Quality Evaluation of Gilts and Sow’s Oocytes During In Vitro Maturation Based on Mitochondria Distribution

Iuliu TORDA, Irina Ioana SPĂTARU, Gabriel OTAVĂ, Simona MARC*, Oana BOLDURA, Ioan HUȚU, Bianca LUNGU, Beatrice TUDOR, Ovidiu GEORGESCU and Călin MIRCU

Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine of Banat “The King Michael I of Romania”, Timisoara, Romania
*Corresponding author S.Marc: simona.marc@usab-tm.ro

RESEARCH ARTICLE

Abstract
Mitochondria is the most frequently studied cell organelle in relation to development competence of the oocytes both in vivo and in vitro. Developmental competence of oocyte is crucial for embryonic development, the molecular activity until the activation of the embryonic genome being based on the maternal reserves stored in the oocyte. Study was designed to qualitatively evaluate 133 COC class C1 and class C2 gilts and sow’s oocytes according to the distribution of mitochondria after IVM, based on Rhodamine 123 stain solution. Regarding mitochondrial distribution, the percentage of oocytes that had a homogeneous distribution around the germinal vesicle was higher in group C1 than C2 and also higher in sow oocytes than in gilts oocytes. For class 1, the difference was 10.58% in favor of sows, the same trend is maintained for C2, the difference being 4.37%. The results are confirmed by morphological examination, where C1 sow’s oocytes matured 26.09% more, compared to gilts oocytes, a difference maintained for C2 oocytes, being 27.4% more sow’s oocytes IVM compared to gilts. Based on mitochondrial distribution we observed that the stage of sexual development of females influences the IVM of oocytes. For pig’s IVF it’s recommended to use C1 sow oocytes.

Keywords: mitochondria, swine oocytes, IVF.

INTRODUCTION
In vitro fertilization (IVF) is an effective method of assisted reproduction technology (Pilar and Romar, 2002). The first approach using in vitro handling technology in sows was made in 1986 (Mattioli et al., 1989). Swine reproductive biotechnology is an important field for research studies due to supplying porcine stem cells for xenogenic tissues and organs in human regenerative medicine (Ahn et al., 2012). All of these purposes need intensive selection of developmentally competent oocytes, otherwise the majority of swine oocytes submitted to in vitro production (IVP) fail to develop to the blastocyst stage. Selection of competent oocytes is done during the first step of IVP, in vitro maturation (IVM) - one of the most critical step of the process. Maturation of oocyte depends on two essential aspects of cytoplasmatic and nuclear maturation. The incomplete maturation can reduce the success of oocyte developmental competence. In vitro maturation of fully grown mammalian oocytes is characterized by initial germinal vesicle breakdown (GVBD) and rearrangement of microtubule network during the first meiosis (MI), followed by extrusion of the first polar body and block of the oocytes in metaphase of the second meiosis (MII). Only fully matured oocytes are capable of undergoing fertilization and the initiation of zygotic development (Ellederova et al., 2004). The complexity of the phenomena that contribute to in vitro oocyte maturation is reflected in the multiple ways of attesting it. Among the methods
used for this purpose is the appreciation of the distribution of mitochondria. Mitochondria are organelles that play a critical role in generating metabolic energy from eukaryotic cells. They are responsible in producing most of the energy by breaking down carbohydrates and fatty acids, which is converted to ATP (adenosine triphosphate) through the process of oxidative phosphorylation (Christakos et al., 2016). Mitochondria have also ubiquitous roles as regulatory elements in calcium homeostasis because possess a series of Ca\(^2+\) transport influx and efflux channels, interacts with endoplasmic reticulum membranes in order to buffer Ca\(^2+\) in the cytoplasm, role in signal transduction or in oxygen sensing, in regulation of intracellular redox potential, control of apoptosis or may be the mediators of cellular aging (Van Blerkom, 2008; Dumollard, 2007). But overload mitochondrial Ca\(^2+\) stimulates mitochondrial permeability transition pore opening and mitochondrial swelling resulting in mitochondrial injury, apoptosis and in cardiomyocytes cell death (Xu et al., 2020). In fact, alterations in mitochondrial function are recognized as a contributing factor in cardiovascular diseases and researchers are trying to find new potential biomarkers of mitochondrial disease and new therapeutic approaches (Murphy et al., 2016).

At the beginning of oogenesis, the number of mitochondrial progenitors is low, but in the mature oocyte they increase to approximately 1x10\(^4\) to 1x10\(^6\) mitochondria/oocyte. The number of the mitochondria has been suggested to be a critical determinant of developmental competence. Zeng et al. (2007) reported that approximately 50% of human oocytes at germinal vesicle stage (GV) that maturated in vitro to MII exhibited 1st polar body, but no detectable metaphase spindle. When the number of mtDNA copy and the ATP content were counted, researchers found that in oocytes without a detectable metaphase spindle these parameters were lower than for oocytes with metaphase spindle, suggesting that low mtDNA copy number indicates reduced organelle complement, reduced ATP content which corresponds to reduced cytoplasmatic bioenergetic capacity. Furthermore, consequences were seen in low fertilization rate after ICSI or if fertilized, high abnormal embryonic development appeared, suggesting that there is a relationship between the low mtDNA, ATP content and the oocyte/embryo competence. Contrary, Brevini et al. (2005) did not observe differences in the formation of meiotic spindles between high and low competence pig oocytes groups or ATP content at the end of IVM, but extensive relocation of mitochondria to the inner cytoplasm and well-developed cytoplasmic microtubules were observed in high competence oocytes, suggesting that the microtubule cytoplasmic network is important in mitochondria relocation and this can be independent from meiotic spindle formation or ATP content.

In embryonic development, mitochondria are inherited maternally and independently of the nuclear genome. Till the stage of gastrulation, when the replication starts again, the mitochondrial population of the embryo is based on mitochondria that exist in the oocyte. Another important aspect is that the distribution of mitochondria in the mature oocyte will be maintained during early development, which means that blastomeres with different mitochondrial load will appear that may potentially confer a different developmental fate (Dumollard, 2007).

Because these organelles are so important for the future embryo and that they are inherited maternally, the aim of this study was to qualitatively evaluate pig oocytes according to the distribution of mitochondria after in vitro maturation.

**MATERIALS AND METHODS**

Sow ovaries (n=20) and gilts ovaries (n=20) were obtained from slaughterhouse and transported to the laboratory in 0.9% NaCl solution supplemented with antibiotics (Pen/Strep, 17-602F, Lonza), at 35°C within two hours. The medium used in washing COC (cumulus-oocyte complex) was TL-HEPES medium (pH 7.4) supplemented with 0.1% PVA, the medium being prepared in the laboratory, as follows: 5M NaCl, 2M KCl, 1M HEPES pH 7.4, 1M CaCl\(_2\), 1M MgCl\(_2\) and 0.3g% glucose. COCs were aspirated by puncture procedure from medium to large follicles with 18G needle attached to a 5 ml syringe. Maturation medium was TCM-199 supplemented with cysteine (0.57 mM), 10% FCS (fetal calf serum), 10% porcine follicular fluid and 10 U/l/ml hormones (PMSG and hCG). Classification of COCs based on morphological aspects was done under stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4°C): 1\(^{st}\) class - CO (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous, 2\(^{nd}\) class - CH (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation) and 3\(^{rd}\) class - CI (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm). Only class I (C1) and II (C2) oocytes were used. Maturation was performed at a temperature of 39°C and at an atmosphere of 5% CO\(_2\) for 22h in a hormones culture medium, then continuing the maturation for 22h without hormones under the same conditions, signs as expansion and mucification of cumulus cells were observed. The COCs were matured according to there their morphological class (36 COCs in C1 sow, 24 COCs in C1 gilts, 35 COCs in C2 sow, 38 COCs in C2 gilts). Preparation of oocytes for staining consisted in enzymatic denudation (hyaluronidase 0.3%) by repeated manual pipetting until the cumulus cells were completely removed, then successive washing with PBS and staining with 1 μM Rhodamine 123 to evaluate the mitochondrial distribution. The 1 μM Rhodamine 123 solution was obtained from 1 mM Rhodamine 123 (0.4 mg Rhodamine 123 in 1 ml DMSO) stock solution by dilution with PBS. Prior to use, the dye solution was incubated at 37°C. The oocytes were kept for 30 minutes in dye at 37°C, washed twice with PBS, fixed in 10% formalin, then mounted on a microscopic blade and
RESULTS AND DISCUSSIONS

Evaluation of *in vitro* oocyte development based on mitochondrial distribution using Rhodamine 123 dye revealed dispersed cytoplasmic localization of mitochondria before maturation and a centered localization around the genetic material after 44 hours of *in vitro* maturation; there are differences depending on the morphological class of the oocytes and their source. Thus, in first-class sow oocytes, most had a mitochondrial distribution around the germinal vesicles (Figure 1). The percentage of oocytes that had a homogeneous distribution of the mitochondria around the germinal vesicle was higher in group C1 than C2 and also higher in sow oocytes than in gilts oocytes.

For class 1, the difference was 10.58% in favor of sows, the same trend is maintained for C2, the difference being 4.37%. The results are confirmed by morphological examination, where C1 sow's oocytes were with 26.09% more matured, compared to gilts oocytes, a difference maintained for C2 oocytes, being 27.4% more sow's oocytes IVM compared to gilts (Figure 2 – Figure 3).
The distribution of the mitochondria during pig oocyte maturation proceeds from a peripheral location and become disperse, more heterogeneous and granulated, being during the early stages of division distributed around the nuclei (El Shourbagy, 2006). Variation in oocyte mtDNA copy number in pig oocyte (10,000-700,000) and also variable distribution after fertilization of mtDNA between blastomeres explain why some blastomeres survive and divide and other become fragmented, with low ATP generation or even mtDNA-driven atresia (El Shourbagy et al., 2006). It is known that in humans (Zeng et al., 2007) but also in cattle, mice or pigs there is a distinct pattern of mitochondrial distribution between mature and immature oocytes, so that they form networks throughout the cell, with the distribution model specific for different stages of the cell cycle. The distribution of mitochondria is possible correlated with the mtDNA copy number, because those oocytes with low mtDNA may be unable to form the networks required for developmental competence. Studies of El Shourbagy et al. (2006) revealed that is a significant difference in mtDNA copy number between fertilised and unfertilised oocytes and unequal mitochondrial segregation between blastomeres during early cleavage stage. Furthermore supplementation of BCB negative oocytes (high G6PD level) with mitochondria from maternal relatives generated a higher fertilisation rate after IVF and ICSI.

In vitro maturation is less effective in gilt oocytes compared to sow oocytes. The reduced ability to mature properly in vitro is related to their limited exposure to hormones (Pinkert et al., 1989), but also to the fact that they are more susceptible to oxidative stress (Yuan et al., 2012). Oocytes must maintain a balance between the generation and elimination of reactive oxygen species, and studies made by Yuan et al. (2012) have shown that gilt oocytes cannot cope with redox balance as effectively as sow oocytes, especially due to low expression of glutaredoxin 2 (GLRX2), protein disulfide isomerase 4 (PDIA4), thioredoxin reductase (TXNRD) and increased glutathione content in sow oocytes. Cumulus cells and follicular fluid also play an important role in maintaining redox balance. Abnormal patterns of redox gene expression and disruption of redox homeostasis contribute to decreased competence in the development of sow oocytes and in vitro maturation. The balance between reactive oxygen species and glutathione plays an important role in oocyte quality (Yuan et al., 2012). There is a complex energy-supply system in oocytes under follicle-stimulating hormone stimulation through which the oocytes acquire developmental competence. The results of Hashimoto et al. (2019) revealed that forskolin and 3-isobutyl-1-methylxanthine treatment of prophase-stage bovine oocytes induced the expression of genes required for glycolysis, fatty acid degradation, and the mitochondrial electron transport system and improved mitochondrial functions and ATP levels in oocytes without involving nuclear maturation. Based on important role of mitochondria in oocyte’s acquiring developmental competence and supporting the early stages of embryonic development, mitochondrial assessment of oocyte prior to any oocyte protocol may enhance success rates of assisted reproductive technologies.

CONCLUSIONS

In conclusion, the stage of sexual development of females influences the in vitro morphological maturation of oocytes. As indicated also by distribution of mitochondria, the oocytes from sows should be used for IVF in swine.

Author Contributions: S.M. and C.M. conceived and designed the analysis; I.T, G.O. and B.L. collected the data; O.B, B.T., O.G. and I.H. contributed data or analysis tools; S.M., I.S. and B.L. performed the analysis; S.M. and I.S. wrote the paper.
Funding Source: This research was funded by UEFISCDI, project number PN-III-P1-1.2-FPRD-2017.

Acknowledgments
Activities under this work were carried out in the Research Laboratory Complex "Horia Cernescu" - financed by project "A bio-economical approach of the antimicrobial agents - use and resistance", in the frame of contract PCCDI 7/19.03.2018, code: PN-III-P1-1.2-FPRD-2017.

Conflicts of Interest
The authors declare that they do not have any conflict of interest.

REFERENCES