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Implications of glycolytic and pentose phosphate pathways on the oxidative status and active mitochondria of the porcine oocyte during IVM

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A B S T R A C T

Glycolysis and the pentose phosphate pathway (PPP) were modulated in porcine cumulus-oocyte complexes during IVM by the addition of inhibitors and stimulators of key enzymes of the pathways to analyze their influence on the oxidative status, active mitochondria, and maturation of the oocyte. The influence of pharmacologic and physiological inhibitors of glycolysis (Sodium fluoride and ATP) and PPP (6-Aminonicotinamide and nicotinamide adenine dinucleotide phosphate) was validated by assessing glucose and lactate turnover and brilliant cresyl blue staining in oocytes. Inhibitors of glycolysis and PPP activity significantly perturbed nuclear maturation, oxidative metabolism (Redox Sensor Red CC-1), and active mitochondria (Mitotracker Green FM) within oocytes ($P < 0.05$). In comparison, physiological stimulators of glycolysis (adenosine monophosphate) and PPP (nicotinamide adenine dinucleotide phosphate) did not affect any of evaluated parameter. In the absence of modulators, fluctuations in the oocyte oxidative activity and active mitochondria were observed during porcine IVM. The inhibition of glycolysis and PPP modified the pattern of oxidation and mitochondrial fluctuation, resulting in impaired meiotic progression. We demonstrated the relationship between carbohydrate metabolism in COC and oocyte redox status necessary for porcine oocyte IVM.

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1. Introduction

In the pig, the addition of glucose to the maturation medium accelerates the meiotic progression of oocytes [1] and increases the percentage of oocytes that complete nuclear maturation, reaching the metaphase II (MII) nuclear stage [2,3]. In addition, glucose metabolism is important for oocyte cytoplasmic maturation, which in turn is necessary for embryo development [4].

The glycolytic pathway is one of the main fates for glucose consumed by murine, bovine, and porcine cumulus-oocyte complexes (COCs) [4–8]. Cumulus cells metabolize glucose, producing glycolytic metabolites pyruvate and/or lactate, which can be further metabolized by the oocyte [8–10]. In somatic cells, the major regulatory point of the glycolytic pathway is the enzyme phosphofructokinase 1 (EC 2.7.1.11), with adenosine monophosphate (AMP) and ATP having important positive and

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negative allosteric regulating roles, respectively [11,12]. Sodium fluoride (NaF) is also a well known inhibitor of the pathway, inactivating the glycolytic enzyme enolase (EC 4.2.1.11; [13]). The intermediary metabolism of glucose also produces the reducing equivalent NADH. Within cumulus cells, NADH is produced by glyceraldehyde 3-phosphate dehydrogenase (glycolysis) and by the oocyte via the reaction catalyzed by α-ketoglutarate dehydrogenase and malate dehydrogenase. In addition to being a cofactor for anabolic pathways, NADH is a key redox molecule and is important in both cytosolic and mitochondrial redox regulation [14]. The redox state describes a complex relationship between oxidized and reduced forms of a large number of molecules, including NAD(P):NAD(P)H, FAD:$FADH_2$ and reduced glutathione:glutathione disulfide (for reviews, see [15,16]).

Alternatively, glucose can be oxidized via the pentose phosphate pathway (PPP), which is linked to the regulation of oocyte nuclear maturation [3,5]. In somatic cells, the major regulatory point of the PPP is glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49), with the NADP:NADPH ratio having an important regulatory role [17]. Furthermore, G6PDH is competitively inhibited by nicotinamide adenine dinucleotide phosphate (NADPH) [18]. 6-Aminonicotinamide (6-AN) is a pharmacologic inhibitor of the PPP that suppresses the two nicotinamide adenine dinucleotide phosphate (NADP)-requiring enzymes of the pathway, namely G6PDH and 6-phosphogluconate dehydrogenase [19]. 6-Aminonicotinamide can replace the nicotinamide moiety of pyridine nucleotides, with the resulting metabolite inhibiting the pyridine nucleotide–linked reactions in a competitive manner [19,20].

The PPP has several metabolic goals: (1) to produce NADPH for reductive synthesis; (2) to yield ribose-5-phosphate as a nucleotide precursor; and (3) to prevent oxidative stress throughout the glutathione and thioredoxin systems, and thus regulating the redox intracellular state [21]. Other sources of NADPH are the reactions catalyzed by the NADP-dependent isocitrate dehydrogenase and malic enzyme. However, in G6PDH-deficient cell lines, the activity of these enzymes is not sufficient to replace the PPP derived NADPH [22]. Conversely, in mouse oocytes, the main source of NADPH seems to be the NADP-dependent isocitrate dehydrogenase [14].

We hypothesize that COC carbohydrate metabolism is one of the main contributing factors for oocyte oxidative status and directly influences the amount of active mitochondria required for the maturation of the oocyte. The aim of the present study was to investigate the effect of inhibitors and stimulators of glycolysis and PPP during porcine oocyte IVM on the oxidative status, active mitochondria, and maturation of the oocyte.

2. Materials and methods

2.1. Materials

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

2.2. Recovery of COCs

Ovaries from slaughtered gilts were transported in a warm environment (28 °C–33 °C) for the 2 to 3 hours journey to the laboratory. Ovaries were washed in 0.9% (wt:vol) NaCl containing 100,000 IU/L penicillin and 100 mg/L streptomycin. cumulus-oocyte complexes were aspirated from 3 to 8 mm antral follicles by using a 10 mL syringe and an 18-gauge needle, and oocytes surrounded by a dense cumulus were selected.

2.3. Oocyte IVM

Cumulus-oocyte complexes were cultured in medium 199 (Earle's salts, L-glutamine, 2.2-mg/L sodium bicarbonate; GIBCO, Grand Island, NY, USA) supplemented with 10% (vol:vol) porcine follicular fluid, 0.57-mM cysteine, 50-mg/L gentamicin sulfate, and 0.5-mg/L porcine follicle-stimulating hormone (Folltropin-V; Bioniche, Belleville, Ontario, Canada) plus 0.5-mg/L porcine luteinizing hormone (Lutropin-V; Bioniche; control medium) under mineral oil at 39 °C for 44 hours in a 5% CO_2 atmosphere [23]. Glycolytic antagonists (5-mM NaF and 10-mM ATP) and agonists (40-mM AMP) and PPP antagonists (0.025-mM 6-AN, 0.125-mM NADPH) and agonists (12.5-mM NADP) were added separately to control culture media. The concentrations of each modulator were chosen on the basis of 50% inhibition of the respective pathway in a previous work [24].

To investigate the effects of manipulating carbohydrate metabolism in COCs on subsequent meiotic progression, the oocyte nuclear morphology was evaluated at 0, 24, 32, 40, and 44 hours of maturation. These time points were chosen because they are temporally associated with key events of the maturation process, namely germinal vesicle breakdown, metaphase I (MI), extrusion of the first polar body, and MII, respectively [25]. To evaluate meiotic progression, COCs were incubated in 1-g/L hyaluronidase in PBS medium for 5 minutes at 37 °C, and the oocytes were mechanically denuded by gentle pipetting. Oocytes were fixed for 15 minutes (2% glutaraldehyde in PBS), cultured with 1% Hoechst 33342 in PBS for 15 minutes, washed in PBS containing 1-mg/mL polyvinylpyrrolidone, and mounted on glass slides. Oocytes were examined under an epifluorescence microscope using 330 to 380-nm (excitation) and 420-nm (emission) filters at × 250 and × 400 magnification and allocated in the different meiotic stages according to nuclear configuration. Twenty to 21 COCs were used for each treatment in three replicates to evaluate the effect of glycolysis modulators on nuclear morphology and three additional replicates for PPP modulators.

2.4. Evaluation of glycolytic activity in COCs

To evaluate glycolytic activity in COCs during IVM, glucose consumption and lactate production per COC was determined. Twenty-eight to 30 COCs were used for each treatment in three replicates. Cumulus-oocyte complexes were individually matured in 20-μL droplets of culture medium for 44 hours, and the glucose and lactate concentrations in the spent maturation medium were

assessed. Lactate concentration was measured using a spectrophotometric assay on the basis of oxidation of this compound by lactate oxidase and the subsequent determination of the hydrogen peroxide formed [26], and glucose concentrations in the spent maturation medium were determined in a similar manner, except using glucose oxidase [26,27]. Twenty-microliter droplets of maturation medium without cells were included in each experiment to provide glucose and lactate reference concentrations, and glucose consumption and lactate production were expressed as nmol/COC/44h.

2.5. Evaluation of PPP activity in COCs

To evaluate PPP activity during IVM in COCs, the Brilliant Cresyl Blue (BCB) test for immature oocytes was performed [28] with some modifications to be adapted to the porcine oocyte IVM. Twenty-eight to 30 COCs were used for each treatment in three replicates. Groups of 30 COCs were matured in 600-μL droplets of culture medium for 41 hours and then transferred for the last 3 hours of IVM to the same culture medium containing 4.8 μM of BCB. At the completion of the culture, oocytes were denuded as previously described and separated into two different groups according to their cytoplasmic coloration: BCB-positive oocytes (with blue cytoplasmic coloration) indicate a low activity of PPP, whereas BCB-negative oocytes (with no blue cytoplasmic coloration) indicate a high activity of PPP.

2.6. Evaluation of oxidative activity and active mitochondria in oocytes

The oxidative activity and quantification of active mitochondria in the oocyte were evaluated at 0, 24, 32, 40, and 44 hours of maturation. Thirty to 40 COCs were used for each treatment in three replicates to evaluate the effect of glycolysis modulators and three additional replicates for PPP modulators. Cumulus cells were removed mechanically by repeated pipetting in PBS with 1-g L^{-1}hyaluronidase, and the zona pellucida was dissolved with 5-g L^{-1} pronase for 1 minute. Oocytes were coincubated with 1-nM Redox Sensor red CC-1 and 0.5-nM Mitotracker green FM (Invitrogen/Molecular Probes, Eugene, Oregon, USA), for 30 minutes at 37 °C in the dark and then washed twice in PBS. Stained oocytes were mounted on glass slides, and images were captured with an epifluorescence microscope (Zeiss, Germany) at × 400 magnification using excitation and/or emission ∼540/600 nm filters for Redox Sensor Red CC-1 and excitation and/or emission ∼490/520 nm filters for Mitotracker green FM. All microphotographs were analyzed using Image J 1240 software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA), measuring the brightness of each oocyte (Fig. 1).

2.7. Statistical analysis

The proportion of COCs with an active PPP and oocyte meiotic maturation were compared using a chi-squared test for nonparametric data. Data for glucose uptake, lactate production, oxidative activity, and active mitochondria are expressed as the mean ± standard error of the mean. In studies evaluating glucose uptake and lactate production, comparisons were made by analysis of variance followed by the Bonferroni post-test. Oxidative activity and active mitochondria were compared using a two × five factorial design. In all tests, $P < 0.05$ was considered significant.

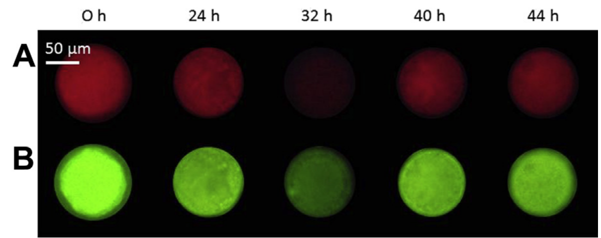

Fig. 1. Oocytes stained with two fluorescent probes, namely (A) Redox Sensor red CC-1 (Molecular Probes, Eugene, OR, USA) and (B) Mitotracker green FM (Molecular Probes), after different times of maturation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Glycolytic activity in COCs and oocyte maturation

Lactate, the end product of glycolysis and glucose were measured in IVM medium to assess the activity of glycolysis in porcine COCs in the presence of the different modulators. When matured in the presence of NaF and ATP, glucose uptake by COCs was at least 2-fold lower than the control, respectively, (Table 1; $P < 0.05$). Likewise, lactate production was also reduced when COCs were exposed to NaF and ATP (Table 1; $P < 0.05$). NaF having the most profound effect on glucose and lactate levels inducing 3.2 less glucose consumption and 3.2-fold less lactate production compared to the control group. However, AMP supplementation did not affect glucose and lactate levels (Table 1).

Inhibition of glycolysis with NaF and ATP resulted in delayed progression of nuclear maturation (Table 2). In the presence of NaF and ATP, the percentage of oocytes remaining at germinal vesicle (GV) stage after 24 hours of

Table 1
Effect of glycolysis modulators on COC glycolytic pathway activity.

Metabolite measurement	Control	NaF	ATP	AMP
Lactate production (nmol per COC 44 h)	81.0 ± 13.2^a	25.4 ± 5.9^b	41.4 ± 13.3^b	83.2 ± 14.4^a
Glucose uptake (nmol per COC 44 h)	29.8 ± 5.1^a	8.1 ± 2.2^b	7.4 ± 3.6^b	28.5 ± 3.5^a

Data show the mean ± standard error of the mean lactate production and glucose uptake per COC during 44 h of culture in the presence of 5-mM NAF, 10-mM ATP, and 40-mM AMP (n = 28–30 COCs for each treatment in three replicates). Different superscript letters indicate significant differences in the same line.
Abbreviations: COC, cumulus-oocyte complex; NaF, sodium fluoride.

Table 2
Effect of glycolysis modulators on oocyte nuclear morphology.

Duration of maturation (h)	Control				NaF				ATP				AMP			
	GV	GVBD	MI	MII	GV	GVBD	MI	MII	GV	GVBD	MI	MII	GV	GVBD	MI	MII
0	98.3^a	1.7^a	0^a	0^a	96.7^a	3.3^a	0^a	0^a	98.3^a	1.7^a	0^a	0^a	100^a	0^a	0^a	0^a
24	15^a	65^a	20^a	0^a	35^b	56.7^a	8.3^a	0^a	70^c	30^b	0^b	0^a	18^a	70^a	12^a	0^a
32	5^a	30^a	65^a	0^a	15^a	46.7^a	38.3^b	0^a	55^b	45^a	0^c	0^a	3^a	32^a	65^a	0^a
40	1.7^a	1.7^a	33.3^a	63.3^a	5^a	16.7^b	36.7^a	41.7^b	50^b	20^b	26.7^a	3.3^c	1.7^a	0^a	35^a	63.3^a
44	1.6^a	1.6^a	8.2^a	88.5^a	5^a	0^a	43.3^b	51.7^b	50^b	15^b	0^c	35^b	0^a	1.6^a	9.8^a	88.6^a

Data show the percentage of oocytes at each stage of development ($n = 20–21$ oocytes for each treatment in three replicates). Different superscript letters indicate significant differences in the percentage of oocytes at the same time point and the same nuclear stage between treatments.
Abbreviations: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II; NaF, sodium fluoride.

culture was significantly higher than that in control and AMP groups (Table 2; $P < 0.05$). At 32 hours of maturation, the percentage of oocytes at MI was lower in the presence of NaF and ATP than that in control and AMP groups ($P < 0.05$). At the end of maturation period (44 hours), the percentage of oocytes at MII was lower in the presence of NaF and ATP than that in control and AMP groups ($P < 0.05$), with NaF blocking progression from MI in 43.3% of oocytes, and ATP blocked 50% of oocytes from progressing beyond the GV stage (Table 2).

3.2. The impact of glycolysis in COCs on oocyte oxidative activity

To determine the impact of glycolytic activity in COCs on oxidative status within the oocyte, denuded oocytes were stained with Redox Sensor Red to quantify oxidative activity during maturation. Oxidative activity within oocytes fluctuated throughout maturation in control group and was significantly lower between 24 and 32 hours, with the lowest activity observed at 32hours. Oxidative activity then recovered to levels similar to 0 hours by 40 and 44 hours ($P < 0.05$). A similar pattern of oxidative activity was seen with the addition of NaF. However, oxidative activity remained low at 40 and 44 hours of maturation ($P < 0.05$, Fig. 2A). ATP supplementation significantly reduced oxidative activity from 24 to 44 hours ($P < 0.05$, Fig. 2B). Oxidative activity within the oocyte did not change from control group in presence of AMP (Fig. 2C).

3.3. Glycolytic activity in COCs and oocyte active mitochondria

To determine the impact of glycolytic activity in COCs on the mitochondrial quantification within the oocyte, the fluorescence intensity of Mitotracker Green within the oocytes was analyzed at different time points. Oocyte active mitochondria showed a similar pattern of variations as oxidative activity throughout maturation in control group, with mitochondrial fluorescence decreasing after 24 hours, was at its lowest at 32 hours and then increased to levels similar to 24 hours by 40 and 44 hours ($P < 0.05$). The addition of NaF induced the decrease in mitochondrial fluorescence at 40 and 44 hours of maturation ($P < 0.05$, Fig. 3A), whereas in the presence of ATP, mitochondrial fluorescence decreased at 24, 40, and 44 hours ($P < 0.05$, Fig. 3B). Mitochondrial fluorescence within the oocyte did not change from control group in presence of AMP (Fig. 3C).

3.4. PPP activity in COCs and oocyte maturation

BCB was used to evaluate PPP activity in COCs after 44 hours culture in the presence of PPP modulators, with BCB-negative oocytes (clear) indicating active PPP within the oocyte. 6-Aminonicotinamide and NADPH induced a decrease in percentage of COCs with active PPP compared to that of the control group (control = 91.9% vs. 6-AN = 58.3% and NADPH = 50% BCB-/total oocytes; $P < 0.05$). However, NADP supplementation did not influence the proportion of BCB- oocytes (85.2% BCB-/total oocytes; $n = 28–30$ oocytes for each treatment in three replicates).

The presence of PPP inhibitors 6-AN and NADPH delayed the resumption of meiosis, with significantly more oocytes remaining at the GV stage after 24 hours of culture and MI stage at 32 hours compared to that of the control and NADP groups ($P < 0.05$). After 44 hours of culture, the percentage of oocytes at MII were lower in the presence of 6-AN and NADPH than that in control and NADP groups ($P < 0.05$), with a large proportion of oocytes remaining at the MI stage when exposed to either of the PPP inhibitors (Table 3).

3.5. PPP activity in COCs and oocyte oxidative activity

Denuded oocytes were stained with Redox Sensor Red to quantify oxidative activity at different time points. The presence of PPP inhibitors significantly reduced oxidative activity within oocytes compared to that of the control group (Fig. 4). 6-Aminonicotinamide supplementation decreased in oocyte oxidative activity between 24 and 44 hours of maturation ($P < 0.05$, Fig. 4A), whereas in the presence of NADPH, oxidative activity decreased at 24, 40, and 44 hours ($P < 0.05$, Fig. 4B). Oxidative activity within the oocyte did not change from control group in presence of NADP (Fig. 4C).

3.6. PPP activity in COCs and oocyte active mitochondria

To determine the impact of PPP activity in COCs on the mitochondrial fluorescence of the oocyte, the intensity of Mitotracker Green within the oocytes was analyzed at different time points. Oocyte mitochondrial fluorescence showed the same pattern of variations as oxidative activity throughout maturation in control group ($P < 0.05$). The addition of 6-AN and NADPH induced lower mitochondrial fluorescence at 24, 40, and 44 hours of maturation

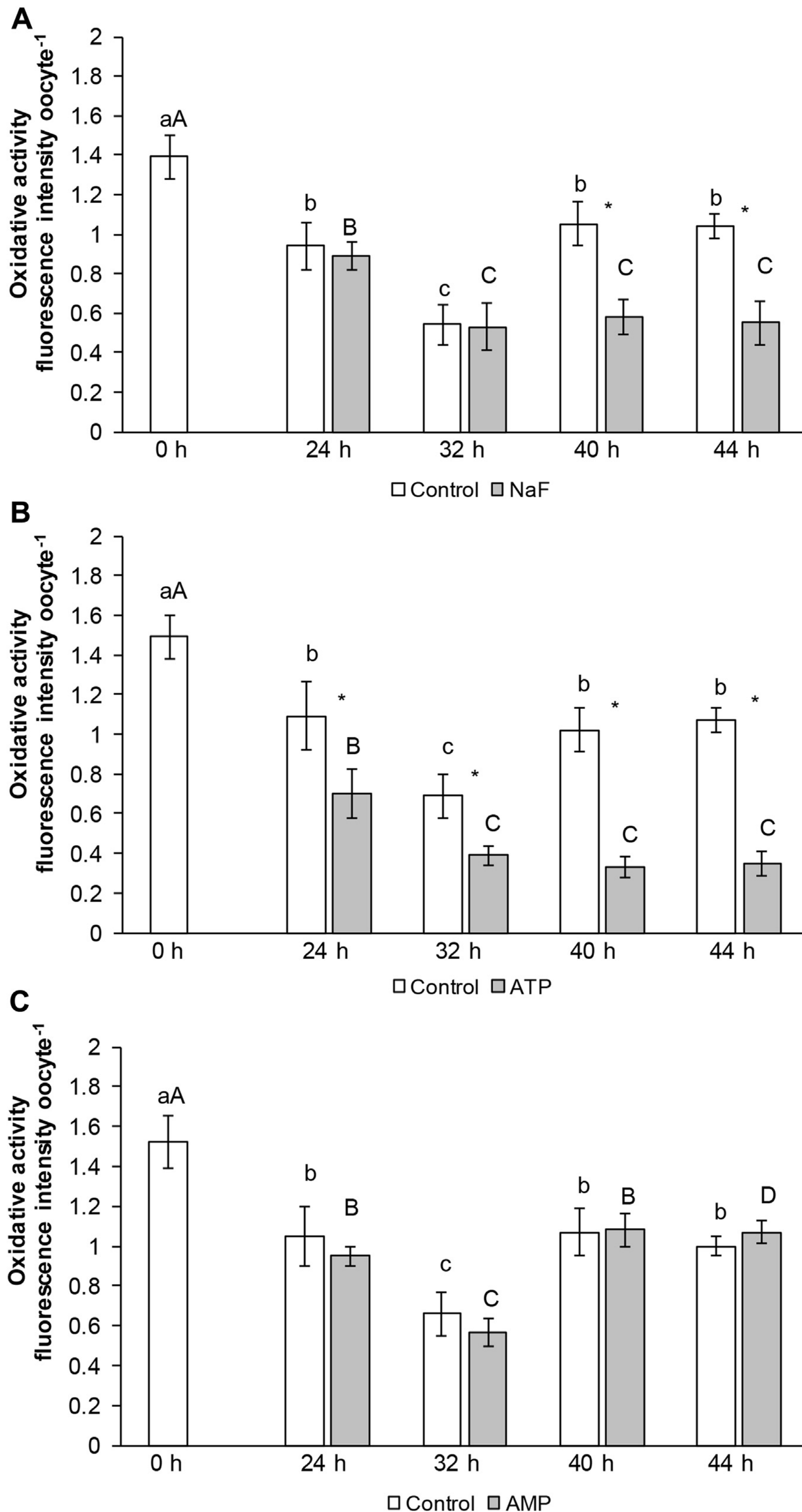

Fig. 2. Oxidative activity within oocytes matured in the presence of (A) 5-mM sodium fluoride (NaF), (B) 10-mM ATP, and (C) 40-mM AMP. Data are the mean ± standard error of the mean (n = 30–40 cumulus-oocyte complexes for each treatment in three replicates). Bars of the same color with different letters differ significantly ($P < 0.05$). *Indicates differences at the same time point between treatments ($P < 0.05$).

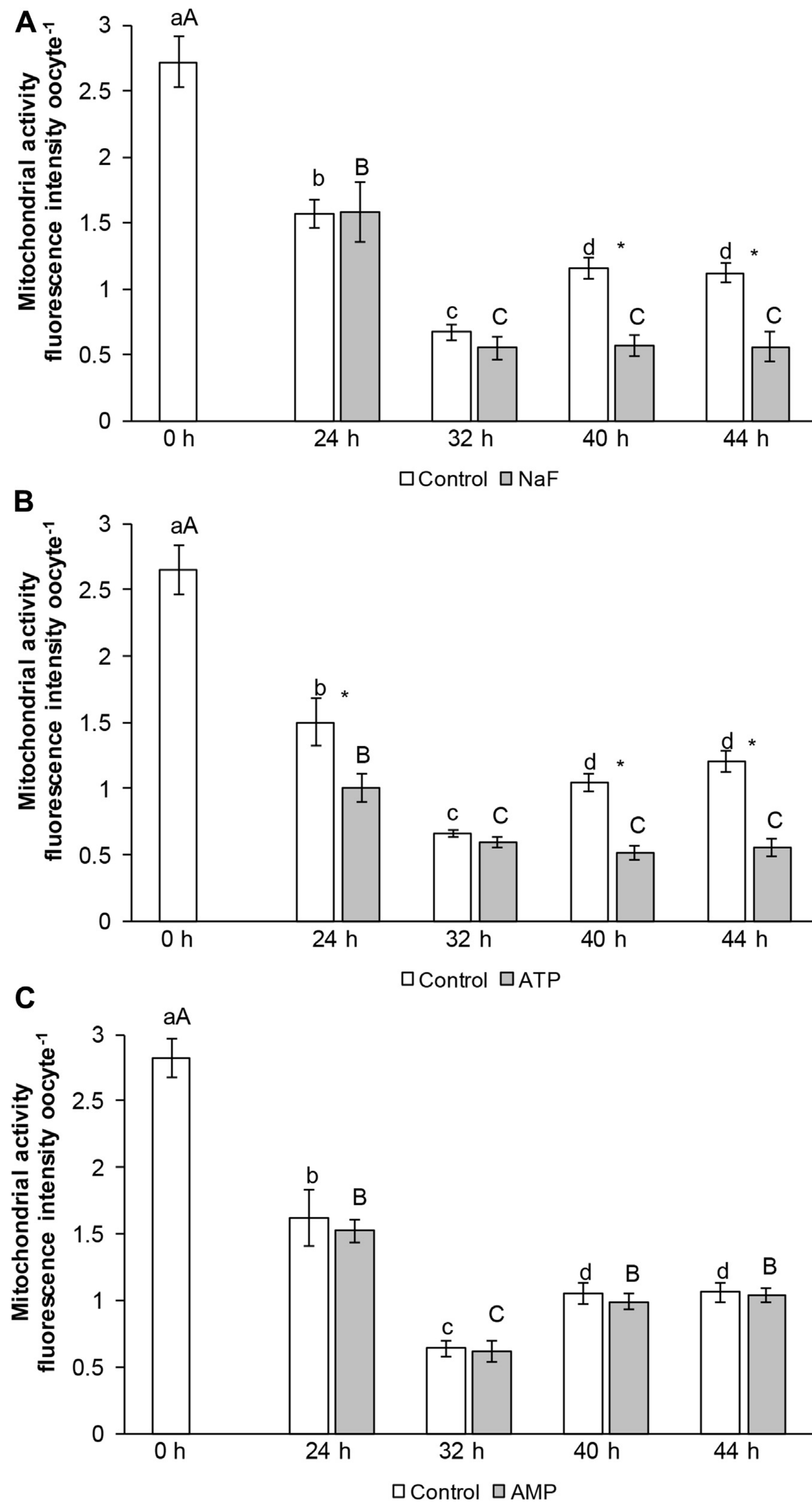

Fig. 3. Mitochondrial fluorescence within oocytes matured in the presence of (A) 5-mM sodium fluoride (NaF), (B) 10-mM ATP, and (C) 40-mM AMP. Data are the mean ± standard error of the mean (n = 30–40 cumulus-oocyte complexes for each treatment in three replicates). Bars of the same color with different letters differ significantly ($P < 0.05$). *Indicates differences at the same time point between treatments ($P < 0.05$).

Table 3
Effect of PPP modulators on nuclear morphology.

Duration of maturation (h)	Control				6-AN				NADPH				NADP			
	GV	GVBD	M I	M II	GV	GVBD	M I	M II	GV	GVBD	M I	M II	GV	GVBD	M I	M II
0	96.7^a	3.3^a	0^a	0^a	96.7^a	3.3^a	0^a	0^a	98.3^a	1.7^a	0^a	0^a	98.3^a	1.7^a	0^a	0^a
24	10.0^a	66.7^a	23.3^a	0^a	71.7^b	21.7^b	6.7^{ab}	0^a	54.1^c	32.8^b	13.1^a	0^a	16^a	71^a	13^a	0^a
32	3.3^a	28.3^a	68.3^a	0^a	15^b	55^b	30^b	0^a	15^b	40^{ab}	45^b	0^a	4.2^a	32.8^a	63^a	0^a
40	1.7^a	1.7^a	30^{ab}	66.7^a	5^a	40^b	45^a	10^c	0^a	40^b	20^b	40^b	1.7^a	2.5^a	32.5^a	63.3^a
44	1.7^a	1.7^a	8.3^a	88.3^a	1.7^a	10^a	30^b	58.3^b	4.9^a	8.2^a	29.5^b	57.4^b	0^a	2^a	8.3^a	89.7^a

Data show the percentage of oocytes at each stage of development (n = 20–21 oocytes for each treatment in three replicates). Different superscript letters indicate significant differences in the percentage of oocytes at the same time point and the same nuclear stage between treatments.
Abbreviations: 6-AN, 6-aminonicotinamide; GV, germinal vesicle; GVBD, germinal vesicle breakdown; M I, metaphase I; M II, metaphase II; PPP, pentose phosphate pathway.

($P < 0.05$, Fig. 5A and B). Mitochondrial fluorescence within the oocyte did not change from control group in presence of NADP (Fig. 5C).

4. Discussion

The addition of glucose to the maturation medium accelerates the meiotic progression and increases the MII rate of porcine oocytes cultured *in vitro* [1–3]. The present study describes the effects of glycolytic and PPP modulators during porcine oocyte IVM on the oocyte active mitochondria, oxidative activity, and maturation rate of the oocytes. The modulation of glycolysis and PPP in COCs demonstrated the impact of these pathways on oxidative status and active mitochondria within the oocyte and on the subsequent oocyte maturation.

Fluctuations in oxidative metabolism and active mitochondria of porcine oocytes were observed during IVM for the first time. In addition, both parameters exhibited a similar pattern of variations during maturation. Fluorescence analysis showed a fall in oxidative metabolism and mitochondrial fluorescence from the beginning until 32 hours of maturation, followed by an increase at 40 hours of IVM. It has been proposed that the metabolic activity of the oocyte increases in the latter half of the meiotic maturation process [7], therefore, oxidative activity and oocyte active mitochondria may be reflecting the metabolic activity at this maturation time.

Inhibition of glycolysis by pharmacologic and physiological modulators, NaF and ATP, was confirmed by the decrease in lactate production and glucose consumption by porcine COCs. The reduced glycolytic activity, in presence of inhibitors, seems to provoke lower oxidative activity and mitochondrial fluorescence within the porcine oocyte during IVM, compared with untreated COCs. This inhibition also affected oocyte maturation rate. The percentage of oocytes at MII after 44 hours of culture was lower in the presence of both inhibitors, a large proportion of oocytes blocked at the MI stage with NaF and in GV stage with ATP. Previously, we saw the decrease on oocyte maturation rate by the inhibition of glycolysis [24]. Thereafter, we hypothesize that lack of substrate provision to oocyte by cumulus cells is mediating this phenomenon. It is known that glycolysis is high in cumulus cells to allow the generation of ATP and produce pyruvate, lactate, malate, and/or oxalacetate, which are readily used as oxidative substrates by the oocyte [6,9,29,30]. However, oocyte use mainly aerobic metabolic pathways, and glycolysis is almost undetectable in this cell. Therefore, in the present study, we intend to assess strictly the oocyte metabolism by measuring oxidative and mitochondrial activities. Now, we can further propose that the observed decrease in oocyte maturation rate is due to a lack of substrates provided from cumulus glycolytic activity to the gamete during the inhibition of the pathway, which impairs oxidative status and oocyte active mitochondria. On the other hand, AMP did not modify neither glycolytic activity in COCs nor oxidative status and active mitochondria in oocytes. The concentration of AMP used in this study was similar or higher than that reported previously to be effective for stimulating phosphofructokinase 1 [31–33].

Our findings showed that the addition of the pharmacologic and physiological inhibitors of PPP, 6-AN, and NADPH to the maturation media is effective to diminish the percentage of COCs with high activity of the pathway. The low PPP activity seems to reduce the oxidative metabolism and mitochondrial fluorescence of the oocyte compared with untreated COCs. Furthermore, a close relationship between PPP activity and maturation process in the porcine oocyte has been proposed [16]. PPP is a primary factor for the progression of nuclear maturation [1], and the flux of glucose throughout the PPP influences the resumption of oocyte nuclear maturation in mouse COC [34] and the progression of all stages of meiosis, including the resumption of meiosis, MI–MII transition, and the resumption of meiosis after fertilization [16,35]. However, the results reported in this study show for the first time the implications of oxidative metabolism and oocyte active mitochondria on the meiotic progression of the oocyte.

In addition, the activity of PPP is important in the regulation of cell redox levels [21] and in events related to the resumption of meiosis [15,16]. In hamster oocytes, the PPP is important not only for preventing cell oxidative stress by regenerating reduced from oxidized glutathione but also for meiotic spindle morphology by protecting the spindle against oxidative damage [36]. Mitochondrial activity is essential for oocyte competence, and the ATP content of oocytes generated from the reducing equivalents derived from carboxylic acid metabolism through the tricarboxylic acid cycle is highly correlated with oocyte competence [14,37]. However, in the present work we found the participation of oocyte oxidative status in the meiotic progress of

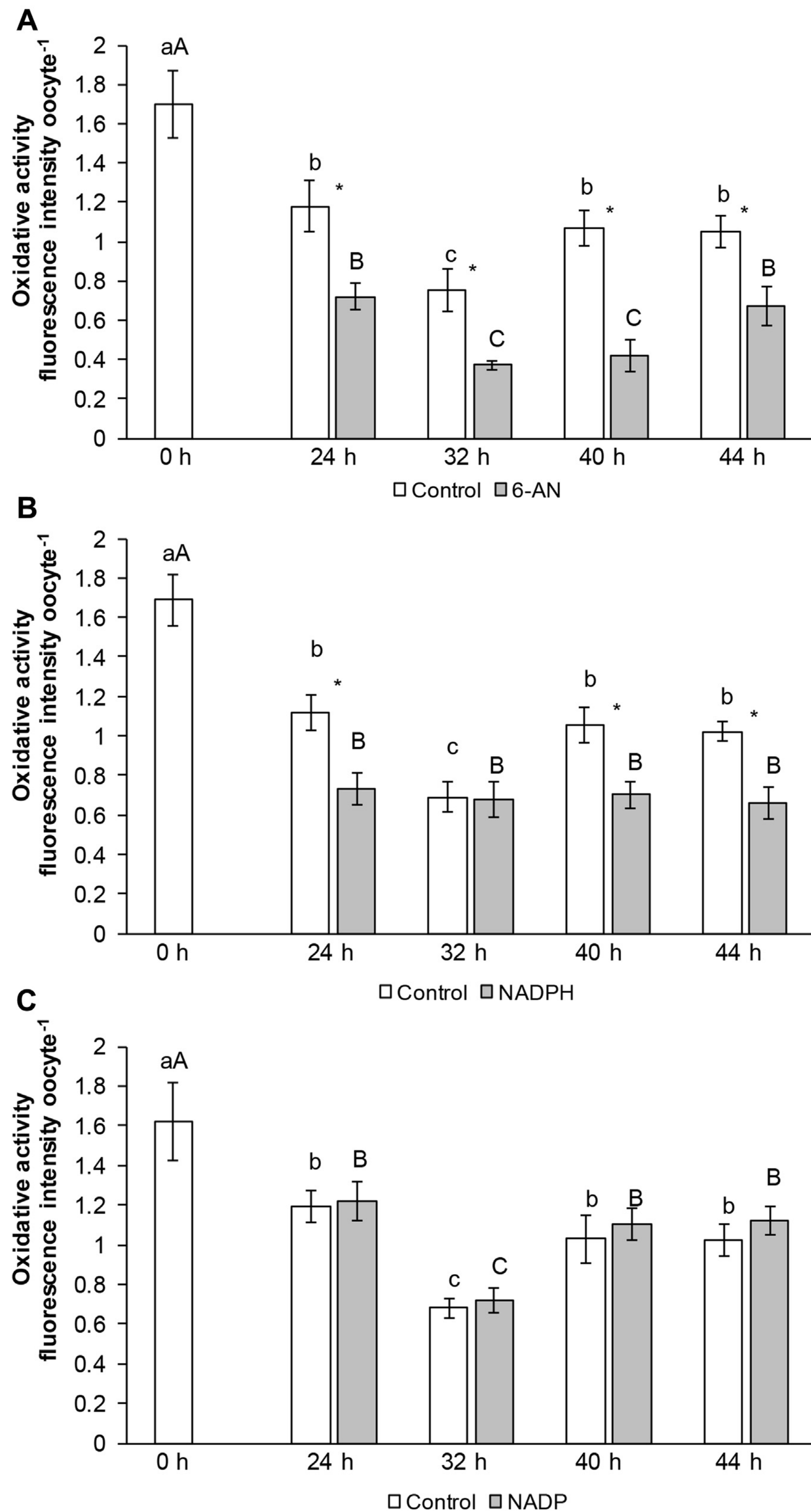

Fig. 4. Oxidative activity within oocytes matured in the presence of (A) 0.025-mM 6-aminonicotinamide (6-AN), (B) 0.125-mM NADPH, and (C) 12.5-mM NADP. Data are the mean ± standard error of the mean (n = 30–40 cumulus-oocyte complexes for each treatment in three replicates). Bars of the same color with different letters differ significantly ($P < 0.05$). *Indicates differences at the same time point between treatments ($P < 0.05$).

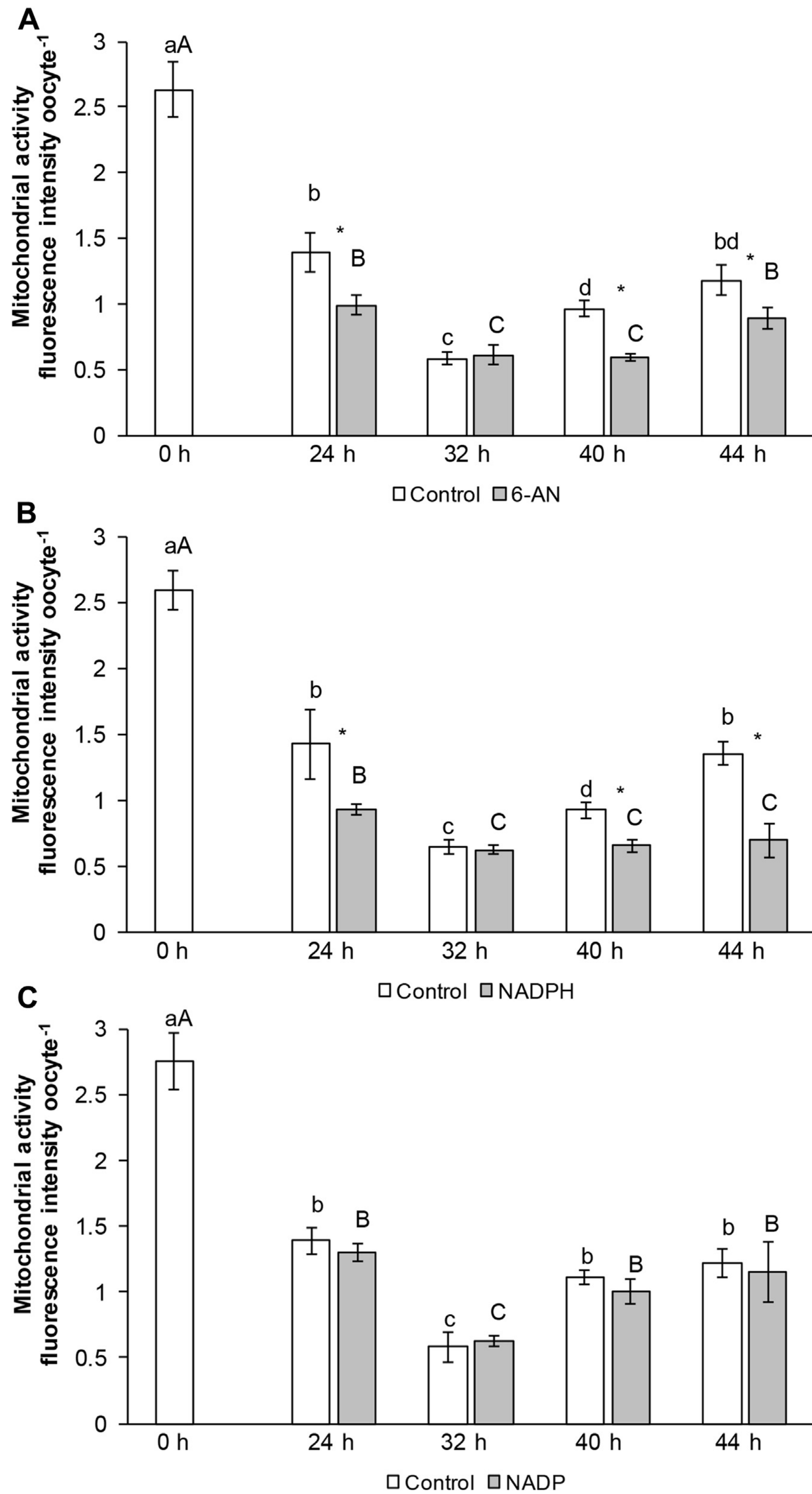

Fig. 5. Mitochondrial fluorescence within oocytes matured in the presence of (A) 0.025-mM 6-aminonicotinamide (6-AN), (B) 0.125-mM NADPH, and (C) 12.5-mM NADP. Data are the mean ± standard error of the mean (n = 30–40 cumulus-oocyte complexes for each treatment in three replicates). Bars of the same color with different letters differ significantly ($P < 0.05$). *Indicates differences at the same time point between treatments ($P < 0.05$).

the oocyte. The addition of NADP, a physiological stimulator of PPP, in the IVM medium had caused no effect on the percentage of COCs with high activity of this metabolic route. PPP activity seems to be high during porcine oocyte maturation, and NADP supplementation seems to be unable to further stimulate this pathway. Therefore, we did not observe any modification to either oxidative activity or mitochondrial fluorescence in the presence of NADP, despite the high concentration used here compared to the one reported to be effective in stimulating the enzyme glucose-6-phosphate dehydrogenase [18] and the PPP [38].

We observed the effect of ATP and NADPH in the cumulus cell and oocyte metabolism. The granulosa-cumulus-oocyte mass is considered a structural and functional syncytium. This notion is on the basis of cell-to-cell communication mediated by gap junctions; these junctions mediate the coupling granulosa cells to cumulus and cumulus to oocyte [39,40]. The crossing of small molecules such as choline, uridine, inositol, nucleosides, nucleotides, and so forth through these gap junctions has been reported [41–45]. The pull out of COCs by aspiration from antral follicles may produce a precocious disruption of the coupling between the COC and granulosa cells, maintaining the permeability of the gap junctions and allowing ATP and NADPH incorporation by the COC.

In conclusion, we have reported for the first time the fluctuations in the oocyte oxidative status and oocyte active mitochondria during porcine oocyte IVM. The pattern of fluctuation is modified by the inhibition of glycolysis and PPP in COCs; furthermore, this condition impaired meiotic progression. We demonstrated the relationship between carbohydrate metabolism in COC and oocyte redox status necessary for porcine oocyte maturation. Increasing knowledge in metabolic oocyte requirements during IVM will allow the improvement of culture conditions to increase the embryo production rates obtained with current technologies.

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Competing interests

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

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