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IMPLICATIONS OFGLYCOLYTIC 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 g~~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~~ to ~~analizing~~ analyze their ~~participation~~ 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 Mito Tracker Green FM, respectively.~~ pharmacological and physiological inhibitors of glycolysis (NaF and ATP) and PPP (6-AN and NADPH) we 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~~did not 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.

Comment [MM1]: Mitotracker Green measures mitochondria mass (number), independent of membrane potential (MMP), so it actually doesn't indicate activity. Rosamine based mitotracker dyes (i.e. Mitotracker Red CMX Ros) do measure MMP.

Comment [MM2]: Is this right???

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.

Glucose/lactate results? BCB?

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 c~~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~~ glucose can be ~~alternatively~~ oxidized ~~through~~ via the pentose phosphate pathway (PPP), which ~~appears to be~~is linked to the regulation of oocyte nuclear maturation [3,5]. 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 [17]. ~~It was also proposedthat~~Furthermore, ~~–~~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~~n~~ 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 catalysed by the NADP-dependent isocitrate dehydrogenase (NADP-IDH) and malic enzyme.~~;~~ ~~however~~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 [22]. Conversely, in mouse oocytes, the main source of NADPH seems to be the NADP-IDH[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

Comment [MM3]: Throughout you need to add replicate and sample numbers at the end of each of the experiments.

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 (28-33ºC) 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 39ºC for 44 h in a 5% CO_2 atmosphere [23]. G

~~Different compounds were added to the control medium. Modulators of g~~lycolytic~~sis~~ 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, ie 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)~~ ~~andfinally~~ 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~~ and the glucose and lactate ~~content~~ concentrations in~~of~~ the spent maturation medium were~~as~~ 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, gg~~lucose ~~uptake~~ concentrations ~~per COC was 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~~ 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~~ 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 ~~activitie~~mass ~~s~~ in oocytes

The oxidative ~~and mitochondrial~~ activity and mitochondria mass~~ies~~ were evaluated at 0, 24, 32, 40 and 44 h of maturation. ~~The c~~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 RedoxSensor 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~~were captured ~~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 ooctes? 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 pentration is a lot stronger. You can also get a deep red version if you want to use it in conjuction with Redox sensor red.

To measure MMP, the oocyes need to be live. Fixing,and permeablisation would be killing these oocytes

RedoxSensor Red CC-1 and excitation/emission~490/520 nm filters for MitoTracker green FM. All microphotographs were analysed using Image J 1240 software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA), measuring the brightness of each oocyte.

Comment [MM6]: Magnification? Did you have an standards or any way to control for different replicates/runs?

Comment [MM7]: Mean or maximum intensity?

Stats?

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~~The maturation~~ in the presence of NaF and ATP, glucose uptake by COCs was at least 2-fold lower than the control ~~induced a decrease in the lactate production and in the glucose uptake per COC~~ respectively (Tabel 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. ~~; h~~However, AMP supplementation did not ~~showed no effect~~affect glucose and lactate levels (Table 1).

Comment [MM8]: Add some figures, either the raw data, fold changes etc to make results appear stronger.

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~~ was 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 a~~50% of oocytes exposed to ATP blocked at the GV stage~~nd in germinal vesicle with ATP~~ (Table 2).

The impact of ~~Glycolytic~~ Glycolysis ~~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 O~~oxidative activity within oocytes fluctuated throughout maturation in control group, and was significantly lower between 24-32 h, with the lowest activity seen at 32h. ~~showing a fall until 32 h, followed by an increase at 40 and 44 h~~Oxidative activity then recovered to levels similar levels as 0 h by 40 and 44 h (P<0.05). A similar pattern of oxidative activity was seen with t~~T~~he 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~~mass

To determine the impact of glycolytic activity in COCs on the mitochondrial ~~activity~~ mass within the oocyte, the fluorescence intensity of Mito Tracker Green within the oocytes was analyzed at different time points. Oocyte mitochondrial ~~activity~~ mass showed ~~the~~ a similar ~~same~~ pattern of variations ~~than~~ 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~~ mass at 40 and 44 h of maturation ($P<0.05$, Fig 2a), whereas in the presence of ATP mitochondrial ~~activity~~ mass decreased at 24, 40 and 44 h ($P<0.05$, Fig. 2b). Mitochondrial ~~activity~~ mass 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~~However, NADP supplementation did not ~~showed no effect (Table 3)~~influence the proportion of BCB- oocytes (85.2% BCB-/total oocytes).

~~In t~~The presence of PPP inhibitors 6-AN and NADPH delayed the resumption of meiosis, with significantly more ~~the percentage of~~ 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~~at the MI stage when exposed to ~~with both~~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~~ decreased 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~~mass

To determine the impact of PPP activity in COCs on the mitochondrial ~~activity~~ mass of the oocyte, the fluorescence intensity of Mito Tracker Green within the oocytes was analyzed at different time points. Oocyte mitochondrial ~~activity~~ mass showed the same pattern of variations ~~than in~~as oxidative activity throughout maturation in control group (P<0.05). The addition of 6-AN and NADPH induced ~~the decrease~~lower m~~in m~~itochondrial ~~activity~~ mass at 24, 40 and 44 h of maturation (P<0.05, Fig 4a and 4b). Mitochondrial ~~activity~~ mass 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 activity~~ies~~ 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, f~~Fluctuations in oxidative metabolism and mitochondrial ~~activities~~ mass 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~~ mass 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 [7], therefore, oxidative and mitochondrial activities may be reflecting the metabolic activity at this maturation time.

~~Glycolysis i~~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~~ mass 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 ~~in~~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[24]; however, now we can further propose the participation of oxidative status and mitochondrial ~~function~~ mass 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 g~~Glycolysis is high in cumulus cells ~~order~~ to allow the generation ofe ATP and produce pyruvate, lactate, malate and/or oxalacetate, which are readily used as oxidative substrates by the oocyte [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 phosphofructokinase1[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~~and the flux of glucose throughout the PPP influences the resumption of oocyte nuclear maturation in mouse COC [34]~~.~~ and ~~It has also beenproposed 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~~ 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

Comment [MM9]: Im not sure what this sentence means

Comment [MM10]: A little repetative

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~~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

385 Oxidative activity within oocytes matured in the presence of (a) 5 mM NaF, (b) 10 mM
386 ATP and (c) 40 mM AMP. Data are the mean ± s.e.m. (n = 30-40 COCs for each treatment
387 in three replicates). Bars of the same colour with different letters differ significantly
388 (P<0.05). * Indicates differences at the same time point between treatments (P<0.05).

389

390 **Figure 2**

391 Mitochondrial activity within oocytes matured in the presence of (a) 5 mM NaF, (b) 10 mM
392 ATP and (c) 40 mM AMP. Data are the mean ± s.e.m. (n = 30-40 COCs for each treatment
393 in three replicates). Bars of the same colour with different letters differ significantly
394 (P<0.05). * Indicates differences at the same time point between treatments (P<0.05).

395

396 **Figure 3**

397 Oxidative activity within oocytes matured in the presence of (a) 0.025 mM 6-AN, (b) 0.125
398 mM NADPH and (c) 12.5 mM NADP. Data are the mean ± s.e.m. (n = 30-40 COCs for
399 each treatment in three replicates). Bars of the same colour with different letters differ
400 significantly (P<0.05). * Indicates differences at the same time point between treatments
401 (P<0.05).

402

403 **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 ($P<0.05$).

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