturation media without gonadotropins, indicating higher glycolytic activity under these maturation conditions. Previously, we reported that glycolysis can be stimulated by the addition of the physiological stimulator AMP to maturation media without gonadotropin supplementation (Gutnisky *et al.* 2012). In the present study, augmented glycolytic activity in the presence of NADP could be explained by the requirement for both NADPH and ATP by the COC. In somatic cells, it has been reported that the flux of glucose through the PPP has four different patterns according to requirements for NADPH, ribose 5-phosphate and ATP. When the cell requires both NADPH and ATP, the end-products of the PPP, namely glyceraldehyde 3-phosphate and fructose 6-phosphate, enter the glycolytic pathway in order to obtain ATP (Berg *et al.* 2002). Although oocytes matured with NADP completed meiotic and cytoplasmic maturation processes, embryo development was impaired, suggesting that high concentrations of NADP may alter the developmental capacity of bovine oocyte.

As reported previously (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 maturation compared with 0 and 9 h 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 medium, oxidative activity remained low throughout maturation, whereas 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 may be due to a change in the NADPH : NADP ratio caused by the inhibition of G6PDH. The activity of this enzyme is important in the regulation of cell redox levels (Tian *et al.* 1998) and in events related to the resumption of meiosis (Harvey *et al.* 2002; Herrick *et al.* 2006). In hamster oocytes, it has been suggested that the PPP is important not only for preventing cell oxidative stress throughout the glutathione system, but also for the maintenance of meiotic spindle morphology by protecting the spindle against oxidative damage (Zuelke *et al.* 1997).

In contrast, the addition of NADP to the maturation medium changed the pattern of oxidative activity compare with control, accelerating the time of the oxidative burst to 9 h maturation. The addition of NADP to the IVM medium may result in precocious activation of the PPP, in this way altering the redox state towards a more oxidative state. In agreement, it has been reported that fully grown oocytes at the GV stage have low PPP activity (Rodríguez-González *et al.* 2002). This shift in the redox state within the oocyte towards a more oxidised state may contribute to the subsequent impaired embryo developmental competence. We propose that a precocious oxidative burst causes the oxidation of some target molecules implicated in events related to embryo development. It has been observed that several transcription factors involved in diverse developmental processes are regulated by the intracellular redox potential (Imai *et al.* 2000; Dickinson and Forman 2002; Rahman *et al.* 2004; Liu *et al.* 2005; Funato *et al.* 2006). The recent discovery that these factors can be sensitive to oxidation (e.g. reactive oxygen species, *S*-glutathionylation, a high NAD(P) : NAD(P)H ratio) is generating new insights into the regulation of embryo development (Dumollard *et al.* 2007). In agreement, it has been reported that low intracellular glutathione concentrations in oocytes may be responsible for the lower embryo developmental capacity in cattle and pig oocytes (de Matos and Furnus 2000; Brad *et al.* 2003).

Inhibition of PPP activity in COCs with 6-AN or NADPH also modified mitochondrial activity during maturation, following the same pattern observed for oxidative activity. These results, together with the high positive correlation between both parameters, confirm that mitochondrial activity and oxidative activity are closely related events. Furthermore, the presence of NADP increased mitochondrial activity at 9 h culture, as was also observed for oxidative activity. Coincidentally, it has been suggested that cytosolic redox state is linked to mitochondrial activity and mitochondrial redox state in ovine embryos (Lieber 1991; Thompson *et al.* 1993). Conversely, it cannot be denied that the addition of PPP modulators may also be affecting NADP-IDH and therefore impacting on the oxidative status and mitochondrial activity of the oocyte. Previously, observed that the activity of both G6PDH and NADP-IDH is similar in cumulus cells and oocytes in the bovine (Cetica *et al.* 2002, 2003).

During maturation, mitochondria migrated from the cortical area towards a more central distribution in untreated oocytes (control group), as reported previously (Gutnisky *et al.* 2012). Inhibition of PPP activity with 6-AN or NADPH also inhibited migration of the mitochondria, suggesting that mitochondrial activity is linked to mitochondrial redistribution. In agreement, it has been 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 mitochondrial migration, which may be related to developmental competence because we have demonstrated that NADP affects the blastocyst rate. In line with these results, it has been proposed that regulation of mitochondrial distribution and segregation may be involved in the complexity of the segmentation axis during early cleavage (Tarazona *et al.* 2006).

In conclusion, we have reported that PPP activity in bovine COCs is necessary for successful meiotic and cytoplasmic maturation of the bovine oocyte. Fluctuations in the oxidative and mitochondrial activities in the oocyte 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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References

Alm, H., Torner, H., Lohrke, B., Viergutz, T., Ghoneim, I. M., and Kanitz, W. (2005). Bovine blastocyst development rate *in vitro* is influenced by selection of oocytes by brillant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology* **63**, 2194–2205. doi:10.1016/J.THERIOGENOLOGY.2004.09.050

Arzondo, M. M., Caballero, J. N., Marin Briggiler, C. I., Dalvit, G., Cetica, P. D., and Vazquez-Levin, M. H. (2012). Glass wool filtration of bull cryopreserved semen: a rapid, effective method to obtain a high percentage of functional spermatozoa. *Theriogenology* **78**, 201–209. doi:10.1016/J.THERIOGENOLOGY.2012.02.001

Barham, D., and Trinder, P. (1972). An improved colour reagent for the determiantion of glucose by the oxidase system. *Analyst* **97**, 142–145. doi:10.1039/AN9729700142

Berg, D. K., Thompson, J. G., and Asher, G. W. (2002). Development of *in vitro* embryo production systems for red deer (*Cervus elaphus*). Part 2. The timing of *in vitro* nuclear oocyte maturation. *Anim. Reprod. Sci.* **70**, 77–84. doi:10.1016/S0378-4320(01)00200-7

Bhojwani, S., Vajta, G., Callesen, H., Roschlau, K., Kuwer, A., Becker, F., Alm, H., Torner, H., Kanitz, W., and Poehland, R. (2005). Developmental competence of HMC(TM) derived bovine cloned embryos obtained from somatic cell nuclear transfer of adult fibroblasts and granulosa cells. *J. Reprod. Dev.* **51**, 465–475. doi:10.1262/JRD.17025

Bhojwani, S., Alm, H., Torner, H., Kanitz, W., and Poehland, R. (2007). Selection of developmentally competent oocytes through brilliant cresyl blue stain enhances blastocyst development rate after bovine nuclear transfer. *Theriogenology* **67**, 341–345. doi:10.1016/J.THERIOGENOLOGY.2006.08.006

Brad, A. M., Bormann, C. L., Swain, J. E., Durkin, R. E., Johnson, A. E., Clifford, A. L., and Krisher, R. L. (2003). Glutathione and adenosine triphosphate content of *in vivo* and *in vitro* matured porcine oocytes. *Mol. Reprod. Dev.* **64**, 492–498. doi:10.1002/MRD.10254

Cetica, P., Pintos, L., Dalvit, G., and Beconi, M. (2002). Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation *in vitro*. *Reproduction* **124**, 675–681. doi:10.1530/REP.0.1240675

Cetica, P., Pintos, L., Dalvit, G., and Beconi, M. (2003). Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation *in vitro*. *Reproduction* **126**, 753–763. doi:10.1530/REP.0.1260753

Comizzoli, P., Urner, F., Sakkas, D., and Renard, J. P. (2003). Up-regulation of glucose metabolism during male pronucleus formation determines the early onset of the s phase in bovine zygotes. *Biol. Reprod.* **68**, 1934–1940. doi:10.1095/BIOLREPROD.102.011452

de Matos, D. G., and Furnus, C. C. (2000). The importance of having high glutathione (GSH) level after bovine *in vitro* maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. *Theriogenology* **53**, 761–771. doi:10.1016/S0093-691X(99)00278-2

Dickinson, D. A., and Forman, H. J. (2002). Glutathione in defense and signalling: lessons from a small thiol. *Ann. N. Y. Acad. Sci.* **973**, 488–504. doi:10.1111/J.1749-6632.2002.TB04690.X

Downs, S. M., and Utecht, A. M. (1999). Metabolism of radiolabeled glucose by mouse oocytes and oocyte–cumulus cell complexes. *Biol. Reprod.* **60**, 1446–1452. doi:10.1095/BIOLREPROD60.6.1446

Downs, S. M., Humpherson, P. G., and Leese, H. J. (1998). Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway. *Biol. Reprod.* **58**, 1084–1094. doi:10.1095/BIOLREPROD58.4.1084

Dumollard, R., Ward, Z., Carroll, J., and Duchen, M. R. (2007). Rugulation of redox metabolism in the mouse oocyte and embryo. *Development* **134**, 455–465. doi:10.1242/DEV.02744

Fleming, W. N., and Saacke, R. G. (1972). Fine structure of the bovine oocyte from the mature graafian follicle. *J. Reprod. Fertil.* **29**, 203–213. doi:10.1530/JRF.0.0290203

Funato, Y., Micchiue, T., Asashima, M., and Miki, H. (2006). The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt–β-catenin signalling through disheveled. *Nat. Cell Biol.* **8**, 501–508. doi:10.1038/NCB1405

Gordon, I. (1994). Oocyte recovery and maturation. In 'Laboratory Production of Cattle Embryos'. pp. 31–142. (Cab International: Wallingford, UK.)

Gupte, S. A., Arshad, M., Viola, S., Kaminski, P. M., Ungvari, Z., Rabbani, G., Koller, A., and Wolin, M. S. (2003). Pentose phosphate pathway coordinates multiple redox-controlled relaxing mechanisms in bovine coronary arteries. *Am. J. Physiol. Heart Circ. Physiol.* **285**, H2316–H2326.

Gutnisky, C., Dalvit, G. C., Pintos, L. N., Thompson, J. G., Beconi, M. T., and Cetica, P. D. (2007). Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte *in vitro* maturation, fertilisation and embryo development. *Reprod. Fertil. Dev.* **19**, 488–497. doi:10.1071/RD06134

Gutnisky, C., Morado, S. A., Dalvit, G. C., Thompson, J. G., and Cetica, P. D. (2012). Glycolytic pathway activity: effect on *in vitro* maturation and oxidative metabolism of bovine oocyte. *Reprod. Fertil. Dev.* [Epub ahead of print]. doi:10.1071/RD12193

Harvey, A. J., Kind, K. L., and Thompson, J. G. (2002). REDOX regulation of early embryo development. *Reproduction* **123**, 479–486. doi:10.1530/REP.0.1230479

Herrick, J. R., Brad, A. M., and Krisher, R. L. (2006). Chemical manipulation of glucose metabolism in porcine oocytes: effects on nuclear and cytoplasmic maturation *in vitro*. *Reproduction* **131**, 289–298. doi:10.1530/REP.1.00835

Imai, S., Johnson, F. B., Marciniak, R. A., Mc Vey, M., Park, P. U., and Guarente, L. (2000). Sir2: an NAD-dependent histone deacetylase that connects chromatin silencing, metabolism, and aging. *Cold Spring Harb. Symp. Quant. Biol.* **65**, 297–302. doi:10.1101/SQB.2000.65.297

Köhler, E., Barrach, H.-J., and Neubert, D. (1970). Inhibition of NADP dependent oxidoreductases by the 6-aminonicotinamide analogue of NADP. *FEBS Lett.* **6**, 225–228. doi:10.1016/0014-5793(70)80063-1

Kruip, T., Cran, D., Beneden, T., and Dieleman, S. (1983). Structural changes in bovine oocytes during final maturation *in vivo*. *Gamete Res.* **8**, 29–47. doi:10.1002/MRD.1120080105

Lieber, C. S. (1991). Alchool, liver, and nutrition. *J. Am. Coll. Nutr.* **10**, 602–632.

Liu, H., Colavitti, R., Rovira, I. I., and Finkel, T. (2005). Redox dependent transcriptional regulation. *Circ. Res.* **97**, 967–974. doi:10.1161/01.RES.0000188210.72062.10

Nelson, D. L., and Cox, M. M. (2005) Glycolysis, gluconeogenesis and the pentose phosphate pathway. In 'Lehninger. Principles of Biochemistry', 4th edn. (Eds K. Ahr and M. Ryan.) pp. 522–557. (W.H. Freeman and Co.: New York.)

Özer, N., Bilgi, C., and Ögüs, H. (2002). Dog liver glucose-6-phosphate dehydrogenase: purification and kinetic properties. *Int. J. Biochem. Cell Biol.* **34**, 253–262. doi:10.1016/S1357-2725(01)00125-X

Pandolfi, P. P., Sonati, F., Rivi, R., Mason, P., Grosveld, F., and Luzzatto, L. (1995). Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO J.* **14**, 5209–5215.

Rahman, I., Marwick, J., and Kirkham, P. (2004). Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-κB and pro-inflammatory gene expression. *Biochem. Pharmacol.* **68**, 1255–1267. doi:10.1016/J.BCP.2004.05.042

Rodríguez-González, E., Lopez-Bejar, M., Velilla, E., and Paramio, M. T. (2002). Selection of prepubertal goat oocytes using the brilliant cresyl blue test. *Theriogenology* **57**, 1397–1409. doi:10.1016/S0093-691X(02)00645-3

Sato, H., Iwata, H., Hayashi, T., Kimura, K., Kuwayama, T., and Monji, Y. (2007). The effect of glucose on the progression of the nuclear maturation of pig oocytes. *Anim. Reprod. Sci.* **99**, 299–305. doi:10.1016/J.ANIREPROSCI.2006.05.008

Stanton, R. C. (2012). Glucose 6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB Life* **64**, 362–369. doi:10.1002/IUB.1017

Stojkovic, M., Machado, S., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P., and Wolfi, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture. *Biol. Reprod.* **64**, 904–909. doi:10.1095/BIOLREPROD64.3.904

Sutton, M. L., Cetica, P. D., Beconi, M. T., Kind, K. L., Gilchrist, R. B., and Thompson, J. G. (2003). Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. *Reproduction* **126**, 27–34. doi:10.1530/REP.0.1260027

Sutton-McDowall, M. L., Gilchrist, R. B., and Thompson, J. G. (2005). Effect of hexoses and gonadotrophin supplementation on bovine oocyte nuclear maturation during *in vitro* maturation in a synthetic follicle fluid medium. *Reprod. Fertil. Dev.* **17**, 407–415. doi:10.1071/RD04135

Sutton-McDowall, M. L., Gilchrist, R. B., and Thompson, J. G. (2010). The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* **139**, 685–695. doi:10.1530/REP-09-0345

Takahashi, Y., and First, N. L. (1992). *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**, 963–978. doi:10.1016/0093-691X(92)90096-A

Tarazona, A. M., Rodriguez, J. I., Restrepo, L. F., and Olivera-Angel, M. (2006). Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced *in vitro*. *Reprod. Domest. Anim.* **41**, 5–11. doi:10.1111/J.1439-0531.2006.00615.X

Tarkowski, A. K. (1966). An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics* **5**, 394–400. doi:10.1159/000129914

Thompson, J. G. (2006). The impact of nutrition of the cumulus oocyte complex and embryo on subsequent development in ruminants. *J. Reprod. Dev.* **52**, 169–175. doi:10.1262/JRD.17089

Thompson, J. G., Bell, A. C., Pugh, P. A., and Trevit, H. R. (1993). Metabolism of pyruvate by pre-elongation sheep embryos and effect of pyruvate and lactate concentrations during culture *in vitro*. *Reprod. Fertil. Dev.* **5**, 417–423. doi:10.1071/RD9930417

Tian, W. N., Braunstein, L. D., Pang, J., Stuhlmeier, K. M., Xi, Q. C., Tian, X., and Stanton, R. C. (1998). Importance of glucose-6-phosphate dehydrogenase activity for cell growth. *J. Biol. Chem.* **273**, 10 609–10 617. doi:10.1074/JBC.273.17.10609

Trinder, P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann. Clin. Biochem.* **6**, 24–27.

Tyson, R. L., Perron, J., and Sutherland, G. R. (2000). 6-Aminonicotinamide inhibition of the pentose phosphate pathway in rat neocortex. *Neuroreport* **11**, 1845–1848. doi:10.1097/00001756-200006260-00009

Wakefield, S. L., Lane, M., Schulz, S. J., Hebart, M. L., Thompson, J. G., and Mitchell, M. (2008). Maternal supply of omega-3 polyunsaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse. *Am. J. Physiol. Endocrinol. Metab.* **294**, E425–E434. doi:10.1152/AJPENDO.00409.2007

Zuelke, K. A., Jones, D. P., and Perreault, S. D. (1997). Glutathione oxidation is associated with altered microtubule function and disrupted fertilization in mature hamster oocytes. *Biol. Reprod.* **57**, 1413–1419. doi:10.1095/BIOLREPROD57.6.1413