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Relationship between porcine cumulus-oocyte complex energy charge and glycolysis during *in vitro* maturation*

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The aim of the present work was to evaluate the relationship between COC energy and glycolysis in the porcine species. COCs were individually matured in IVM media supplemented with Gonadotropins, Gonadotropins + 0.1 mM ATP and Gonadotropins + 1 mM ATP and without supplementation (control) to modulate glycolytic activity. Glucose uptake and lactate production per COC were enhanced by the presence of FSH and LH in the maturation medium, even in the presence of 0.1 mM ATP. However, 1 mM ATP was effective to inhibit gonadotropin stimulation on glucose consumption, lactate production and nuclear maturation ($p<0.05$). Endogenous ATP concentration was similar in immature COCs and those matured in absence of FSH + LH. However, endogenous ATP concentration increased after 48 h of culture in the presence of gonadotropins ($p<0.05$). Based on these results we determined if glycolytic activity is inhibited by a rise in the COC energy charge. Glucose uptake and lactate production increased from 24 to 48 h of culture ($p < 0.05$), but no differences in these parameters were observed between 48 and 72 h of in vitro

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culture. This study confirmed the stimulation of glycolysis by gonadotropins in porcine COCs and the inhibition of this pathway by the addition of exogenous ATP. We demonstrated the relationship between the gonadotropic stimulation of glycolytic activity and an increase in ATP content in porcine COC during IVM. However, the endogenous ATP content reached in the matured COC seems not to reduce glycolytic activity in the COC.

KEY WORDS: ATP / glucose / maturation / phosphofructokinase

Glycolysis is a universal central pathway of glucose catabolism, the pathway with the largest flux of carbon in most cells. During the sequential reactions of glycolysis, some of the free energy released from glucose is conserved in the form of ATP and NADH. In the overall glycolytic process, one molecule of glucose is converted to two molecules of pyruvate. Two molecules of ADP and two of phosphate are converted to two molecules of ATP. Four electrons, as two hydride ions, are transferred from two molecules of glyceraldehyde 3-phosphate to two of NAD [Nelson and Cox 2005a]. The flux of glucose through the glycolytic pathway is regulated to maintain nearly constant ATP levels (as well as adequate supplies of glycolytic intermediates that serve biosynthetic roles). The required adjustment in the rate of glycolysis is maintained by a complex interplay among ATP consumption, NADH regeneration and allosteric regulation of some regulatory enzymes of the pathway (hexokinase, phosphofructokinase-1 -PFK-1- and pyruvate kinase) and by second-to-second fluctuations in the concentration of key metabolites that reflect the cellular balance between ATP production and consumption [Nelson and Cox 2005a]. Hexokinase is allosterically inhibited by its product, glucose 6-phosphate, thus it is regulated by the cellular concentration of the sugar if it accumulates. PFK-1 and pyruvate kinase are closely linked to cell energy levels. ATP is not only a substrate for PFK-1, but also a negative modulator of the enzyme. The high cellular ATP concentration signals that ATP is being produced faster than it is being consumed; ATP inhibits PFK-1 by binding to an allosteric site and lowering the affinity of the enzyme for its substrate, fructose 6-phosphate (F 6-P). ADP and AMP, which increase in concentration as consumption of ATP outpaces production, act allosterically to relieve this inhibition by ATP. These effects combine to produce a higher enzymatic activity when ADP or AMP accumulates and a lower activity when ATP accumulates. High concentrations of ATP allosterically inhibit also pyruvate kinase [Nelson and Cox 2005b].

Energy metabolism is crucial for oocyte maturation, because progression through this dynamic process requires large amounts of energy from various substrates, including carbohydrates, amino acids and lipids [Collado-Fernandez *et al*. 2012, Songsasen 2012]. In the antral follicle, oocytes are enclosed within a cumulus, forming a cumulus-oocyte complex (COC).

Coordinated communications between the oocyte and surrounding cumulus cells (CCs) occur within the COC through paracrine signals that interact with membrane receptors to activate different intracellular pathways [Eppig *et al*. 1997, Salustri 2000]. A direct interaction between these cell types takes place via cytoplasmic extensions of CCs that penetrate the zona pellucida and create contacts with the oocyte membrane,

allowing the formation of gap junctions. Intracellular communications establish metabolic coupling between the oocyte and CCs through bidirectional exchange of small molecules including ATP, ions and nutritive elements such as pyruvate and various metabolites, including lipids. CCs therefore participate in oocyte growth and nuclear and cytoplasmic maturation as well as maintenance of meiotic arrest before fertilization [Conti 2010].

Glucose is considered the most important external energy source metabolised by COCs. The glycolytic pathway is one of the main fates for glucose consumed by murine, bovine, and porcine COCs [Downs and Utecht 1999, Cetica *et al*. 2002, Preis *et al*. 2005, Krisher *et al.* 2007, Alvarez *et al*. 2012]. Glucose consumed by CCs during in vitro maturation (IVM) is used in oxidative catabolism through glycolysis to obtain energy (ATP) and oxidative substrates for the oocyte [Cetica *et al*. 2002, Sutton *et al.* 2003, Gutnisky *et al*. 2013]. Once in the oocyte, full oxidation of this substrates takes place inside the mitochondria, in the Krebs cycle and respiratory chain pathways, providing additional ATP molecules to the cell [Steeves and Gardner 1999, Cetica *et al.* 2002, Sutton-McDowall *et al.* 2010].

Inhibition of glycolytic activity in the COC was shown to decrease lactate production and diminish the meiotic maturation rate through alteration of mitochondrial activity in bovine and porcine oocytes [Alvarez *et al.* 2013, Gutnisky *et al*. 2013]. An addition of AMP to culture medium increased glucose uptake and lactate production by bovine COCs during IVM [Gutnisky et al. 2013]. However, in porcine COCs the stimulatory effect of AMP on the glycolytic pathway was not found [Alvarez *et al.* 2013]. Gonadotropins increased lactate production in the follicles of rats, mice and humans by increasing the glycolytic activity of the cumulus and granulosa cells [Billig *et al*. 1983, Harlow et al. 1987, Boland *et al*. 1993]. In bovine and porcine COCs, glycolytic activity is also increased in the presence of gonadotropins [Zuelke and Brackett 1992, Sutton *et al*. 2003, Gutnisky *et al*. 2007, Alvarez *et al.* 2012].

The aim of the present work was to evaluate the relationships between COC energy and glycolysis in the porcine species; specially, the variation in ATP content in relation to glycolytic pathway activity and the reciprocal effect of ATP on this metabolic pathway.

Material and methods

Materials

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

Recovery and classification of cumulus-oocyte complexes

Ovaries from slaughtered gilts were transported in a warm environment (28-33°C) for the 2- to 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. Cumulus-oocyte

complexes were aspirated from 3- to 8-mm antral follicles using a 10-ml syringe and an 18-gauge needle, and oocytes surrounded by a dense cumulus were selected for in vitro culture.

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 (control medium) under mineral oil at 39°C for 48 h in a 5% CO_2 atmosphere [Abeydeera *et al.* 2001]. To determine if glucose utilization is regulated by gonadotropins (stimulator of glycolysis) and ATP (physiological inhibitor of glycolysis), COCs were cultured in the control medium supplemented with 0.5 mg/l porcine follicle-stimulating hormone (FSH) (Folltropin-V; Bioniche, Belleville, ON, Canada) + 0.5 mg/l porcine luteinising hormone (LH) [Amaral *et al*.] (Lutropin-V, Bioniche), FSH + LH + 0.1mM ATP, or FSH + LH + 1mM ATP.

Evaluation of glucose uptake and lactate production

To evaluate glucose uptake and lactate production, COCs were matured individually in 20 µl droplets of culture medium. They were then removed and oocyte meiotic maturation was assessed. The glucose and lactate contents of the spent maturation medium of each droplet were then determined individually. The glucose and lactate concentrations were measured using a spectrophotometric assay. For glucose determination an assay based on the oxidation of the sugar by glucose oxidase and the subsequent determination of the hydrogen peroxide formed was used, while for lactate concentration the assay included lactate oxidase [Barham and Trinder 1972, Gutnisky *et al*. 2007].

Evaluation of oocyte maturation

To evaluate nuclear maturation 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 x 250 and x 400 magnification. Oocytes were considered matured when the presence of the metaphase II chromosome configuration and the first polar body extrusion were observed.

Determination of endogenous ATP concentration in COCs

Endogenous ATP concentration was determined in immature and matured COCs using a commercial kit based on the luciferin-luciferase reaction (Bioluminescent Somatic Cell Assay Kit, Sigma) [Stojkovic *et al*. 2001].

Statistical analysis

Oocyte maturation percentages were compared with a homogeneity test using a chi-square analysis for non-parametric data. Glucose uptake, lactate production and ATP content were compared by analysis of variance, data of these variables are expressed as the mean ±standard error of the mean. $P<0.05$ was considered significant.

Results and discussion

Modulation of glycolysis in porcine COCs

To evaluate glycolytic activity in the presence of gonadotropins and ATP, immature COCs were randomly divided in four groups for IVM: Control, Gonadotropins, Gonadotropins + 0.1 mM ATP and Gonadotropins + 1 mM ATP. Each COC was matured individually in 20 microliter droplets and glucose uptake and lactate production were determined from the spent maturation media. Twenty-microliter droplets of the maturation medium without cells were included in each experiment to provide glucose and lactate reference concentrations.

COC energy charge during *in vitro* maturation

To evaluate the energy charge in COCs during IVM, Immature COCs were randomly divided into three groups: Immature, Control and Gonadotropins. COCs of the Control and Gonadotropins groups were matured individually in 20 microliter droplets for 48 h. ATP levels were assessed in immature COCs and matured COCs with and without gonadotropin addition.

Relation between COC energy charge and glycolytic activity

According to the results obtained in experiments 1 and 2, we determined if glucose utilisation is inhibited by a rise in the COC energy charge. Immature COCS were randomly divided into two groups for IVM: Control and Gonadotropins. The medium was refreshed every 24 h to avoid substrate consumption interference. COCs were placed in a new droplet of the culture medium every 24 h, while glucose uptake and lactate production were determined as described above after 0-24, 24-48 and 48-72 h of culture (day 1, 2 and 3 of culture).

Modulation of glycolysis in porcine COCs

To evaluate the potential to modulate glucose utilisation by porcine COCs we used gonadotropins as the stimulator and ATP as the inhibitor of glycolytic activity. Glucose uptake and lactate production per COC were enhanced by the presence of FSH and LH in the maturation medium (Control: glucose uptake 28.5±3.5 nmoles COC^{-1} 48^{-1}, lactate production 68.4±8.4 nmoles COC^{-1} 48^{-1} vs. FSH+LH: Glucose uptake 65.2±4.9 nmoles COC^{-1} 48^{-1} lactate production 166 5.5±12.9) even in the presence of 0.1 mM ATP (FSH+LH+0.1 mM ATP : glucose uptake 63.5±5 nmoles

COC^{-1} 48^{-1}, lactate production 151.92±12 nmoles COC^{-1} $48^{-1)}$. However, 1 mM ATP was effective to inhibit gonadotropin stimulation on glucose consumption and lactate production ATP (FSH+LH+1 mM ATP : glucose uptake 25.4±4.5 nmoles COC^{-1} 48^{-1}, lactate production 55,8±7.5 nmoles $COC^{-1}48^{-1}$), reaching similar values to the control group (Fig. 1, $p<0.05$). Percentages of oocytes reaching metaphase II after maturation were significantly lower in the presence of FSH + LH + 1mM ATP (29.6%) than in other treatments (control 64.7%, FSH + LH 75.7%, FSH + LH + 0.1mM ATP 72.3%) ($p<0.05$; n = 28-30 COCs for each treatment in three replicates).

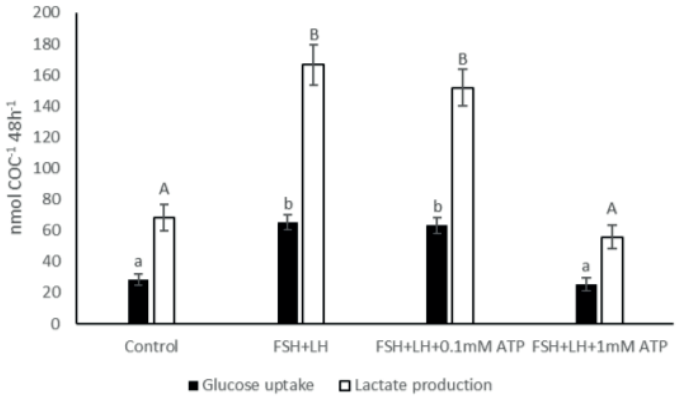

Fig. 1. Glucose uptake and lactate production per COC during 48 h of culture in the presence of FSH and LH, FSH and LH + 0.1 mM ATP, and FSH and LH + 1 mM ATP (n = 28-30 COCs for each treatment in three replicates). Data show the mean ±standard error of the mean. Bars with different superscript letters indicate significant differences in a, b glucose uptake and A, B lactate production ($p<0.05$).

COC energy charge during *in vitro* maturation

Endogenous ATP content was evaluated in porcine COCs during IVM in the presence or absence of gonadotropins. Endogenous ATP concentration was similar in immature COCs and those matured in the absence of FSH + LH (ATP concentration: Immature: 0.16±0.015 pmoles COC^{-1} 48 h^{-1} vs Control: 0.1±0.01 pmoles COC^{-1} 48 h^{-1}). However, energy charge, represented by the endogenous ATP concentration, increased after 48 h of culture in the presence of gonadotropins (FSH+LH: 0.2±0.018 pmoles COC^{-1} 48 h^{-1} (Fig. 2, $p<0.05$; n = 30-40 COCs for each treatment in three replicates). Energy charge was not assessed in those groups supplemented with exogenous ATP.

Relation between COC energy charge and glycolytic activity

As ATP content increased in COCs matured in the presence of gonadotropins (experiment 2) and exogenous ATP decreased glycolysis in the porcine COC (experiment 1), *in vitro* culture was extended up to 72 h (three days of culture) to assess the effect of the COC energy charge on glycolytic activity. Glucose uptake

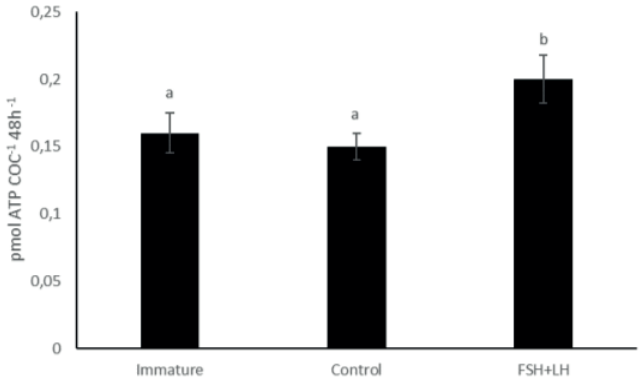

Fig. 2. ATP content per COC immediately after COC aspiration (Immature) and after 48 h of culture in the absence or presence of FSH and LH (n = 30-40 COCs for each treatment in three replicates). Data show the mean ±standard error of the mean. a, b Bars with different superscript letters indicate significant differences ($p<0.05$).

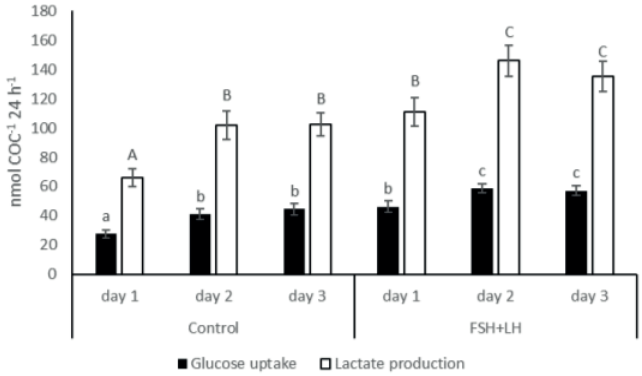

Fig. 3. Glucose uptake and lactate production per COC every 24 h of culture in the absence or presence of FSH and LH (n = 30 COCs for each treatment in three replicates). Data show the mean ±standard error of the mean. Bars with different superscript letters indicate significant differences in a, b, c glucose uptake and A, B, C lactate production ($p<0.05$).

and lactate production increased in the second day of culture in the presence or absence of gonadotropins (day 1, Control: glucose uptake 27.5±2.5 nmoles COC^{-1} 48^{-1} and lactate production 66.6±6 nmoles COC^{-1} 48^{-1}; FSH+LH: glucose uptake 46.2±4.1 nmoles COC^{-1} 48^{-1} and lactate production 110.88±9.84 nmoles COC^{-1} 48^{-1} vs day 2: Control: glucose uptake 41.1±3.6 nmoles COC^{-1} 48^{-1} and lactate production 101.64±9.7 nmoles COC^{-1} 48^{-1}; FSH+LH: glucose uptake 58.7±3.3 nmoles COC^{-1} 48^{-1} and lactate production 145.9±10.9 nmoles COC^{-1} 48^{-1} ($p<0.05$). On the other hand, there were no differences in glucose uptake and lactate production between day 2 and day 3 of in vitro culture in both groups (Fig.3; n = 30 COCs for each treatment in three replicates).

As was stated above, the aim of this work was to verify if glycolytic stimulation in porcine COCs increases the COC ATP content and study if the increase in the ATP level can induce a negative feedback on this pathway.

To evaluate the relationship between COC energy charge (ATP) and glycolysis in porcine COCs, we first modulate this pathway during oocyte IVM. In experiment 1 the presence of gonadotropins in the IVM medium showed a stimulatory effect on glycolytic activity determined by an increase in glucose uptake and lactate production. Only a high concentration of ATP (1 mM) was sufficient to inhibit this effect in porcine COCs. In a previous study on enzymatic extracts of bovine COCs we demonstrated that a low concentration of ATP is necessary for PFK-1 enzymatic activity, while a high concentration inhibits its activity, thus ATP is a substrate of the enzyme in addition to a negative allosteric effector [Gutnisky *et al*. 2017]. These two different binding sites have different Km for ATP, the catalytic binding site (with a binding constant of ~0.15 mM) and the allosteric inhibitor binding site (with a binding constant of ~2.5 mM) [Costa Leite *et al*. 2007, Marinho-Carvalho *et al*. 2009, Marcondes *et al*. 2010, Al Hasawi *et al.* 2014].

With another experimental model Yuan *et al*. [2016] reduced the amount of ATP in porcine oocytes by suppressing glucose metabolic pathways through treatment with dehydroepiandrosterone (DHEA), a pentose phosphate pathway (PPP) inhibitor, or iodoacetate (IA), an inhibitor of glycolysis. This suggests that glucose plays a key role in IVM of porcine oocytes, promoting cytoplasmic maturation by supplying ATP, possibly through the PPP and glycolytic metabolic pathways [Yuan *et al*. 2016].

Similarly, during IVM of mouse COCs in the presence of glucose, treatment with either DHEA or IA significantly reduced the oocyte ATP level. In turn, supplementation of F 6-P overcame the inhibitory effect of DHEA on ATP production, but had no effect on the inhibition induced by IA. These results suggested that both PPP and glycolysis produced ATP during oocyte maturation and that PPP provided F 6-P and glyceraldehyde 3-phosphate for glycolysis to generate ATP. PPP produced similar amounts of ATP, but significantly more NADPH than the level in glycolysis during oocyte maturation. The observations on the effect of F 6-P during oocyte maturation further confirmed that PPP in cumulus cells generated ATP and improved blastocyst development by providing glycolysis with intermediate products such as F 6-P. The authors suggested that whereas glycolysis promoted ooplasmic maturation mainly by supplying energy, PPP facilitated ooplasmic maturation to a greater extent by both supplying energy and reducing oxidative stress [Xie *et al*. 2016].

The next step was to study the effect of ATP on glycolytic pathway activity. In experiment 2 we observed an increase in endogenous COC ATP content when glycolysis was stimulated by FSH and LH during in vitro maturation. This suggests that the higher glucose uptake observed with gonadotropin supplementation was followed by a rise in the endogenous ATP level increasing COC´s energy charge.

In mouse oocytes different authors found controversial results. Nazmara *et al*. [2014] reported that ATP contents of mouse GV oocyte was not significantly different

than those MII obtained after IVM [Nazmara *et al*. 2014]. However, Yu *et al*. [2010] observed three distinct increases in cytosolic ATP levels temporally associated with discrete events of oocyte maturation. These changes in cytosolic ATP levels were mirrored by changes in mitochondrial ATP levels, suggesting that mitochondrial ATP production is stimulated during oocyte maturation [Yu *et al*. 2010].

A significant increase of ATP content in oocytes during maturation has been previously reported in the pig [Brevini *et al*. 2005] and cattle [Stojkovic *et al*. 2001]. This ATP increase is correlated with the success rates in embryo development in cattle [Stojkovic *et al*. 2001], although not in the pig [Brevini *et al*. 2005]. The degree of mitochondrial activity and levels of ATP production in bovine oocytes have been shown to increase during IVM [Stojkovic *et al.* 2001, Tarazona *et al.* 2006, Machatkova *et al.* 2012, Gutnisky *et al*. 2013]. ATP content was also higher in "better" oocytes graded on their morphology and exhibiting higher blastocyst rates [Stojkovic *et al*. 2001]. These data suggest that the status and activity of mitochondria in the mammalian oocyte are determining factors of oocyte quality.

Finally, to evaluate the effect of the COC ATP level on the glycolytic pathway activity, we extended COC culture up to 72h. In experiment 3 the culture was extended to 24h from the time where the increase in endogenous ATP was observed in order to assess the effect of COC energy charge on glycolytic activity. We expected the additional 24h to show the ATP negative feedback on glycolysis evidenced by a decrease in uptake and lactate production. Additionally, the gamete is entering a latency stage at that time point, due to completion of oocyte maturation. Despite the rise in COC energy charge, there was an increase in glycolytic activity and this higher activity was also maintained in the 48-72h period of culture. It is evident that the increase in endogenous COC ATP content and the completion of oocyte maturation do not inhibit glycolysis in porcine mature COCs.

In a similar experimental design of extended in vitro maturation, the mitochondrial activity and ATP content in bovine oocytes gradually increased from 20 to 30h and continued to increase up to 40h of culture [Koyama *et al*. 2014]. These data and our findings suggest that metabolic activity in COCs is maintained at a high level despite the completion of oocyte maturation. In agreement with these findings, it has been reported that glycolytic activity of the bovine oocytectomised cumulus is similar to that of the intact COC, suggesting that glycolysis in cumulus cells is independent of the presence of the oocyte [Sutton *et al*. 2003].

Our study confirmed the stimulation of glycolysis by gonadotropins in porcine COCs and the inhibition of this pathway by the addition of exogenous ATP. We demonstrated the relationship between the gonadotropic stimulation of glycolytic activity and the increase in ATP content in porcine COC during IVM. However, the endogenous ATP content reached in the matured COC seems not to decrease glycolytic activity in the COC.

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