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IMPLICATIONS OF GLYCOLYTIC AND PENTOSE PHOSPHATE PATHWAYS ON THE OXIDATIVE STATUS AND MITOCHONDRIAL ACTIVITY OF THE PORCINE OOCYTE DURING IN VITRO MATURATION

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Running title: carbohydrates and oxidative metabolism in porcine IVM

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Abstract

The glycolysis and the pentose phosphate pathway (PPP) were modulated in porcine cumulus-oocyte complexes (COCs) during in vitro maturation (IVM) by the addition of inhibitors and stimulators of key enzymes of the pathways, with the aim of analyzing their participation and influence on the oxidative status, mitochondrial activity and maturation of the oocyte. The influence of Glycolysis and PPP in COCs were evaluated by lactate production–glucose uptake and by the Brilliant Cresyl Blue test, respectively. Oocyte oxidative and mitochondrial activities were evaluated by Redox Sensor Red CC-1 and MitoTracker Green FM, respectively. Pharmacological and physiological inhibitors of glycolysis (NaF and ATP) and PPP (6-AN and NADPH) were validating by assessing glucose and lactate turnover and brilliant cresyl blue staining in oocytes. Modulators of glycolysis and PPP activity significantly perturbed nuclear maturation, oxidative metabolism (Redox Sensor Red CC-1) and mitochondrial mass (MitoTracker Green FM) within oocytes (P < 0.05). In comparison, oocyte nuclear maturation rate and oxidative and mitochondrial activities decreased in the presence of the pharmacological (NaF) or the physiological (ATP)-inhibitors of glycolysis (P<0.05). The pharmacological (6-AN) and the physiological (NADPH) inhibitors of PPP induced a decrease in the oocyte nuclear maturation rate and oxidative and mitochondrial activities (P<0.05). The physiological stimulators of glycolysis (AMP) and PPP (NADP) caused no effect on any of evaluated parameter. In the absence of modulators, we found fluctuations in the oocyte oxidative activity and mitochondrial activities mass were observed during porcine IVM.
The inhibition of glycolysis and PPP modified the pattern of oxidation and mitochondrial fluctuation, and this condition resulting in impaired meiotic progression. We demonstrated the relationship between carbohydrate metabolism in COC and oocyte redox status necessary for porcine oocyte IVM.

Key words: glycolysis, pentose phosphate pathway, oxidative status, mitochondria, oocyte, pig.

Introduction

In the porcine species 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]. Additionally, glucose metabolism is important for oocyte cytoplasmic maturation, which in turn is necessary for embryo development [4].

The glycolytic pathway has been proposed as is one of the main fates for the glucose consumed by murine, bovine and porcine cumulus-oocyte complexes (COCs) [4-8]. Evidence suggests that cumulus cells metabolize glucose, producing glycolytic metabolites, mainly pyruvate and/or lactate, which are can be incorporated and further metabolized by the oocyte during maturation [8-10]. In somatic cells, the major regulatory
point of the glycolytic pathway is the enzyme phosphofructokinase 1 (EC 2.7.1.11), with AMP and ATP having important positive and 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, this NADH is produced metabolite is mainly synthesized by cumulus cells in the glycolytic pathway 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 used as 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 oxidised 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 alternatively oxidized through the pentose phosphate pathway (PPP), which appears to be 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]. It was also proposed that G6PDH is competitively inhibited by NADPH [18]. 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 [19]. 6-AN 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\cite{21}. 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-sufficient to replace the PPP production of derived NADPH \cite{22}. Conversely, in mouse oocytes, the main source of NADPH seems to be the NADP-IDH\cite{14}.

We hypothesize that COC carbohydrate metabolism is one of the main contributing factors for oocyte oxidative status and directly influences mitochondrial activity 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 in vitro maturation on the oxidative status, mitochondrial activity and maturation of the oocyte.

Materials and Methods

Materials

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

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

Oocyte in vitro maturation

COCs were cultured in medium 199 (Earle’s salts, L-glutamine, 2.2 mg/L sodium bicarbonate; GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) porcine follicular fluid, 0.57 mM cysteine, 50 mg/L gentamicin sulphate 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 h in a 5% CO₂ atmosphere [23].

Different compounds were added to the control medium. Modulators of glycolysis antagonists (5 mM NaF and, 10 mM ATP) and agonists (40 mM AMP) and Modulators of 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

Comment [MM4]: Need to know how many COCs and the volume used for standard IVM, i.e. all the experiments except glucose/lactate
modulator were chosen based on the 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 h of maturation. These time points were chosen because they are temporally associated with key events of the maturation process, namely germinal vesicle breakdown (GVBD), 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 min at 37ºC and the oocytes were mechanically denuded by gentle pipetting. Oocytes were fixed for 15 min (2% glutaraldehyde in PBS), cultured with 1% Hoechst 33342 in PBS stained for 15 mins (1% Hoechst 33342 in PBS) and finally 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 250x and 400x magnification and allocated in the different meiotic stages according to nuclear configuration.

Number of replicates and the number of COCs used were treatment? This needs to be added at the end of each experiment.

Evaluation of glycolytic activity in COCs
To evaluate glycolytic activity in COCs during IVM, glucose consumption and lactate production per COC was determined. COCs were individually matured in 20-µl droplets of culture medium for 44 h, then removed from the droplets and glucose and lactate content concentrations in the spent maturation medium were assessed. Lactate concentration was measured using a spectrophotometric assay based on the oxidation of this compound by lactate oxidase and the subsequent determination of the hydrogen peroxide formed [26] and.

Additionally, glucose uptake concentrations per COC were measured in a similar manner by determining the glucose content of in the spent maturation medium but were determined in a similar manner, except using glucose oxidase [26,27].

Twenty-microlitre 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.

Was a standard curve produced at the same time?

(Have you thought about analysing glucose/lactate after shorter culture times? Just curious!)

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. 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 which had been added with containing 4.8 µM of BCB. At the completion of the culture, oocytes were denuded as previously described and finally separated into two different groups according to their cytoplasmic colouration: BCB-positive oocytes (with blue cytoplasmic colouration) indicate a low activity of PPP, whereas BCB-negative oocytes (with no blue cytoplasmic colouration) indicate a high activity of PPP.

Evaluation of oxidative and mitochondrial activity and mitochondria mass in oocytes

The oxidative and mitochondrial activity and mitochondria mass were evaluated at 0, 24, 32, 40 and 44 h of maturation. The cumulus cells were removed mechanically by repeated pipetting in PBS with 1 g L⁻¹ hyaluronidase and before the zona pellucida was dissolved with 5 g L⁻¹ pronase for 1 min. The dual stains of Redox Sensor red CC-1 and Mito Tracker green FM were used in this experiment. Oocytes were coincubated with a final concentration of 1nM Redox Sensor red CC-1 and 0.5 nM Mito Tracker green FM (did you get both from Invitrogen/Molecular Probes?), for 30 min at 37˚C in the dark and then washed twice in PBS. Stained oocytes were then mounted on glass slides and fluorescence was measured using digital microphotographs obtained with an epifluorescence microscope (company), using excitation/emission ~540/600 nm filters for

Comment [MM5]: Could you please send me a few images of the stained oocytes? We've never been able to get mitotracker green to work. For future work, I highly recommend using a rosamine based dye such as Mitotracker Red CMXRos as Fluoro is dependent on MMP, it works well with live cells and oocyte penetration is a lot stronger. You can also get a deep red version if you want to use it in conjunction with Redox sensor red. To measure MMP, the oocytes need to be live. Fixing and permeabilisation would be killing these oocytes.
Results

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. This induced a decrease in the lactate production and in the glucose uptake per COC 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 9.6-fold less lactate production compared to the control group. However, AMP supplementation did not show any effect on 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 h of culture were significantly higher than in control and AMP groups (Table 2; P<0.05). At 32 h of maturation the percentage of oocytes at MI were lower in the presence of NaF and ATP than in control and AMP groups (P<0.05). At the end of maturation period (44 h) the percentage of oocytes at MII were lower in the presence of NaF and ATP than in control and AMP groups (P<0.05), with NaF inducing a 4.3% of oocytes to be blocked at MI and remaining mainly in MI with NaF, 50% of oocytes exposed to ATP blocked at the GV stage and in germinal vesicle with ATP (Table 2).

The impact of Glycolytic activity in COCs and 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 at different time points during maturation. Oocytes exhibited variations in oxidative activity within oocytes fluctuated throughout maturation in control group, and was significantly lower between 24-32 h, with the lowest activity seen at 32 h, showing a fall until 32 h, followed by an increase at 40 and 44 h. Oxidative activity then recovered to levels similar as 0 h by 40 and 44 h (P<0.05). A similar pattern of oxidative activity was seen with the addition of NaF. However, induced the decrease in oxidative activity remained low at 40 and 44 h of maturation (P<0.05, Fig 1a). ATP supplementation
significantly reduced, while in the presence of ATP, oxidative activity decreased from 24 to 44 h (P<0.05, Fig. 1b). Oxidative activity within the oocyte did not change from control group in presence of AMP (Fig. 1c.).

Glycolytic activity in COCs and oocyte mitochondrial activity

To determine the impact of glycolytic activity in COCs on the mitochondrial activity within the oocyte, the fluorescence intensity of Mito Tracker Green within the oocytes was analyzed at different time points. Oocyte mitochondrial activity showed a similar pattern of variations as oxidative activity throughout maturation in control group, with mitochondrial mass decreasing after 24h, was at its lowest at 32 h and then increased to levels similar to 24h by 40 and 44 h (P<0.05). The addition of NaF induced the decrease in mitochondrial activity at 40 and 44 h of maturation (P<0.05, Fig 2a), whereas in the presence of ATP mitochondrial activity decreased at 24, 40 and 44 h (P<0.05, Fig. 2b). Mitochondrial activity within the oocyte did not change from control group in presence of AMP (Fig. 2c.).

PPP activity in COCs and oocyte maturation

BCB stain was used to evaluate PPP activity in COCs following 44 h cultures in the presence of PPP modulates, with BCB- oocytes (clear) indicating active PPP within the
oocyte. 6-AN and NADPH induced a decrease in percentage of COCs with active PPP compared to 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 show any effect (Table 3) on the proportion of BCB- oocytes (85.2% BCB-/total oocytes).

In the presence of PPP inhibitors 6-AN and NADPH delayed the resumption of meiosis, with significantly more oocytes remaining at the GV germinal vesicle stage after 24 h of culture and MI stage at 32h were compared to higher than in the control and NADP groups (P<0.05). At 32 h of maturation the percentage of oocytes at MI were lower in the presence of 6-AN and NADPH than in control and NADP groups (P<0.05). Following 44 h of culture, at the end of maturation period of 44 h the percentage of oocytes at MII were lower in the presence of 6-AN and NADPH than in control and NADP groups (P<0.05), with a large proportion of oocytes remaining mainly in the MI stage when exposed to either of the PPP inhibitors (Table 4).

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 the control group (figure 3). The addition of 6-AN supplementation induced the decrease in oocyte oxidative activity from between 24 to 44 h of maturation (P<0.05, Fig 3a), whereas in the presence of NADPH, oxidative activity...
decreased at 24, 40 and 44 h (P<0.05, Fig. 3b). Oxidative activity within the oocyte did not change from control group in presence of NADP (Fig. 3c).

**PPP activity in COCs and oocyte mitochondrial activity**

To determine the impact of PPP activity in COCs on the mitochondrial activity of the oocyte, the fluorescence intensity of Mito Tracker Green within the oocytes was analyzed at different time points. Oocyte mitochondrial activity showed the same pattern of variations than oxidative activity throughout maturation in control group (P<0.05). The addition of 6-AN and NADPH induced the decrease mitochondrial activity at 24, 40 and 44 h of maturation (P<0.05, Fig 4a and 4b). Mitochondrial activity within the oocyte did not change from control group in presence of NADP (Fig. 4c.).

**Discussion**

The present study describes the effects of glycolytic and PPP modulators during porcine oocyte IVM on the mitochondrial mass and oxidative activities and maturation rate of the oocytes. The modulation of glycolysis and PPP in COCs demonstrated the impact of these pathways on oxidative and mitochondrial activities within the oocyte and on the subsequent oocyte maturation.
In the present study, fluctuations in oxidative metabolism and mitochondrial activities 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 activities from the beginning until 32 h of maturation, followed by an increase at 40 h of IVM. It has been proposed that the metabolic activity of the oocyte increases in the latter half of the meiotic maturation process\cite{7}, therefore, oxidative and mitochondrial activities may be reflecting the metabolic activity at this maturation time.

Inhibition of glycolysis by pharmacological 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 activities 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 h of culture was lower in the presence of both inhibitors, remaining mainly a large proportion of oocytes blocked at the MI stage with NaF and in germinal vesicle GV stage with ATP. We have already demonstrated the effect of glycolysis inhibition in COCs on oocyte maturation rate\cite{24}; however, now we can further propose the participation of oxidative status and mitochondrial function in this phenomenon. This is probably due to a lack of substrates provided from cumulus glycolytic activity to the gamete during the inhibition of the pathway. It has been suggested that glycolysis is high in cumulus cells in order to allow the generation of ATP and produce pyruvate, lactate, malate and/or oxalacetate, which are readily used as oxidative substrates by the oocyte \cite{6,9,29,30}. On the other hand, AMP
cannot did not modify neither glycolytic activity in COCs nor oxidative and mitochondrial activities in oocytes. The concentration of AMP used in this study was similar or higher than that reported previously to be effective for stimulating phosphofructokinase [31-33].

Our findings showed that the addition of the pharmacological and physiological inhibitors of PPP, 6-AN and NADPH, to the maturation media are 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 activities mass of the oocyte, compared with untreated COCs. We observed in a similarly to previous work the effect of PPP inhibition in COCs on oocyte maturation rate [24]. Furthermore, a close relationship between PPP activity and the maturation process in the porcine oocyte has been proposed [16]. It has been suggested that the PPP is a primary factor for the progression of nuclear maturation [1]. Accordingly, it was demonstrated that the flux of glucose throughout the PPP influences the resumption of oocyte nuclear maturation in mouse COC [34], and 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 fertilization [16,35]. However, the results reported in this study shows for the first time the implications of oxidative metabolism and mitochondrial activities mass 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, 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 [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 (TCA) cycle is highly correlated with oocyte competence[14,37]. However, in the present work we had also demonstrated the participation of oocyte oxidative status in the meiotic progress of 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 cannot observe any modification neither oxidative activity nor mitochondrial activity in the presence of NADP; despite the concentration utilized in the present work was higher than the ones reported to be effective in stimulate the enzyme glucose-6-phosphate dehydrogenase [18] and the PPP[38].

In conclusion, we have reported for the first time the fluctuations in the oocyte oxidative and mitochondrial activities 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.

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

Funding

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Figure legends

Figure 1
Oxidative activity within oocytes matured in the presence of (a) 5 mM NaF, (b) 10 mM ATP and (c) 40 mM AMP. 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). * Indicates differences at the same time point between treatments (P<0.05).

Figure 2

Mitochondrial activity within oocytes matured in the presence of (a) 5 mM NaF, (b) 10 mM ATP and (c) 40 mM AMP. 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). * Indicates differences at the same time point between treatments (P<0.05).

Figure 3

Oxidative activity within oocytes matured in the presence of (a) 0.025 mM 6-AN, (b) 0.125 mM NADPH and (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). * Indicates differences at the same time point between treatments (P<0.05).

Figure 4

Comment [MM11]: Sample number and replicates needs to be added to the methods section
Mitochondrial activity within oocytes matured in the presence of (a) 0.025 mM 6-AN, (b) 0.125 mM NADPH and (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). * Indicates differences at the same time point between treatments (P<0.05).

Bibliography


