ULTRASTRUCTURAL ENERGETIC MODEL OF PORCINE OOCYTES WITH DIFFERENT MEIOTIC COMPETENCE

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ABSTRACT

The principal objective of the study was to characterize energetic components of matured porcine oocytes with different meiotic competence. Lipid droplets (LDs), activity of mitochondria and adenosine triphosphate (ATP) content represent the constituents important for cytoplasmic maturation of mammalian oocytes. This research provides detection of LDs, ATP content and rearrangement of mitochondria in matured porcine oocytes with different meiotic competence. In porcine oocytes mitochondria surround LDs forming so-called metabolic units. Meiotic competence of porcine oocytes is directly influenced by the size of follicle from which oocytes are derived. The meiotically higher competent (MHC) and less competent (MLC) oocytes were isolated separately from medium (6-9 mm) and small follicles (<5 mm) respectively by aspiration and cutting of ovarian cortex. LDs were stained by molecular probe Nile red. To determine localization of the lipid droplets protein, oocytes were labeled with an antibody against the lipid droplet specific protein ADRP. For vizualization of mitochondria we used special probe MitoTracker® Orange. The ATP content (pmol per oocyte) was detected using FL-ASC assay kit. After maturation the total area of lipid droplets was analogous between MLC and MHC oocytes (28.8% vs. 29.8%). The total number of lipid droplets was higher in MHC (296.6±109.9) oocytes in comparison to MLC oocytes (277.64±100.6). In MHC oocytes significantly higher ($P<0.05$) proportion of oocytes with metabolic units was discovered. The ATP content was higher ($P<0.05$) in MHC oocytes in comparison to MLC oocytes.

In conclusion, we have shown that porcine oocytes with higher meiotic competence have greater amount of lipid droplets, higher levels of metabolic units and ATP content in comparison to less competent oocytes.

Key words: oocytes, meiotic competence, lipids, mitochondria, ATP

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INTRODUCTION

In biomedical applications including the production of pharmaceutical preparations and as donors of organs for xenotransplantation pigs have become a progressively significant species (Kątska-Książkiewicz, 2006). Because of their physiological resemblances to humans, the maturation of porcine oocytes and production of porcine embryos via *in vitro* procedures is necessary for basic and also biomedical research (Abeydeera, 2002). Improvement of applicable structures for *in vitro* oocyte maturation requires information of cytoplasmic factors, primarily encompassing energy metabolism involved in the achievement of oocyte developmental competence during maturation period and follicular growth. During oocyte growth, developmental competence has shown to be accomplished gradually (Trounson et al., 2001), nonetheless oocytes originating from large follicles have greater meiotic and developmental competence than oocytes isolated from small follicles (Machatkova et al., 2004). Intracellular lipids play essential roles in energy storage, cell configuration and in metabolic function of biological membranes (Kim et al., 2001) and also could arrange into distinctive formations during oocyte maturation and early embryo development (Romek et al., 2011). The oocytes require energy from lipid droplets (LDs) units during intensive processes of their growth and maturation. LDs are multifunctional organelles containing a core of neutral lipids (triacylglycerols-TG and sterol esters-SE) which are constrained by a monolayer of phospholipids and lipid associated proteins (Bartz et al., 2007; Yang et al. 2012; Zechner et al., 2009). The protein layer of LDs can fluctuate among droplets within a cell, in different cell types and during various metabolic occasions. The perilipin family (PAT) of lipid droplets proteins consists of various members with different functions: perilipin, adipophilin or adipocyte differentiation-related protein (ADRP), S3-12, tail-interacting protein of 47 KDa (TIP47) and oxidative tissues-enriched PAT protein (OXPAT) located at the lipid droplets superficies (Bickel et al., 2009). The activity of LDs involves managing of fat mobilization and indicates direct interaction with mitochondria, peroxisomes and ER (Beller et al., 2010). Mitochondria are specific organelles involved in forming of ATP over metabolic process of fats and carbohydrates in the cell cytoplasm (Wilding et al., 2001).

MATERIAL AND METHODS

Oocyte collection. The ovaries were obtained from naturally cyclic sows at a local slaughterhouse. The meiotically higher competent (MHC) and less competent (MLC) oocytes were isolated by rupture of follicular wall, separately from small (<5 mm) follicles by cutting of the ovarian cortex and from medium (6-9 mm) follicles by aspiration with a medical syringe.

Oocyte maturation. The oocytes were matured in 500 µl of TCM-199 medium (Earle's salts) with the addition of 0.20 mM sodium pyruvate, 0.57 mM cysteamine, 50 IU ml penicillin, 50 µg/ml streptomycin (Sigma Chemicals Co., Prague, Czech Republic), 10% BFS (bovine fetal serum, Sigma Chemicals Co.) and gonadotropins (PG 600 15 IU, Intervet, Holland) in a 4-well multi-dish (Nunc, Intermed, Denmark) at 39°C in atmosphere of 5% CO_2. At 44h after the start of *in vitro* maturation, oocyte were stained with moleculare probes and examined for polar body extrusion, an indicator of complete meiotic maturation. For each experiment a group of oocytes was randomly selected. After being labeled these oocytes were used to optimize image acquisition with the confocal microscope.

Oocyte staining Experiment 1. Detection of lipids and ADRP protein

Oocytes in mature (MII) stage were denuded of cumulus cells manually in TCM-199 medium containing 1% NBCS (Newborn calf serum) and 0.1% (w/v) hyaluronidase (Sigma Aldrich). After washing all processed oocytes were fixed in a 500 µl 3.7% paraformaldehyde solution for 60 min at room temperature. They were washed in phosphate buffered saline (PBS) and permeabilized with

1% TRITON X-100. The membrane was blocked using 5% Rabbit serum in 0,01% Tween 20 with 0,4% BSA, and incubated over night at 4 °C using primary antibody against ADRP (Santa Cruz Biotechnology) followed by incubation for 1h with specific secondary antibody CY5 goat anti-rabbit (Jackson Immunoresearch) for visualization of ADRP protein. The lipids of the oocytes were stained in PBS supplemented with 0.4% BSA and 1μM Nile red (Sigma Aldrich) for 10 min at room temperature. After staining, oocytes were washed in PBS and mounted on glass slides, without oocyte compression, using Vectashield medium (Vector Lab) containing 1 μM of DNA dye Sytox blue for identification of nuclear stage. The oocytes were stored below 0ºC until examination.

Experiment 2. Detection of mitochondria and measurement of ATP content

For mitochondria staining, oocytes were incubated in MitoTracker® Orange in holding medium for 30 minutes at 38°C in 5% CO2, washed two to three times in PBS and fixed in 3,7% paraformaldehyde in PBS for 24 hours. After staining, oocytes were washed in PBS and mounted on glass slides, without oocyte compression, using Vectashield medium (Vector Lab) containing 1 μM of DNA dye (Sytox Orange) for identification of nuclear stage. The oocytes were stored below 0ºC until examination. The concentration of ATP in samples was estimated using a bioluminescent assay kit (FL-ASC, Sigma) which is based on luciferin-luciferase reaction with ATP. The oocytes were rinsed in PBS and transposed individually, with a 50µl PBS, into 200 µl plastic tubes. After adding somatic cell reagent (FL-SAR), oocytes were threated with ice-cold assay mix (FL-AAM reagent). The luminoscence intesity was detected using Luminoskan plate reader (type 391A; Labsystems, Helsinki, Finland). The ATP content of individual oocyte was calculated by the formula created from a linear regression of the standard curve.

Oocyte examination

The oocytes were examined with the use of a laser scanning confocal microscope (Leica TCS SP2 AOBS; Leica, Heidelberg, Germany) equipped with Ar and HeNe lasers. The 488 nm excitation band and 540–600 nm detector were used for lipid droplets visualization, 638 nm excitation band and 638-710 nm detector were used for detection of ADRP protein and 458 nm excitation band and 464-487 nm detector for detection of chromatin. The 40x Leica HCX PL APO CS objective, pinhole, offsets, gain and AOBS were adapted. These parameters were kept throughout the whole experiment. The oocytes were scanned in equatorial optical section, microphotographs were saved and processed using the NIS – Elements AR 3.00 software.

Statistical analysis . All data were subjected to one-way ANOVA, and the significance of difference among means was determined by the Fisher's least significant difference (LSD) test (StatSoft, Inc. 2011. STATISTICA, data analysis software system, version 10.). Differences at $P<0.05$ were considered statistically significant.

RESULT AND DISCUSSION

Experiment 1 was conducted to determine lipid composition and specificity of adipose differentiation-related protein in matured porcine oocytes with different meiotic competence.

The onset of maturation process subordinate on the implementation on many important cytoplasmic factors. Modifications correlated with ultrastructure of the growing oocytes linked to aggregation of lipid droplets are precondition of energy for meiotic resumption. In this experiment we examined the capability of oocytes obtained from two follicular size classes to resume and complete meiosis. Several authors have already confirmed that the quality of oocytes, their maturation, fertilization and embryo development is directly influenced by the size of follicles from which oocytes are isolated (Bolamba and Sirard, 2000; Marchal et al., 2002; Machatkova et al. 2008). According to Homa et al. (1986) the lipid content in porcine oocytes promoted growth and development with a potential role in the regulation of maturation process. In our research, the total area covered with

lipid droplets in matured oocytes was lower in meiotically less competent (MLC) oocytes in comparison to meiotically higher competent (MHC) oocytes, but did not significantly differ (Table 1.), which implies that meiotically higher competent oocytes contain a higher number of energy LDs units.

Table 1. The total area covered with LDs in MLC and MHC oocytes after maturation

Grade of meiotic competence	*n*	*Area of lipid droplets (%)*
MLC	105	28,8±7,5 [a]
MHC	89	29,8±8,4 [a]

Data with same superscripts are not significantly different.

Lipid droplets are important cytoplasmic markers and have major role in energy matabolism during the process of maturation of porcine oocytes (Kikuchi et al., 2002). Nimura et al. (2002) have reported that the large number of small lipid droplets concentrated in mature porcine oocytes were an energy source for fertilization and early embryo development. Hiraga et al. (2013) assessed lipid droplets as an important cytomplasmic parameter for *in vitro* maturation of porcine oocytes with high developmental competence. As shown in table 2. the total number of lipid droplets was higher in oocytes derived from medium follicles in comparison to oocytes derives from small follicles, these data indicated that meiotically higher competent oocyte have a higher number of LDs.

Table 2. The total number of LDs in MLC and MHC oocytes after maturation

Grade of meiotic competence	*n*	*Number of lipid droplets*
MLC	105	277,64±100,6 [a]
MHC	89	296,6±109,9 [a]

Data with same superscripts are not significantly different .

Proteomic research have described that LDs are surrounded by structural proteins, proteins that mediate membrane traffic and proteins which are included in the biosynthesis and decomposition of lipids (Bartz et al., 2007). Jiang and Serrero (1992) first recognized ADRP protein in differentiation process of adipocyte cells. ADRP is a 50 kDa protein and a member of PAT family, associated with the surface of intracellular lipid droplets and included in fatty acid metabolism (Bickel et al., 2009). ADRP regulated lipid storage in different types of cells (Kim et al. 2005) and has important role in lipid droplet formation (Immamura et al. 2002). To complete the specifity of the neutral lipid dye for LDs in porcine oocytes, we labeled oocytes with an antibody against the lipid droplet specific protein ADRP. Figure 1 shows that the LDs specifically are wrapped by ADRP protein in cytoplasm of porcine oocytes.

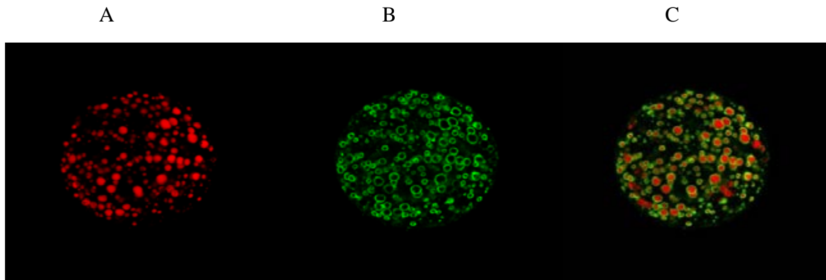
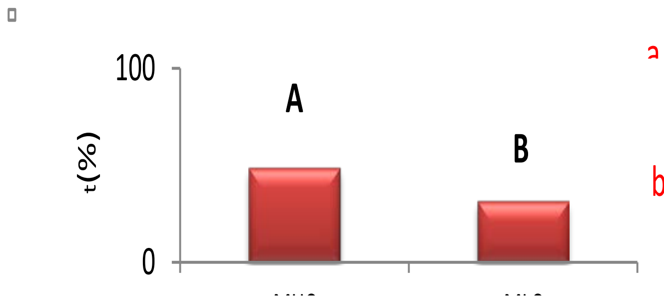


Figure 1. Representative equatorial sections of porcine oocytes stained for cytoplasmic lipids (A), adipose defferentiation-related protein (B) and merge (C), imaged with confocal microscopy. Scale bar represents 20µm.

Several authors estimated that of ADRP localizes to neutral lipids in different types of cells. Aardema et al. (2011) determined ADRP on LDs of bovine oocytes. Brasaemle et al. (1997) estimated ADRP localization with LDs by immunocytochemical method, in cultured murine 3T3-L1 adipocytes, murine MA-10 Leydig cells, Chinese hamster ovary (CHO) fibroblasts and human HepG2 hepatoma cells. Heid et al. (1998) confirmed presence of ADRP on LDs in Sertoli cells of testes, cirrhotic liver, mammary gland and adrenal cortex.

Experiment 2. Evaluation of mitochondrial distribution and ATP content in porcine oocytes with different meiotic competence

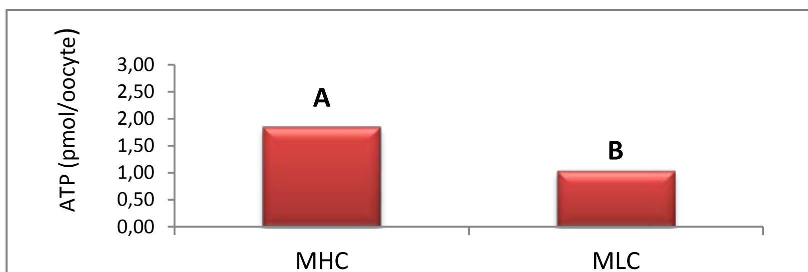
To appraise energy status of porcine oocytes, we examined mitochondrial distribution and ATP content between two different quality classes of oocytes, after maturation *in vitro*. Mitochondria are closely correlated with LDs as a specific metabolic units. Sturmey et al. (2006) determined, by FRET method, that mitochondria are related to lipids within 10 nm, however close relationship between these two organelles promotes rapid transport of free fatty acids from LDs to the mitochondria. In rabbit oocytes mitochondrial activity increases analogically with development of follicular size (Kanaya et al., 2007). Egerszegi et al. (2010) observed a higher mitochondrial activity in developmentally more competent porcine oocytes in contrast to developmentally lesser oocytes. Sun et al. (2001) studied mitochondrial changes in distribution during maturation of porcine oocytes and their data indicate that the oocyte maturation, fertilization and early embryo development are closely related to alterations in allocation of mitochondria. In our experiment we detected significantly higher ($P<0,05$) proportion of metabolic units in MHC oocytes after maturation (Figure 2.).



Values with different superscripts are significantly different (P< 0.05).

Figure 2. Proportion of matured oocytes with metabolic units. a) Representative image of porcine oocyte labeled for mitochondria without metabolic units and b) with metabolic units. Scale bar represents 20 µm.

Mitochondria are dominant cell producer of ATP included in cytoplasmic modulation (Shourbagy et al., 2006). ATP composition represent a significant marker for oocyte maturation (Stojkovic et al., 2001), as well as the increase of ATP amount is associated with meiotic competence of oocytes (Machatkova et al., 2012). The meiotic maturation happens in the presence of extensive sweep of ATP in human and mouse oocytes, but in human oocytes superior possibility for reaching embryogenesis is related to embryos developing from oocytes with content of ATP > or = 2 pmol per oocyte (Van Blerkom et al., 1995). In present research ATP content was higher (P< 0.05) in MHC oocytes compared to MLC oocytes (Figure 3).



Values with different superscripts are significantly different (P< 0.05).

Figure 3. ATP content in MHC and MLC matured porcine oocytes after maturation.

Nagano et al. (2006) investigated maturational capability and ATP amount in bovine oocytes, their result indicate that morphological structure of bovine oocytes is closely associated to their ATP levels. Several authors have already reported increasing of ATP content during maturation process in bovine oocytes (Brevini et al., 2005; Stojkovic et al., 2001). Machatkova et al. (2012) observed that ATP content increase significantly from germinal vesicle to metaphase II stage in different bovine oocytes categories.

CONCLUSIONS

In this research, the meiotically higher competent porcine oocytes contain a superior amount of estimated energy components in comparison to meiotically less competent oocytes. These findings also indicate an important role of lipids in porcine oocyte maturation and suggest the possible role of lipid droplets associated protein ADRP. Further research is needed to explore the detailed functions of this protein in porcine oocytes. In summary, we have identified basic energy parameters in porcine oocytes, which are directly in interaction with their quality.

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