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Influence of the Cumulus and Gonadotropins on the Metabolic Profile of Porcine Cumulus–Oocyte Complexes During *In Vitro* Maturation

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Contents

The aim of this work was to examine the influence of the cumulus and gonadotropins on the metabolic profile of porcine cumulus oocyte complexes (COCs) during *in vitro* maturation. Immature COCs were assigned to morphological classes A_1 (with a dense cumulus), A_2 (with a translucent cumulus), B_1 (with the corona radiata), B_2 (with only some remaining cumulus cells) and matured with or without gonadotropins. Glycolysis and ammonia production were higher in the A class COCs; gonadotropins increased both, especially in the A_1 COCs ($p < 0.05$). The A class COCs had the highest initial protein contents and at the end of *in vitro* maturation. Furthermore, hormonal stimulation induced a similar increase in protein contents of both A classes ($p < 0.05$). The neutral lipid content and reactive oxygen species (ROS) levels were similar in the immature oocytes of the COCs of all classes. A reduction was seen in both these variables when maturation proceeded either in the presence or absence of gonadotropins. The cumulus type surrounding the oocyte is related to the metabolism of carbohydrates and amino acids by the COC during *in vitro* maturation under gonadotropic stimulation. Oocyte lipolytic activity and ROS production appear to be independent of the surrounding cumulus and the presence of gonadotropins.

Introduction

The available information on the metabolic profile of porcine COCs during *in vitro* maturation is limited (Sturmey and Leese 2003). It has been suggested that, in rodents, the cumulus cells provide nutritional support, passing glycolytic metabolites to the oocyte during growth and maturation (Leese and Barton 1984; Buccione et al. 1990). Metabolizing bovine cumulus cells may also provide pyruvate and lactate to the oocyte as substrates for further oxidation (Cetica et al. 1999, 2002; Sutton et al. 2003). It has been proposed that oocytes use Krebs cycle metabolites from the cumulus cells to obtain energy during *in vitro* maturation (Rieger and Loskutoff 1994; Cetica et al. 2003).

In rodents as well as bovine and porcine species, amino acids are involved in events related to the maturation capacity of oocytes (Kito and Bavister 1997; Cetica et al. 2003; Hong et al. 2004). Certainly, the presence of different amino acids in the maturation medium of bovine and porcine COCs increases the efficiency of *in vitro* fertilization and the rates of pronucleus and blastocyst formation, suggesting that amino acids improve cytoplasmic maturation (Rose-Hellekant et al. 1998; Lim et al. 1999; Hong et al. 2004). Protein synthesis in bovine oocytes increases during the first 12 h of *in vitro* culture (Wu et al. 1996), approximately at the time of the germinal vesicle's breakdown (Tomek et al. 2002), while in bovine cumulus cells, it is relatively constant over the full 24 h of maturation (Wu et al. 1996). Yet, it has also been suggested that bovine oocytes use amino acids as an energy source (Rieger and Loskutoff 1994; Cetica et al. 2003). Although, in a less detailed report, protein synthesis has been described in porcine oocyte and cumulus cells during *in vitro* maturation (Ball et al. 1985),porcine oocytes contain more lipid droplets than those of humans, equines or rodents (Kikuchi et al. 2002). Indeed, the approximate lipid content of a porcine oocyte is 161 ng, almost three times that of ruminant oocytes (McEvoy et al. 2000). The major lipids in immature porcine oocytes are triacylglycerols (Homa et al. 1986), and the reduction in the triacylglycerol content of bovine and porcine oocytes maturing *in vitro* suggests these compounds might be used as an energy source during this process (Kim et al. 2001; Sturmey and Leese 2003).

In vitro culture systems usually have higher oxygen tensions than *in vivo* environments, leading to an increase in the concentration of reactive oxygen species (ROS) (Luvoni et al. 1996). Oxidative modification of cell compounds by ROS causes damage that impairs normal cell function (Halliwell and Gutteridge 1988). Certainly, oxidative stress during *in vitro* culture provokes alterations in bovine, murine and porcine oocytes that impair their maturation and developmental competence (Tatemoto et al. 2000; Fatehi et al. 2005; Choi et al. 2007). It has been suggested that cumulus cells protect pig oocytes from ROS damage via increasing the glutathione content of the gamete (Tatemoto et al. 2000).

Gonadotropins exert their effects via receptors located on cumulus cells. Their coupling leads to biochemical changes that induce the resumption of oocyte meiotic activity as well as signalling that leads to oocyte cytoplasmic maturation. In pigs, gonadotropins increase the number of oocytes with a mature nucleus and cytoplasm, and the expansion of the surrounding cumulus (Mattioli et al. 1991; Schoevers et al. 2003; Alvarez et al. 2009). While the metabolic response of porcine COCs to hormones have remained largely unexamined, metabolic changes in response to gonadotropins are known to occur in those of other species; for example, these hormones increase 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 COCs too, glycolytic activity is increased in the presence of gonadotropins (Zuelke and Brackett 1992; Sutton et al. 2003; Gutnisky et al. 2007).

We hypothesize that inefficient *in vitro* porcine embryo production could partly be due to the current lack of knowledge regarding the metabolic variables involved in oocyte maturation. The gonadotropic stimulation of cumulus cells might improve the metabolism of glucose, amino acids and lipids by COCs, as well as reduce ROS formation during *in vitro* maturation. More in-depth knowledge of porcine COC metabolism would contribute towards the establishment of better *in vitro* maturation protocols. The aim of this work was to determine the influence of the cumulus and gonadotropins on the metabolic profile of porcine COCs during *in vitro* maturation.

Materials and Methods

Materials

Unless otherwise specified, all chemicals used were purchased from the 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 2–3-h of 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 were allotted to one of four different classes according to morphological criteria [as determined by stereomicroscopy and described in (Alvarez et al. 2009)]: (A_1) oocytes surrounded by a dense cumulus; (A_2) oocytes surrounded by a translucent cumulus; (B_1) oocytes surrounded by the corona radiata and (B_2) oocytes with only some remaining cumulus cells.

Oocyte *in vitro* maturation

The differently classed 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) foetal bovine serum (GIBCO), 0.57 mM cysteine, 50 mg/l gentamicin sulphate and 0.5 mg/l porcine follicle-stimulating hormone (FSH) (Folltropin-V; Bioniche, Belleville, ON, Canada) plus 0.5 mg/l porcine luteinizing hormone (LH) (Lutropin-V; Bioniche) – or without these gonadotropins (Control) – under mineral oil at 39°C for 48 h in a 5% CO_2 atmosphere (Abeydeera et al. 2001). The number of COCs used in each experiment was the minimum possible for the measurement technique employed.

After incubation, oocyte meiotic maturation was determined by the presence of metaphase II as described in (Alvarez et al. 2009), or *in vitro* fertilization was performed.

In vitro fertilization

In vitro fertilization was performed using fresh semen from a Yorkshire boar of proven fertility. Sperm-rich fractions were collected by the gloved-hand method. Sperm samples were washed twice in phosphate-buffered saline (PBS) plus 3 g/l bovine serum albumin (BSA) by centrifugation at $400 \times \mathbf{g}$ for 5 min. The pellets were resuspended in fertilization-modified Tris-buffered medium (Abeydeera and Day 1997). Samples were filtered through a 20-mg glass wool column (height 10 mm, diameter 4 mm), previously washed with a modified Tris-buffered medium, to obtain the motile fraction (Pereira et al. 2000). Forty mature COCs were denuded by pipetting and inseminated to a final concentration of 5×10^8/l spermatozoa. The co-incubation of gametes was performed in 400-µl droplets of fertilization-modified Tris-buffered medium under mineral oil at 39°C for 18 h in a 5% CO_2 atmosphere. Oocytes were considered cytoplasmically mature when at least one decondensed sperm head and/or a fully formed pronucleus could be identified, as described in (Alvarez et al. 2009).

One-tenth of the oocytes used in each experiment were maintained without exposure to sperm to test for spontaneous parthenogenesis.

Number of cumulus cells in cumulus–oocyte complexes

Ten COCs were matured in 100 µl of culture medium and the number of cumulus cells determined. Ten immature COCs were included in each experiment to measure their initial number of cumulus cells.

For the determination of the number of cumulus cells, cumulus–oocyte complexes were individually suspended in 1 g/l hyaluronidase, 2.5 g/l trypsin, 3.8 g/l EDTA and 3 g/l BSA in PBS, and the cumulus cells were separated by vortex agitation for 10 min at 37°C. The cell count for each COC was estimated using a Neubauer counting chamber.

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 assessed. The glucose and lactate contents of the spent maturation medium were then determined. The glucose concentration was measured using a spectrophotometric assay based on the oxidation of the sugar by glucose oxidase and the subsequent determination of the hydrogen peroxide formed (Barham and Trinder 1972; Gutnisky et al. 2007). The lactate concentration was measured in a similar manner but using lactate oxidase (Barham and Trinder 1972). Twenty-microlitre droplets of maturation medium without cells were included in each experiment to provide glucose and lactate reference concentrations.

To determine the contribution of the oocyte to COC glucose uptake and lactate production, oocytes belonging to class A_1 COCs were denuded as described in (Alvarez et al. 2009) and individually matured in 20 µl of culture medium with FSH & LH.

Total protein content and production of ammonia

Twenty-five COCs were matured in 250 µl of culture medium and total protein contents determined. At the

same time, the ammonia concentration of the spent medium was measured. Oocyte meiotic maturation was evaluated in five COCs. Twenty immature COCs were included in each experiment to measure their initial protein content.

For the determination of the total COC protein content, 20 COCs were frozen–thawed twice in distilled water, dispersed by vortex agitation for 30 min at 37°C with an alkaline solution to a final concentration of 1 N NaOH and centrifuged at 10 000 × ***g*** for 20 min. Biuret and Folin-Ciocalteu reagents were added to the supernatant, and the protein concentration was measured spectrometrically (Lowry et al. 1951; Gutnisky et al. 2007).

The ammonia concentration was measured using a spectrophotometric assay based on the oxidation of NADPH by glutamate dehydrogenase during ammonia consumption. Parallel 250-µl droplets of maturation medium without cells were included in each experiment to provide a reference ammonia concentration.

To check the oocyte contribution to protein content modification and ammonia production recorded for the COCs, 25 oocytes belonging to class A_1 COCs were denuded and then matured in 250 µl of culture medium with FSH & LH. The protein content of the oocyte and ammonia production per oocyte were then determined. Simultaneously, 25 class A_1 COCs were matured in 250 µl of culture medium with FSH & LH and then denuded to measure their protein content. An aliquot of five oocytes was used to check meiotic maturation. Additionally, immature oocytes belonging to class A_1 COCs were denuded and used to determine the initial oocyte protein content.

Correlation between cumulus cell number and total protein content

Fifty-five COCs were matured in 550-µl droplets of culture medium, and total protein and DNA contents were determined. Oocyte meiotic maturation was evaluated in five COCs. Fifty immature COCs were included in each experiment to measure their initial protein and DNA contents.

Fifty COCs were frozen–thawed twice in distilled water and dispersed by vortex agitation for 5 min. They were then divided into two aliquots. One aliquot was used to determine the COC protein content as described earlier. The second was used to determine the cumulus cell number per COC by adding it to a solution of 0.121% (w/v) Tris, 0.037% (w/v) EDTA, 20.44% (w/v) NaCl, 0.1 µg/ml Hoechst 33258, pH 7.4 and incubating for 10 min in darkness. Fluorescence was then measured in a spectrofluorometer at 365 nm excitation and 458 nm emission (Labarca and Paigen 1980; Gutnisky et al. 2007). The quantity of cumulus cells was calculated on the basis that a porcine cell contains, on average, 5.1 pg DNA (Mirsky and Osawa 1961).

Neutral lipid content

To evaluate neutral lipid content, 20 COCs were matured in 200-µl droplets of culture medium. After incubation, oocyte lipid content and meiotic maturation were determined. Twenty immature COCs were included in each experiment to determine the initial oocyte lipid content.

The neutral lipid content was determined as previously described (Genicot et al. 2005) with slight modifications. Denuded oocytes were incubated for 2 h at 37°C in a solution of 10 µg/ml Nile Red, 0.1% (w/v) polyvinyl alcohol and 0.9% (w/v) NaCl, pH 7. Fluorescence was determined using digital photomicrographs obtained with an epifluorescence microscope (excitation: 510 nm and emission: 590 nm). For this, all photomicrographs were analysed using IMAGE J 1240 software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA), measuring the brightness of each oocyte. The neutral lipid content was expressed in arbitrary units per oocyte. Meiotic maturation was evaluated in the same oocytes after lipid measurement.

ROS production

To evaluate ROS formation, 13 COCs were matured in 130-µl droplets of culture medium. Oocyte ROS production and meiotic maturation were determined after incubation. Thirteen immature COCs were included in each experiment to measure the initial oocyte ROS production.

To measure the oocyte ROS level, COCs were collected from the maturation medium, and the oocytes were denuded and incubated at 37°C in PBS supplemented with 0.3% (w/v) BSA for 30 min in the presence of 5 µM 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) (Le Bel et al. 1992; Morado et al. 2009). To measure esterase activity, COCs were collected from the maturation medium, and the oocytes were denuded and incubated at 37°C in PBS supplemented with 0.3% (w/v) BSA for 15 min in the presence of 0.12 µM fluorescein diacetate (FDA).

After exposure to their respective fluorochromes, both oocyte samples were washed in PBS supplemented with 0.3% (w/v) BSA and mounted on glass slides. Fluorescence was measured using digital photomicrographs obtained with an epifluorescence microscope (excitation: 450–490 nm and emission: 520 nm). All photomicrographs were analysed using IMAGE J 1240 software, measuring brightness for each oocyte.

The fluorescence detected by DCHF-DA is dependent on esterase activity. The ratio between the brightness of each oocyte measured by DCHF-DA and the mean brightness detected by FDA in each COC class and treatment was, therefore, considered a better indicator of oocyte ROS levels (Lane et al. 2002; Morado et al. 2009). ROS levels were expressed in arbitrary units per oocyte per min. Meiotic maturation was evaluated in the same oocytes after ROS measurement.

Statistical analysis and experimental design

Non-parametric values were recorded as percentages and analysed using the chi-squared test. Parametric values were reported as means ± SEM; comparisons were made by ANOVA using a factorial design. The Pearson test was used to determine correlations. Significance was set at $p < 0.05$. Statistical analyses were

performed using the STATISTIX 8 program (Analytical Software, Tallahassee, FL, USA).

Results

Effect of cumulus features and gonadotropins on oocyte nucleus and cytoplasm maturation *in vitro*

The percentage of metaphase II oocytes was similar for COCs matured in the presence or absence of gonadotropins and with no differences seen among the different classes (Fig. 1a). Yet, the percentage of oocytes with mature cytoplasm was higher in all classes (except for B_2) in the presence of gonadotropins ($p < 0.05$). The A_1 class COCs matured with gonadotropins showed the highest percentage of oocytes with mature cytoplasm, apparently because of stimulated pronucleus formation ($p < 0.05$). No pronucleus formation was observed in COCs matured in the absence of gonadotropins (Fig. 1 b). No spontaneous parthenogenic activation was observed for any COC class matured without gonadotropins. Yet, some parthenogenic activation was observed in COCs matured with gonadotropins: 0% for A_1 class, 0% for A_2 class, 0% for B_1 class and 6.7% for B_2 class (n = 10–15 oocytes observed for each class).

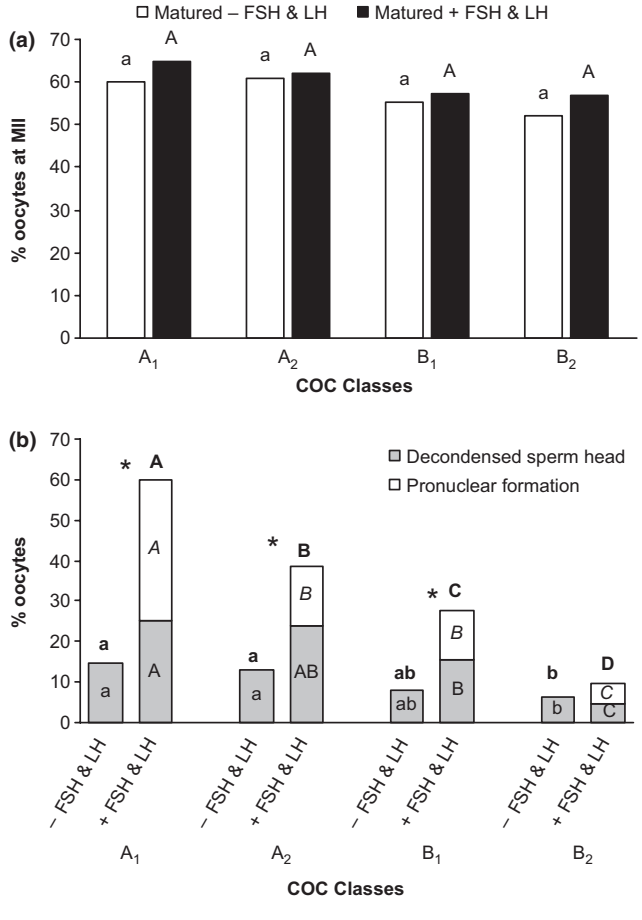

Fig. 1. (a) Percentage of oocytes reaching metaphase II (M II) in different COC classes after maturation with (+FSH & LH) and without gonadotropins (–FSH & LH). [a] or [A] The same superscript over bars indicates no significant difference between classes. No significant differences were seen between COCs of the same class when matured with or without FSH & LH (n = 91–100 oocytes for each bar). Experiments were repeated three times. (b) Percentage of oocytes with mature cytoplasm for different COC classes after maturation with (+FSH & LH) or without gonadotropins (–FSH & LH), as evaluated by sperm head decondensation and/or pronucleus formation. [a, b; a, b; A, B, C, D; *A*, *B*, *C*] and [A, B, C] Different superscripts over bars indicate significant differences between classes in regard to the same evaluation parameter ($p < 0.05$). *Significant differences were seen between COCs of the same class when matured with and without gonadotropins ($p < 0.05$, n = 140–148 oocytes for each bar). Experiments were repeated four times

Effect of cumulus features and gonadotropins on cumulus cell number

The number of cumulus cells was greater in COCs belonging to the A classes than in those belonging to the B classes ($p < 0.05$). Yet, no significant differences were seen between A_1 and A_2 nor between B_1 and B_2 under any of the three oocyte condition sets (immature, matured without FSH & LH and matured with FSH & LH). In both A classes, the number of cumulus cells increased (compared with immature oocyte controls) during maturation in the presence of gonadotropins ($p < 0.05$) and decreased during culture in the absence of these hormones ($p < 0.05$). Yet, no difference was observed in the number of cumulus cells for the COCs of the B classes under any circumstances (Fig. 2).

Effect of cumulus features and gonadotropins on glycolytic activity

Substrate consumption and glycolytic final product formation were measured simultaneously to assess the activity of the metabolic pathway. Glucose uptake and lactate production per COC were higher in the A class than in the B class COCs in the absence of gonadotropins ($p < 0.05$). No difference was detected between classes A_1 and A_2, nor between classes B_1 and B_2. During maturation with gonadotropins, glucose uptake and lactate production increased in the A class COCs ($p < 0.05$), with the highest values for both variables recorded for the A_1 class ($p < 0.05$) (Fig. 3a,b).

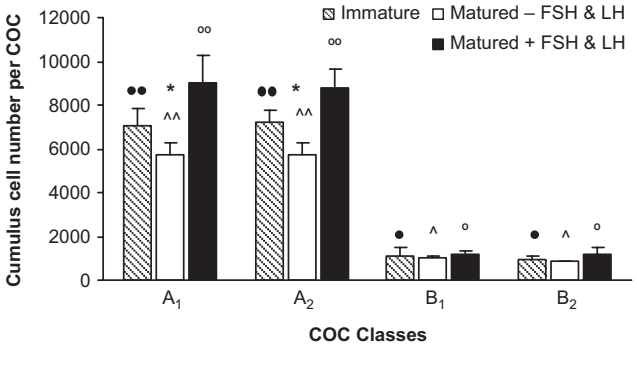

Fig. 2. Cumulus cell number per COC in different classes before (immature) and after maturation with (+FSH & LH) or without gonadotropins (–FSH & LH). [••, •] and [^^, ^] and [ºº, º] Different superscripts over bars indicate significant differences between classes ($p < 0.05$). *Values for all three oocyte conditions (immature, matured – FSH & LH and matured +FSH & LH) are significantly different from one another within the same COC class ($p < 0.05$, n = 30 COCs for each bar). Experiments were repeated three times. Data are presented as mean ± SEM

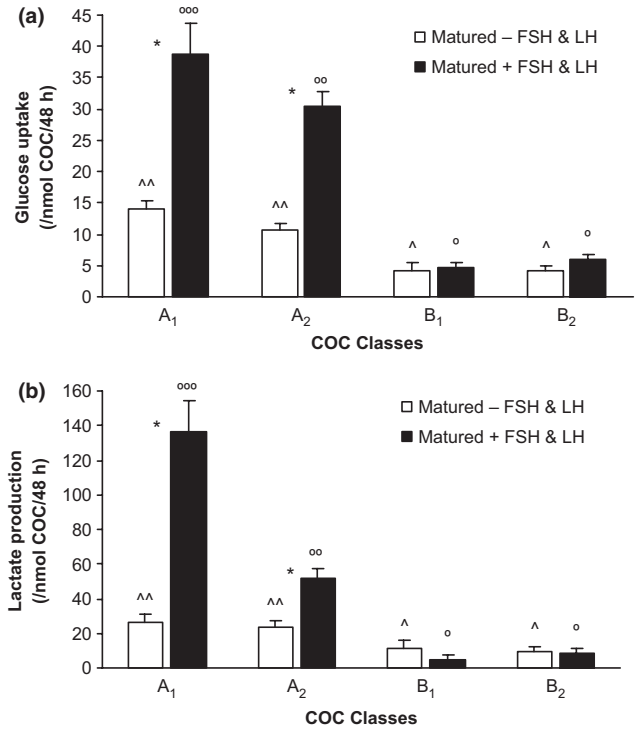

Fig. 3. (a) Glucose uptake by COCs in different classes during maturation with (+FSH & LH) or without gonadotropins (−FSH & LH). ^^, ^ and ººº, ºº, º Different superscripts over bars indicate significant differences between classes ($p < 0.05$). *Values for COCs matured with and without gonadotropins are significantly different within the same class ($p < 0.05$, n = 20–25 replicates for each bar). Data are presented as mean ± SEM. (b) Lactate production by cumulus–oocyte complex (COC) in different classes during maturation with (+FSH & LH) or without gonadotropins (−FSH & LH). ^^, ^ and ººº, ºº, º Different superscripts over bars indicate significant differences between classes ($p < 0.05$). *Values for COCs matured with and without gonadotropins are significantly different within the same class ($p < 0.05$, n = 20–25 replicates for each bar). Data are presented as mean ± SEM

A strong positive correlation was seen between glucose uptake and lactate production per COC ($r = 0.85$; $p = 0.0000$). A glucose/lactate molar rate of around 1 : 2 was recorded for the A_1 class matured without gonadotropins and for the A_2 class matured with or without gonadotropins. For the A_1 class matured with gonadotropins, the molar rate was approximately 1 : 3.5.

After 48 h of *in vitro* culture, neither glucose uptake nor lactate production could be detected in denuded oocytes obtained from A_1 class COCs.

Effect of cumulus features and gonadotropins on amino acid metabolism

The anabolic and catabolic fates of amino acids were determined from the difference in protein content per COC and the production of ammonia via deamination, respectively. Under all three oocyte condition sets, the total protein content per COC was greater for the A classes than the B classes ($p < 0.05$), with no significant difference between A_1 and A_2 nor between B_1 and B_2. The presence of gonadotropins during maturation induced a similar increase in total protein content per COC for the A_1 and A_2 classes ($p < 0.05$). Yet, gonadotropins had no effect on total protein content per COC in the B classes (Fig. 4a). Ammonia production was greater in the A classes than in the B classes ($p < 0.05$), with no significant difference between A_1 and A_2 nor between B_1 and B_2. Ammonia production increased in the A_1, A_2 and B_1 class COCs in the presence of gonadotropins, although the strength of this effect decreased from A_1 to B_1 ($p < 0.05$) (Fig. 4b). A strong, positive correlation was seen between COC total protein content and ammonia production ($r = 0.82$; $p = 0.0000$). In denuded oocytes obtained from A_1 class COCs, no variation was seen in total protein content per oocyte after 48 h of *in vitro* culture (1.3 ± 0.1 μg protein per oocyte, n = 8 replicates) compared with immature oocyte controls (1.4 ± 0.1 μg protein per oocyte, n = 8 replicates). No variation in the total protein content per oocyte matured within the COC was seen either (1.3 ± 0.1 μg protein per oocyte, n = 8 replicates). After 48 h of *in vitro* culture, no ammonia production was observed for denuded oocytes obtained from A_1 class COCs.

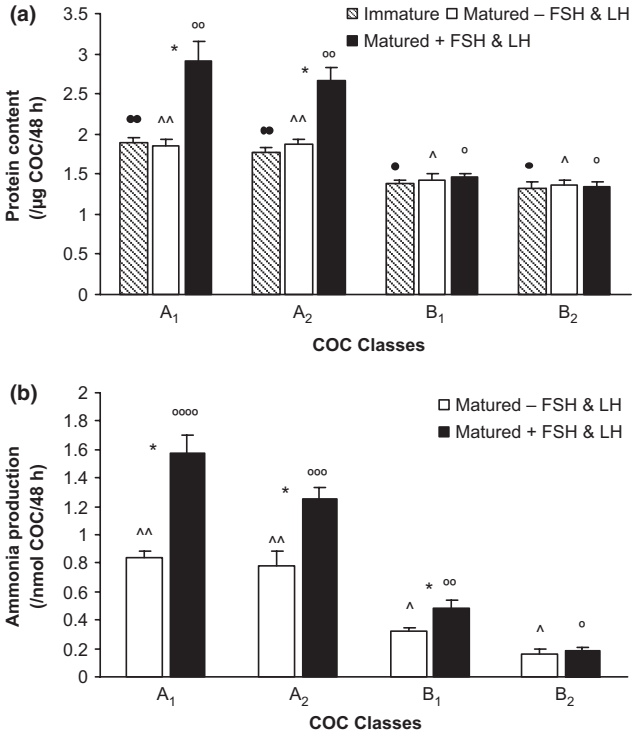

Fig. 4. (a) Total protein content per cumulus–oocyte complex (COC) in different classes before (immature) and after maturation with (+FSH & LH) or without gonadotropins (−FSH & LH). ••, • and ^^, ^ and ºº, º Different superscripts over bars indicate significant differences between classes ($p < 0.05$). *Values for COCs matured with and without gonadotropins are significantly different within the same class ($p < 0.05$, n = 9–11 replicates for each bar). Data are presented as mean ± SEM. (b) Ammonia production by COCs of different classes during maturation with (+FSH & LH) or without gonadotropins (−FSH & LH). ^^, ^ and ºººº, ººº, ºº, º Different superscripts over bars indicate significant differences between classes ($p < 0.05$). *Values for COCs matured with and without gonadotropins are significantly different within the same class ($p < 0.05$, n = 9–11 for replicates for each bar). Data are presented as mean ± SEM

For both immature COCs and COCs matured in the presence of gonadotropins, a strong positive correlation was seen between the cumulus cell number and the total protein content per COC ($r = 0.92$, $p = 0.0000$, $n = 15$ replicates; $r = 0.7$, $p = 0.0026$, $n = 16$ replicates, respectively). Yet, no significant correlation between these variables was recorded for COCs matured in the absence of gonadotropins ($r = 0.28$, $p = 0.2513$, $n = 19$ replicates).

Effect of cumulus features and gonadotropins on lipid metabolism

The neutral lipid content per oocyte decreased because of *in vitro* maturation, with no differences seen among the different COC classes ($p < 0.05$). The presence of gonadotropins during culture did not modify the reduction of the oocyte lipid content in any COC class (Fig. 5).

Effect of cumulus features and gonadotropins on oocyte ROS production

Oocyte ROS production, expressed as the ratio of the brightness recorded in DCHF-DA/FDA fluorescent assays, was similar for all COC classes under all three oocyte condition sets. Oocyte ROS production decreased over *in vitro* maturation, with no differences seen among the different COC classes ($p < 0.05$). The presence of gonadotropins did not modify the reduction in oocyte ROS levels in any COC class (Fig. 6). No difference was seen in esterase activity (determined by FDA) among the COC classes or with respect to oocyte condition sets (data not shown). The percentage of metaphase II oocytes present during the study of glucose uptake, lactate production, protein content, ammonia production, cumulus cell number, lipid metabolism and ROS production did not differ among the different classes matured in the presence or absence of gonadotropins.

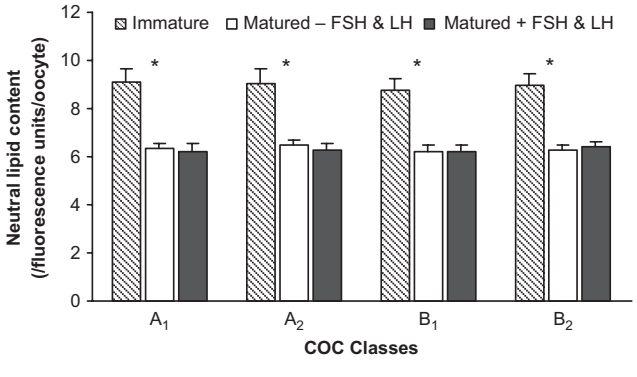

Fig. 5. Neutral lipid content per oocyte in different COC classes before (immature) and after maturation with (+FSH & LH) or without gonadotropins (−FSH & LH). Bars of the same colour are not significantly different between classes. *Values for immature oocytes are significantly different from those matured – FSH & LH and matured + FSH & LH within the same class ($p < 0.05$, $n = 64–78$ oocytes for each bar). Experiments were repeated four times. Data are presented as mean ± SEM

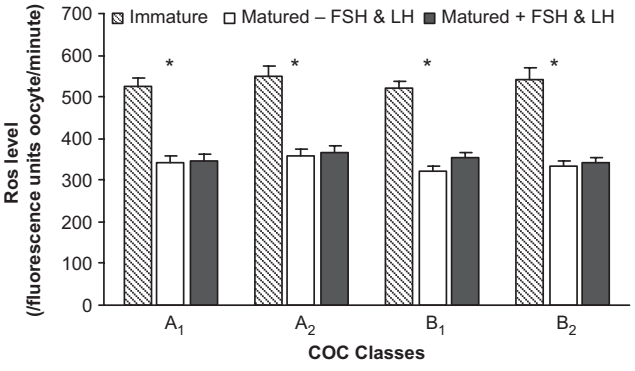

Fig. 6. Level of reactive oxygen species per oocyte in different COC classes before (immature) and after maturation with (+FSH & LH) or without gonadotropins (−FSH & LH). Bars of the same colour are not significantly different between classes. *Values for immature oocytes are significantly different from those matured – FSH & LH and matured + FSH & LH within the same class ($p < 0.05$, $n = 30$ oocytes for each bar). Experiments were repeated three times. Data are presented as mean ± SEM

Discussion

It is generally accepted that a well-developed cumulus surrounding the porcine oocyte and the presence of gonadotropins during *in vitro* maturation improve cytoplasmic maturation. Changes in COC metabolism mediated by the type of cumulus and its response to FSH and LH would be one of the main factors involved.

In the present work, all COC classes showed similar percentages of metaphase II oocytes in the presence and absence of FSH & LH. This suggests that oocyte nuclear maturation can be completed, despite different cumulus features and gonadotropic influences. In the present study, the average percentage of nuclear maturation was approximately 60%. While higher rates can be achieved by oocyte maturational stimulation with gonadotropins, insulin, growth factors and/or dibutyryl cAMP (Coy et al. 2005; Zhang et al. 2010), these compounds may also modulate metabolic pathways and overlap gonadotropic regulation. To avoid this effect, only FSH & LH were added to maturation medium in the present work. The greater pronucleus formation observed in A_1 class COCs matured with hormones suggests a close association exists between cumulus features, the gonadotropic response and oocyte cytoplasmic maturation capacity. Cytoplasmic maturation is a rather complex process compared with meiotic progression; it is possible that the degrees of cumulus participation induced by gonadotropins during culture determined the differences observed among the classes.

Cumulus cell number in A_1 and A_2 COCs decreased over culture time in the absence of gonadotropins. In the rat, it is reported that gonadotropins prevent apoptosis in granulosa cells (Chun et al. 1996). Thus, it may be that the loss of cells observed in the present work was attributed to the apoptosis of cumulus cells in the absence of hormonal stimulation. In contrast, the cumulus cell number in A_1 and A_2 COCs increased during culture when gonadotropins were present. This effect has also been reported in bovine COCs (Gutnisky et al. 2007). Granulosa cell proliferation is reported to

be stimulated by gonadotropins and IGF-I in the pig (Adashi et al. 1985; Hatey et al. 1992).

In the B_1 and B_2 class COCs, cumulus cell number remained constant during culture, both in the presence and absence of gonadotropins. Oocyte-secreted factors might stimulate the cumulus cells nearest to the oocyte to not undergo apoptosis. In agreement with this idea, it has been suggested that, in mouse oocytes, secreted factors stimulate granulosa cell proliferation and follicular development (Vanderhyden et al. 1992).

A strong positive correlation was seen between glucose uptake and lactate production, and a 1 : 2 glucose/lactate molar rate was established for the A_1 COCs when matured without gonadotropins and for the A_2 COCs matured with or without gonadotropins. Thus, breakdown via glycolysis appears to be the main fate of the glucose consumed by porcine COCs during *in vitro* maturation. Glycolysis has been proposed the main glucose metabolism pathway during bovine COC *in vitro* maturation (Sutton et al. 2003).

Higher glycolytic activity was seen in the A_1 and A_2 than in the B_1 and B_2 COCs, but neither glucose uptake nor lactate production was detected for denuded oocytes. This agrees with the low level or absence of glycolytic activity reported for denuded bovine oocytes (Zuelke and Brackett 1992; Rieger and Loskutoff 1994; Sutton et al. 2003). Together, these results suggest that glycolysis is mainly performed in cumulus cells. It is possible that these cells metabolise glucose via the glycolytic pathway to provide the oocyte with metabolites that can be further oxidised (Cetica et al. 1999, 2003; Downs and Utecht 1999).

The A_1 and A_2 class COCs increased their glycolytic activity under gonadotropic stimulation, the highest increase being observed in the first of these classes. These results suggest that gonadotropins stimulate glycolysis in cumulus cells. Yet, the same number of cumulus cells was present in the A_1 and A_2 COCs; thus, the intensity of hormonal stimulation appears to depend on the morphological features of the COC. Bovine COCs are also reported to increase their glycolytic activity in the presence of FSH and/or LH during oocyte *in vitro* maturation (Zuelke and Brackett 1992; Sutton-McDowall et al. 2004).

Total protein content was higher in the A_1 and A_2 than in the B_1 and B_2 class COCs whether immature or mature. Immature COCs showed a high positive correlation between cumulus cell number and total protein content, suggesting that variations in protein content among COCs is mainly because of differences in the number of cumulus cells.

An increase was seen in the total protein content per COC only for the A_1 and A_2 classes in the presence of gonadotropins, suggesting that FSH & LH stimulate protein synthesis mainly in the cumulus cells. In addition, no variation was seen in the total protein content of the denuded oocytes during *in vitro* maturation. Despite the active protein synthesis described in bovine oocytes (Wu et al. 1996; Tomek et al. 2002), it is reported that the total protein content of such oocytes decreases during *in vitro* maturation (Cetica et al. 2001). Yet, the stimulation of protein synthesis by gonadotropins has also been reported in bovine COCs during *in vitro* maturation (Gutnisky et al. 2007). In the present work, the strong, positive correlation between cumulus cell number and total protein content was maintained when maturation proceeded in the presence of gonadotropins. Thus, variations in the total protein content of immature COCs, and of those matured with gonadotropins, may be mainly because of differences in the number of cumulus cells.

During maturation without gonadotropins, the total protein content per COC did not change, and the cumulus cell number decreased. The correlation between the cumulus cell number and protein content was lost after *in vitro* maturation without hormones. These results suggest that synthesised proteins may enter the extracellular matrix during maturation without gonadotropic stimulation.

The production of ammonia observed during COC maturation suggests that COCs catabolise amino acids in the culture medium. Ammonia production was greater in the A_1 and A_2 class COCs. In addition, no production by denuded oocytes was detected after 48 h of culture. These results suggest that amino acid catabolism takes place mainly in cumulus cells. Transaminase activity has been reported in bovine cumulus cells and oocytes (although not deaminase activity), suggesting the participation of transaminases in oxidative substrate provision to the gamete (Cetica et al. 2003).

The A_1, A_2 and B_1 class COCs increased their ammonia production under gonadotropic stimulation, with the highest increase observed for the A_1 COCs. These results suggest that gonadotropins stimulate amino acid catabolism in cumulus cells. As the A_1 and A_2 COCs had the same number of cumulus cells, it would seem that the intensity of hormonal stimulation depends on the morphological features of the COC.

Interestingly, lactate production was greater than expected with respect to glucose uptake during culture of the A_1 COCs in the presence of gonadotropins; these COCs returned a glucose/lactate molar rate of approximately 1 : 3.5. It is possible that gonadotropic stimulation increases lactate production from sources additional to the glycolytic pathway. The stronger ammonia production observed by A_1 COCs in the presence of gonadotropins suggests that, in part, amino acids might be routed towards lactate production. It is possible that in COCs, as in other cell types, several amino acids can be metabolised to form pyruvate, which in turn can be reduced to form lactate (Nelson and Cox 2005).

A strong positive correlation was observed between the total protein content and ammonia production, suggesting a parallel use of amino acids in both protein synthesis and ammonia production. Interestingly, rather than defining the main metabolic fate of amino acids, gonadotropic stimulation seems to increase both anabolic and catabolic reactions involving them.

The presence of cumulus cells during *in vitro* maturation is reported to modify the lipid droplet distribution in porcine oocytes (Cui et al. 2009). In the present work, all the evaluated COC classes showed a similar reduction in the oocyte neutral lipid content during *in vitro* maturation, both in the presence and absence of

gonadotropins. Thus, porcine oocyte lipolysis seems to be independent of the cumulus characteristics and not to be modulated by hormones. Greater lipase activity is reported in bovine oocytes than in cumulus cells, suggesting that lipid metabolism is mainly performed in the female gamete (Cetica et al. 2002).

Immature oocytes from COCs of all classes showed similar ROS levels, as well as similar reductions in these levels after maturation in the presence and absence of FSH & LH. These results suggest that cumulus cells do not regulate oocyte ROS production and that gonadotropins are not involved in their modulation. In bovine oocytes, similar oocyte ROS production was seen after the maturation of the full COC or the denuded oocyte, suggesting that oocyte ROS production is independent of the surrounding cumulus (Cetica et al. 2001). This might be explained by the presence of enzymatic and non-enzymatic antioxidant systems in the oocyte (Sawai et al. 1997; Cetica et al. 2001).

In conclusion, the presence of cumulus cells surrounding the oocyte influences the metabolism of carbohydrates and amino acids, and this is influenced by FSH and LH. Therefore, the gonadotropic stimulation might improve the maturation of oocytes with a complete cumulus by the enhancement of these aspects of porcine COC metabolism. COC morphology influences oocyte cytoplasmic maturation, glycolytic activity, protein synthesis and amino acid catabolism during *in vitro* maturation under gonadotropic stimulation. Despite the similar number of cumulus cells observed in some classes of COCs, the different metabolic responses to gonadotropins suggest that COCs of different morphology possess different metabolic capacities. Metabolic ability seems to be necessary for the maturation of the cytoplasm, and especially for pronucleus formation. Oocyte lipolytic activity and ROS production may not be related to cumulus features or gonadotropic regulation. Therefore, differences in cytoplasmic maturation between COC classes cannot be attributed to the latter aspects of their metabolism.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Gabriel Alvarez performed the experiments, analysed data and drafted paper. Gabriel Dalvit analysed data and drafted paper. Pablo Cetica designed the study, analysed data and drafted paper.

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