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Characteristics of cytoplasmic parameters in porcine oocytes during folliculogenesis

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Abstract

The characteristics of energy status in porcine oocytes as related to their meiotic competence and *in vitro* maturation were studied. Cycling pubertal gilts in the early luteal to the early follicular phases of the ovarian cycle were used as oocyte donors. The oocytes recovered from medium (MF) or small follicles (SF) were considered meiotically more or less competent, respectively. The oocytes were examined before or after maturation by confocal microscopy, a bioluminescent cell assay and Western blotting. Four experiments, each in triplicate, were performed to assess both SF and MF oocytes in terms of metabolic units formed by mitochondria and lipids, ATP and lipid consumption and lipid droplets with adipose differentiation-related protein (ADRP) expression. In conclusion, specific differences in energy characteristics between porcine oocytes with different meiotic competence were found. Meiotically more competent oocytes are more advanced in terms of energy reserves before maturation, while meiotically less competent oocytes are more active in replenishing energy stores during maturation.

Key words: porcine oocytes, *in vitro* maturation, energy status, meiotic competence

Abstrakt

Byly studovány charakteristiky energetického statutu prasečích oocytů ve vztahu k jejich meiotické kompetenci a zrání *in vitro*. Jako dárkyně oocytů byly využity cyklující pubertální prasničky od časné luteální do časné folikulární fáze ovariálního cyklu. Oocyty izolované ze středních folikulů (MF) byly posouzeny jako s vyšší meiotickou kompetencí a oocyty z malých folikulů (SF) jako s nižší meiotickou kompetencí. Oocyty před a po zrání byly vyšetřeny pomocí metod konfokální mikroskopie, bioluminiscenčního testu a Western blottingu. Byly provedeny čtyři experimenty, každý ve třech replikách, k vyhodnocení jak MF tak SF oocytů ve smyslu výskytu metabolických jednotek tvořených mitochondriemi a lipidy, spotřeby ATP a lipidů a výskytu tukových kapének s přítomností adipose differentiation-related proteinu (ADRP). Práce odhalila specifické rozdíly v energetických vlastnostech prasečích oocytů s různou meiotickou kompetencí. Oocyty s vyšší meiotickou kompetencí jsou pokročilejší ve tvorbě energetických rezerv před zráním, zatímco oocyty s nižší meiotickou kompetencí jsou aktivnější v doplňování energetických zásob během zrání.

Klíčová slova: prasečí oocyty, zrání in vitro, energetický status, meiotická kompetence

CONTENT

1 Introduction 9
2 Aim........ 11
3 Literature review........ 12
3. 1 Oogenesis........ 12
3. 1. 1 Proliferation phase........ 13
3. 1. 2 Oocyte growth........ 14
3. 1. 3 Oocyte maturation........ 15
3. 1. 4 Nuclear maturation........ 15
3. 1. 5 Cytoplasmic maturation........ 16
3. 1. 5. 1 Organelle distribution........ 16
3. 1. 5. 2 Cytoskeleton dynamics........ 17
3. 1. 5. 3 Molecular maturation........ 18
3. 1. 6 Maturation in vivo and in vitro........ 19
3. 1. 7 Regulation of oocyte maturation........ 19
3. 2 Meiotic competence........ 20
3. 3 Developmental competence........ 21
3. 4 Folliculogenesis........ 23
3. 5 Mitochondria........ 25
3. 6 Adenosine triphosphate (ATP)........ 29
3. 7 Lipid droplets (LDs)........ 31
3. 8 Adipose differentiation-related protein (ADRP)........ 35
4 Materials and methods........ 38
4. 1 Oocyte donors........ 38
4. 2 Oocyte collection........ 38
4. 3 Oocyte maturation........ 38
4. 4 Experiment 1........ 39
4. 4. 1 Mitochondrial-lipid dual staining........ 39
4. 4. 2 Oocyte examination........ 39
4. 5 Experiment 2........ 40
4. 5. 1 ATP examination........ 40
4. 6 Experiment 3........ 40
4. 6. 1 Lipid droplet staining........ 40

4. 6. 2 Oocyte examination 40
4. 7 Experiment 4 41
4. 7. 1 ADRP staining 41
4. 7. 2 Oocyte examination 41
4. 7. 3 ADRP identification by Western blotting 42
4. 8 Statistical analysis 42
5 Results and discussion 43
5. 1 Evaluation of metabolic units 43
5. 1. 1 Discussion 44
5. 2 Evaluation of ATP content 46
5. 2. 1 Discussion 47
5. 3 Evaluation of lipid content in porcine oocytes 49
5. 3. 1 Experiment A: The relative area of lipid droplets in oocytes derived from small and medium follicles from GV to MII stage as related to the phase 49
5. 3. 2 Experiment B: The relative number of lipid droplets in oocytes derived from small and medium follicles from GV to MII stage as related to the phase 50
5. 3. 3 Experiment C: Relative number of lipid droplets in oocytes derived from small and medium follicles from GV to MII stage, as related to the phase 51
5. 3. 4 Experiment D: Lipid droplets in oocytes derived from small and medium follicles regardless of the phase 53
5. 3. 1 Discussion 55
5. 4 Detection of adipose differentiation-related protein-ADRP 60
5. 4. 1 Discussion 61
6 Conclusions 64
7 References 66
8 List of figures 92
9 List of tables 93
10 List of abberaviations 94
11 List of attachments 97

1 INTRODUCTION

Reproduction is present in every cell, by mitotic division cells of multicellular organisms reproduce and by merging of gametes basic reproductive unit comes to development of new organisms. In plants and animals, there are two main types of cells: somatic and reproductive cells, known as germ cells. Stem cells create somatic cells, while germ cells give rise to gametes. The basic difference among them is reflected in the number of chromosomes, somatic cells contain homologous pairs of chromosomes, whereas gametes contain only unpaired chromosomes. Mitosis or mitotic cell division occurs in somatic cells and represents a fundamental process of living cells. It consists of two interrelated processes: the division of the nucleus and cytokinesis and the changes in the cytoplasm which include proper division of the cell. In mammals, there are two types of gametes referred to as oocytes in the female and sperm in the male. The meiotic process which produces haploid cells – oocytes and sperms and the subsequent maturation of these cells into functioning gametes occur in process of gametogenesis. The creation of a new organism includes the process of formation of gametes (gametogenesis) and their merging (fertilization). The fusion of a maternal and a paternal gamete is a complex and fascinating process.

Mammals are indeed impressive organisms and they have different morphologies with important characteristics which allow scientists to create animal model systems. Establishment of these animal systems is very important for further investigations, because it is very difficult to establish an appropriate model for research in more complicated species of animals and human. In recent years, the major challenge in the field of reproductive biotechnology has been to explore the molecular and cellular mechanisms that are involved in controlling the quality of oocytes. The mammalian oocyte is a specific structure consisting of cytoplasmic organelles which communicate among themselves and are spatially associated. Porcine species represents a good experimental model for elementary and biomedical research because of their physiological and immunological resemblances with humans (Prather et al., 2003; Zhou and Li, 2009). The porcine embryo production *in vitro* is very important in reproductive biotechnology for cloning, transgenesis, since these animals are used as donors of organs for xenotransplatations, embryonic stem cell lines and also as a model of human diseases.

In female fertility, oocyte quality represents an important factor and despite enormous efforts of scientists, the factors and mechanisms affecting its quality has not been sufficiently described yet. In the present research we took into consideration the role of ultrastructural cytoplasmic parameters related to energy metabolism in porcine oocytes with different developmental competence. These energy components directly affect the quality of oocytes.

2 AIM

The aim of the work presented in this thesis was to investigate the role of cytoplasmic energy parameters in porcine oocytes and their comparison among different stages of folliculogenesis. Another aim was to verify a connection between the meiotic competence and observed cytoplasmic parameters. We focused on alterations among lipid composition, mitochondria, ATP consumption and ADRP content during the transition from the germinal vesicle stage to the metaphase II.

Within the stated aim there were these selected partial aims:

- Characterization of changes in metabolic units formation and proportion of oocytes with metabolic units.
- Determination of adenosine triphosphate consumption.
- Quantification of the relative area covered with lipid droplets, the mean number of lipid droplets and ratio of small and large lipid droplets in immature and mature oocytes regardless and related to the phase of folliculogenesis.
- Detection of adipose differentiation-related protein on the surface of lipid droplets in porcine oocytes and changes in its expression.

3 LITERATURE REVIEW

3. 1 Oogenesis

Ovary is the female gonad responsible for differentiation and release of a mature oocyte for fertilization and successful spreading of species. Morphological differentiation of the pig ovary starts since day 24 post-coitum (p.c., a measure of embryonic age), when gonads are populated with primordial germ cells. In sows, differentiation of primitive ovary was reached after about 44 days of embryonic development (Pelliniemi and Lauteala, 1981).

Oogenesis represents formation of female gametes, when oogonia formed from primordial germ cells (PGCs) reach the stage of primary oocytes. The process of oogenesis happens in the ovaries and starts three weeks after fertilization in the early fetal development with formation of PGCs (Edson et al., 2009), stops at birth and continues during puberty in the course of the reproductive life of the female (Rahman et al., 2008). In mammals during the process of oogenesis, oocytes grow and produce great amount of macromolecules and undergo a complex morphological and developmental alteration (Wassarman and Albertini, 1994). These developmental processes need high energy support.

Oogenesis begins with a germ cell – oogonia and this cell undergoes series of mitosis to increase in number and ultimately results in up to one to two million cells in the embryo localized in the utmost layers of the fetal ovaries. PGCs appear during the embryo development and originate extragonadally from yolk sac endoderm and part of the allantois which arises from the posterior part of the primitive sex cords. The PGCs migrate from the epithelial yolk sac through the connective tissue to the genital ridges where they rapidly proliferate. After colonization of the gonad, PGCs undergo a phase of mitotic proliferation with an unfinished cytokinesis and this leads to the creation of germ cell. Sanchez and Smitz (2012) reported that migration, proliferation and colonization of PGCs to the developing gonads are regulated by many factors and depend as well on the interaction of PGCs and their surrounding somatic cells. Primordial germ cells can be clearly identified at 7.5 days post-coitum (d.p.c.) during gastrulation by their high expression of alkaline phosphatase. Until 12.5 dpc, PGCs stay sexually undifferentiated, when they combine with the mesodermally derived somatic components of the gonads to form ovaries or testes. In pigs, these cells were observed on the surface of the emerging gonads between 24-26 days of embryonic

development (Bielanska-Osuchowska, 2006). PGCs increase in number through mitotic cell division, later these cells divide meiotically to produce mature oocytes or sperms.

The process of oogenesis consists of the proliferation of PGCs, growth and maturation phase.

3. 1. 1 Proliferation phase

In the first phase of oogenesis, primitive sex cords (medullary cords) were colonized by PGCs which undergo repeated mitotic divisions to form the primordial oocytes known as oogonia-stem cells. Oogonia, surrounded by somatic epithelial cells originating from genital ridge of mesenchymal cells, condense to form individual primordial follicles, which first develop in the inner areas of the ovarian cortex (Guraya, 2008). Then oogonia interrupt mitotic acitivity and enter meiosis (Goto et al., 1999). After meiosis has been started, the oogonial germ cell is called a primary oocyte. The primary oocyte is surrounded by a layer of follicular cells forming the primary follicle. Guraya (2008) reported that differentiation of oogonia into oocytes is closely followed up by their congregation with pregranulosa or follicular cells which get segregated from the somatic cells of the ovarian blastema.

In fetal ovary, the primary oocyte progress gradually, it enters meiosis I, over the different stages of prophase I and arrests at the diplotene stage of the first prophase with homologous chromosomes (Mandelbaum, 2000). At birth, all oocytes from growing follicles are arrested at the diplotene stage of prophase I. The prophase of the first meiotic division as long inactive phase lasts from birth to reproductive age. The oocytes persist in the arrested stage until a few hours before ovulation. As generally known, oocytes of vertebrates are arrested in prophase of the first meiotic division for several weeks, months or years. The duration of this period depends on the species. The oocytes arrested in the diplotene stage of the first meiotic prophase are managed by a few determinants: follicle size, cumulus cell communication, gonadotropins, steroids, cAMP, cAMP-PDE, adenylate cyclase, PK-C, MAP-K, MPF and gene expression (Chaube, 2001). Cyclic AMP is the intracellular signaling molecule produced by granulosa cells and transported via gap junctions to the oocytes, which maintains the oocyte in the meiotic arrest (Desai et al., 2013).

Marteil et al. (2009) reported that the arrest in prophase of meiosis I correlates with the sensitivity to hormone activity which disenables early nuclear maturation of small oocytes. In the process of oogenesis, oocytes go thorugh different cytoplasmic changes. The first cytoplasmic alterations begins at the late diplotene stage of prophase I (Van Blerkom and

Runner, 1984). In this protracted period, proportionally with increasing of their size, oocytes concentrate molecules of mRNA, increase a store of cytoplasmic enzymes, metabolic substrates, lipids and sugars necessary for their maturation and the starting of embryonic development (Marteil et al., 2009). In mammalian species, the developmental procedure commences when maternal RNA and protein are concentrated during growth and maturation of oocytes (Telford et al., 1990).

3. 1. 2 Oocyte growth

Oocytes have to grow before they achieve their competence to resume nuclear maturation for subsequent fertilization and cleavage (Kanitz et al., 2001). The growth of oocytes represents interactions among the somatic and germ cells on the molecular and cellular level. During growing phase of oocytes, fewest cytological changes take place. Oocytes undergo a complex organization of new genes and products, as well as alteration and distribution of the existing ones. During the follicular development, the growth and differentiation of oocytes are controlled by the activity of granulose cells whose functions are regulated by multiform hormones and factors. For the oocyte growth, the intracellular communication among the oocyte and granulosa cells ensures the nutritional and metabolic support (Guraya, 2008). In the growth phase by means of biochemical reactions and cytoplasmic changes, oocytes gradually acquire competence for nuclear and cytoplasmic maturation (Van Blerkom and Runner, 1984).

Chromosomes of the primary oocyte are composed of two chromatids, and each chromatid contains identical DNA molecules. In this period of growth, the overall level of DNA methylation increases, with the development of follicles from preantral stage to full maturity that results in inhibition of gene activity. In the growth phase of oogenesis, major changes of cytoplasmic organelles take place. In the cytoplasm of the oocyte, the second rearrangement occurrs. In the growth phase the number and size of the lipid droplets also enlarge. In this phase, mitochondria circulate from perinuclear area and become distributed over the whole cytoplasm (Van Blerkom and Runner, 1984). In the cortical stroma, after exit from the resting stage, the oocyte starts to grow rapidly and its amount is enchanced by accumulating organelles, lipids, proteins and RNAs (Fauser et al., 1999). The content of proteins necessary for this rapid growth is produced by the oocyte and the granulosa cells.

3. 1. 3 Oocyte maturation

In the process of oogenesis, the competence of mammalian oocytes is influenced by many factors, but one of the most important is oocyte maturation. For adequate fertilization and embryo development and also for implementation of reproductive biotechnologies methods, the procedure of oocyte maturation is greatly important. Development in the embryonic period depends on events occurring during oocyte maturation. In the course of the complex process of maturation, mammalian oocytes achieve gradually the capability for maintenance of following developmental period. The process of maturation and the timing of oocyte meiotic arrest must be firmly managed, because the mature oocytes have a relatively short life time in the female reproductive tract (Mehlmann, 2005). The period of oocyte maturation varies between species. The processes which occur during oocyte maturation are associated with the energy status of oocytes.

Oocyte maturation is a dynamic and highly coordinated process, in which the oocytes finish the first meiotic division, cytoplasmic alteration and prosper to metaphase II (Mehlmann, 2005). During the maturation of oocytes, meiosis I occurs at the time of ovulation and meiosis II at the time of fertilization (Chappel, 2013). When the mature oocyte is exposed to luteinizing hormone (LH) surge, meiosis I is terminated.

Maturation of mammalian oocytes involves nuclear and cytoplasmic maturation and the final result of these processes is a mature oocyte competent for successful fertilization. A few hours before ovulation oocyte develops the ability to be fertilized. The mammalian capacity of oocytes to resume meiosis and undergo nuclear and cytoplasmic changes has a large influence on subsequent process of fertilization and early mammalian development.

3. 1. 4 Nuclear maturation

The oocyte contains a large germinal vesicle (GV) with a large nucleolus, before maturation starts. Nuclear maturation means the meiotic process of chromosomal reduction to a haploid content, so as to produce a diploid organism upon fusion with sperm (Voronina and Wessel, 2003). Throughout nuclear maturation, redistribution of the cytoskeleton eventuate. This process includes actions correlated with the germinal vesicle breakdown (GVBD), condensation of chromosomes, metaphase I spindle formation, separation of the homologous chromosomes with extrusion of the first polar body and arrest at metaphase II (Josefsberg et

al., 2000). With the second meiotic division, chromatids separate and the second polar body is formed. Finally, the chromatids remaining in the oocyte decondense and a pronucleus forms. Niimura et al. (2002) observed lipid droplets in porcine oocytes and reported that alteration in the size of lipid droplets is associated with nuclear maturation. For completion of nuclear maturation, bovine oocyte need 24 h (Sirard et al., 1989), and porcine 44 h (Kim et al. 2011). After successful nuclear maturation, an oocyte arrested in metaphase II is formed. In this phase the oocyte is physiologically relevant to finish the second meiotic division in the process of fertilization.

3. 1. 5 Cytoplasmic maturation

For cellular functions during maturation, fertilization and early embryo development, the cytoplasm of oocytes ensures useful metabolic conditions for the production of energy indispensable in these processes (Cetica et al., 2002). Cytoplasmic maturation means both the ultrastructural changes occurring in the oocyte from the GV to the MII stage and the acquisition of developmental competence of the oocyte (Duranthon and Renard, 2001). Because of this characteristic, there is considerable interest in the cytoplasmic maturation of mammalian oocytes.

Cytoplasmic maturation is regulated and greatly affected by hormone fluctuations. During maturation, the alteration of hormonal concentration probably has a substantial pertinence for the secretory activity of cumulus cells, a subset of granulosa cells. Accordingly, meiotic or cytoplasmic maturation of oocytes may be affected indirectly by the grade and continuance of hormonal exposure of cumulus cells, surrounding maturing oocytes as cumulus oophorus inside follicle.

Cytoplasmic maturation includes organelle rearrangement, storage of mRNAs, proteins and transcription factors which participate in the process of maturation, fertilization and early embryogenesis (Ferreira et al., 2009). The same author classified cytoplasmic maturation into three phases: organelle distribution, cytoskeleton dynamics and molecular maturation.

3. 1. 5. 1 Organelle distribution

As is known, cytoplasmic organelles undergo rearrangement and changes during the process of maturation. Alterations of organelles in cytoplasm are important for oocyte

capability of going through nuclear maturation and fertilization. The changes in metabolic processes of oocytes and rearrangement of their organelles indicate that maturation is a complex process with precise regulation. Ferreira et al. (2009) reported information about ultrastructural analysis of cytoplasmic organelles. According to their research, mitochondria, ribosomes, endoplasmic reticulum, cortical granules and the Golgi complex occupy different positions during oocyte maturation. Organization activity of these organelles and also chromosome segregation are supported by the microfilaments and microtubules positioned in cytoplasm of mammalian oocytes. Also cytoplasmic maturation markers, proteins, regulators of cell cycle and constituents of enzymatic antioxidant system undergo the process of transcription in this phase.

In oocyte cytoplasm, the lipid droplets and mitochondria are indispensable organelles for production of energy, necessary for maturation, fertilization and embryo development (Ambruosi et al., 2009). Cran (1985) described morphological alterations of cytoplasmic organelles and observed them in the germinal vesicle stage and after maturation. During the germinal vesicle stage, volume fraction and number of lipid droplets and mitochondria remained unchanged, but after maturation qualitative changes were noticed. The same author reported a decline in mitochondrial number during porcine oocyte maturation. In germinal vesicle stage the lipid compound in porcine oocytes can be customized to the high requirements of the cells, and advance growth and development (Homa et al., 1986). The results of Cran (1985) indicate that the number of lipid droplets is connected to the resumption of meiotic maturation, because number of lipid droplets in porcine oocytes considerably increases from 30 h to 50 h after application of human Chorionic Gonadotropin (hCG) hormone, while the total amount of lipids remained unaltered.

3. 1. 5. 2 Cytoskeleton dynamics

The cytoskeleton ensures constituent support and provides combined molecular motor proteins to implement cytoplasmic transport and cell motility. Cytoskeleton includes three types of filaments: microtubules, actin filaments (microfilaments) and intermediate filaments (Marteil et al., 2009).

Microtubules and actin filaments participate in oocyte maturation. Microtubules are polymers of α- and β-tubulin and represent the dynamic structure of the cytoskeleton and perform a crucial role in many of different functions (Brevini et al., 2007). In the cytoplasm of porcine oocytes during *in vitro* maturation, microtubules arise from the cortex to the internal

cytoplasm and evanesce at the end of meiotic maturation (Marteil et al., 2009). Microtubules are engaged in organelle allocation and in chromosome segregation within the meiotic spindle. Immature oocytes do not possess microtubules, while after germinal vesicle breakdown a small microtubules begin to appear near the condensed chromatin (Kim et al., 1996). Interestingly, during maturation transitory mesh of the microtubules has been noted only in oocytes with higher developmental competence.

Microfilaments consist of globular and compacted actin subunits (Ferreira et al., 2009). They were detected in the cytoplasm of oocytes at the germinal vesicle stage, while after germinal vesicle breakdown they were placed near to the female chromatin (Kim et al., 1996). Microfilaments have the function to regulate migration of cortical granules and chromatin motion during oocyte development (Sun and Schatten, 2006). Microfilaments participate in creation of the first meiotic spindle and they play a major role in the meiotic spindle arranging (Schuh and Ellenberg, 2008).

The intermediate filaments contain tetramers of fibrous polypeptides (Ferreira et al., 2009; Marteil et al., 2009). These cytoskeleton components sustain the structural integrity of the oocyte and participate in anchoring of maternal RNA in certain areas of the oocyte. The intermediate filaments support nucleolar fusion (Lian et al., 2014).

3. 1. 5. 3 Molecular maturation

Ferreira et al. (2009) described that molecular maturation occurs during oocyte growth. This process includes the transcription, storage and processing of the mRNAs expressed by the chromosomes, which will be further translated into proteins by the ribosomes. For accumulation of mRNA and proteins, oocytes needs functional transcription engine (Sirard, 2001). The proteins derived from these mRNAs participate in maturation, fertilization, pronucleus formation and early embryogenesis. Tomek et al. (2002) described that mRNAs are accumulated in message ribonucleoprotein (mRNP) complexes, while the general protein synthesis is stimulated during maturation. Their results show that translation increased at the time of GVBD and these processes were followed by polyadenylation of mRNA, afterwards transcription declined and polyadenylated mRNA was accumulated until metaphase II.

3. 1. 6 Maturation in vivo and in vitro

The liberation of a mature oocyte from the follicle with the competence to support regular ermbryonic development is the final stage of maturation *in vivo* (Fulka et al., 1998). Oocyte maturation *in vivo* is triggered by the preovulatory surge of gonadotropins via granulose cells (Eppig, 1991) and also cumulus expansion can be induced with FSH or LH hormones (Hillensjo and Channing, 1980). It is probable that spontaneous maturation *in vivo* lasts longer than *in vitro* maturation induced by hormones. Pincus and Enzmann (1935) first described meiotic maturation of mammalian oocytes *in vitro* in rabbits. A number of studies suggest that oocytes isolated from large antral follicles of many species promptly advance to metaphase II in culture and undergo fertilization in a high percentage of instances. However, their developmental potential after fertilization is low. From these facts, it can be concluded that *in vivo* conditions are greater in comparison with *in vitro*. For the provision of energy during maturation, cumulus cells have an effect on the oocyte, because they manage the nutritive repository, across the control of fatty acid lipolysis and synthesis. During *in vitro* maturation, absence of cumulus cells influence metabolism of lipids and causes substandard maturation (Auclair et al., 2013). Oocytes of different species required different intervals for *in vitro* maturation. The porcine oocytes achieved metaphase II after 44 h of maturation culture (Abeydeera et al., 1998; Gonzales-Figueroa and Gonzales-Molfino, 2005), bovine after 24 h in maturation medium (Luna et al., 2001; Smiljakovic and Tomek, 2006), human from 24 h to 48 h (Roberts et al., 2002) and equine between 24 and 32 h (Hinrichs et al., 1993).

3. 1. 7 Regulation of oocyte maturation

Mammalian oocytes are arrested at prophase of the first meiotic division and maturation of oocytes starts with the resumption of meiosis. Shortly before ovulation, meiosis resumes as reaction to a surge of luteinizing hormone from the pituitary gland (Mehlmann, 2005). The wave of LH hormone activates ovulation of the oocyte and induces oocyte maturation. Subsequent activation of M-phase promoting factor causes meiosis and process of germinale vesicle breakdown (Desai et al., 2013). The first meiotic division ends with the emergence of two haploid cells, oocyte II and the first polar body and this represents process of ovulation. Consequently, the oocytes undergo the first meiotic division and then become arrested at

metaphase of the second meiotic division after ovulation and wait for the process of fertilization (Chaube, 2001).

The regulation of meiosis resumption in porcine oocytes is controlled by a few protein kinases such as MPF, MEK/MAP kinase/p90^{rsk}, PKC, cAMP, Connexin-43 and PI 3-kinase. Assembly of meiotic apparatus of porcine oocytes includes MAP kinase/p90^{rsk}, NuMA and γ-tubulin, polo-like kinase 1(Plk1) and CENP-E (Sun and Nagai, 2003). For meiotic resumption, protein synthesis is substantial in mammalian oocytes.

After ovulation, each oocyte continues to metaphase of meiosis II. The second meiotic division begins with the penetration of a viable sperm. Meiosis II is completed only if fertilization occurs, resulting in a fertilized mature ovum and the second polar body. Meiosis is the process whereby diploid oogonia and spermatogonia divide their number of chromosomes, in purpose of subsequent combining with a haploid cell of opposite gender to procreate new diploid individual (Mehlmann, 2005). Then the oocyte extrudes 23 sister chromatids, forming second polar body and a fertilized zygote that has a normal diploid complement of 46 chromosomes. The proces of pulling chromosomes outside of the oocyte, to create the first and second polar bodies, demands important content of energy, which is ensured by ATP from oxidative phosphorylation in the mitochondria.

However, finalization of oogenesis occurs only after a spermatozoon penetrates to a mature, ovulated oocyte (Van Blerkom and Runner, 1984). After fertilization when sperm penetrated into the oocyte, the development that was started during oogenesis is activated and the process of embryogenesis begins. The initial periods of embryogenesis are directed by maternally inherited constituents positioned inside the oocyte. The process of early embryogenesis becomes dependent on the expression of genetic information derived from the embryonic genome when development proceeds and maternally inherited molecules decay (Telford et al., 1990).

3. 2 Meiotic competence

The meiotic competence represents the ability to resume and complete the first meiotic division and to arrest at the second meiotic metaphase. The ability to resume and complete the first meiotic division is connected to the process of oocyte growth, this is the reason for the complicated process of meiosis. After isolation and oocyte culture, the oocytes do not sustain

spontaneous maturation until the final stage of growth, when oocyte acquire competence for spontaneous maturation *in vivo*. Evaluation of energy cytoplasmic markers during process of *in vitro* maturation and investigation of changes in this section during achievement of meiotic competence for development of oocytes is very important. Beyond meiotic competence, the oocyte has to complete its differentiation by acquiring the ability to support the cytoplasmic maturation and finally the ability to be successfully fertilized and develop into a viable embryo. The final differentiation of meiotically competent oocytes occurs at the end of folliculogenesis. Oocyte capacitation comes in the late period of oocyte differentiation.

Meiotic competence of porcine oocytes has been associated with oocyte size, nucleolar structure and function (Motlik et al., 1984). As generally known, the cells derived from large follicles are more competent compared to those obtained from smaller follicles. In early antral follicles, oocytes become able to resume meiosis but only in growing antral follicles they become able to complete meiosis up to metaphase I and progress to metaphase II (Marchal et al., 2002). Porcine oocytes acquire meiotic competence gradually during growth of follicles (Motlik and Fulka, 1986). Marchal et al. (2002) reported that porcine oocytes acquired meiotic competence in ovarian follicles with a diameter of 3 mm and more. In growing porcine oocytes, acquisition of meiotic competence depends on their capacity to activate MPF and MAP kinase (Kanayama et al., 2002). The acquisition of meiotic competence during follicular growth was also described in mouse (Eppig and Schroeder, 1989) and ruminant oocytes (Mermillod et al., 1999).

3. 3 Developmental competence

Developmental competence means the ability of matured oocytes to undergo fertilization, subsequent embryo cleavage, and embryonic development. Internal ability of development is reflected in the quality of oocytes. This is associated to biochemical and molecular processes which support maturation and fertilization of oocytes and enable subsequent embryo development (Gilchrist et al., 2008). The developmental potential of an embryo is reliant on developmental potential of oocyte from which it derives. Mattioli et al. (1989) for the first time described *in vitro* developmental competence of porcine oocytes. Many immature oocytes are able to terminate meiosis *in vitro*, but only a small proportion of them is competent to continue development to the blastocyst stage (Krisher and Bavister, 1998).

According to Sirard et al. (2006), developmental competence of oocytes consists of a few important factors: meiosis resumption, cleavage following fertilization, capability to develop to the blastocyst stage and ability to induce pregnancy and bring it to term in good health. The cumulus cells surrounding the oocyte have an impact on acquisition of oocyte developmental competence.

The quality and developmental competence of mammalian oocytes are characterized by specific cytoplasmic factors: lipids, microtubules, relocation of mitochondria and ATP production. The mature oocytes with high developmental competence have a different lipid structure in comparison with immature oocytes (Ami et al., 2011). The study of Kim et al. (2001) indicates that the fatty acids located in lipid droplets of bovine oocytes are important for oocyte competence. A low developmental competence is related to deficiency of microtubule network which prevents appropriate relocation of mitochondria (Brevini et al., 2005). Several authors already confirmed differences among morphologically good and poor oocytes indicating that activity and relocation of mitochondria is in correlation with their different deveopmental ability after *in vitro* fertilization. Failure of mitochondrial function and insufficiency of ATP production has been associated to reduced developmental competence (Dalton et al., 2014). The nuclear transfer experiments indicated that decline of developmental competence was linked with cytoplasmic defects (Mermillod et al., 1998).

The follicle ambience and maternal signals, transmitted via granulosa and cumulus cells, are important for the progressive gain of developmental competence and as a support for oocyte growth (Gilchrist et al., 2008). Khatir et al. (1997) reported important information that calf follicular fluid, irrespective of the size and quality of the follicle from which it originates, stimulates the acquisition of developmental competence by adult oocytes during maturation, as does adult follicular fluid, but is inactive on prepubertal oocytes. However they concluded that the follicular environment is not responsible for the low developmental competence of prepubertal oocytes, as these oocytes are unable to respond to the stimulatory components of follicular fluid. Koenig and Stormshak (1993) described differences between pubertal and third-estrous gilts, when embryos delivered during the first-estrus cycle are not fully competent for *in vitro* development. The results of Menino et al. (1989) show that the reduced *in vitro* development of embryos collected from gilts mated at the first estrus may be due to an aberration in blastocoel formation and expansion.

The developmental competence of oocytes matured *in vitro* is lower in comparison with oocytes matured *in vivo*, reducing the general effectiveness of *in vitro* maturation process.

According to Rodriguez and Farin (2004), oocytes matured in the presence of gonadotropic hormones have a better developmental competence. The acquisition of developmental competence of bovine oocytes increases with the age of the calves, and full developmental competence of oocytes is accomplished before puberty (Presicce et al., 1997). Volarcik et al. (1998) reported that the developmental competence of human oocyte decreases with age.

3. 4 Folliculogenesis

The ovarian follicles are basic structural and functional unit of the ovary and they ensure micro-conditions essential for oocyte development and maturation (Abd-Allah, 2010). The process of ovarian follicle development is known as folliculogenesis. The process of folliculogenesis starts during foetal development in many mammalian species, involving porcine (Bielanska-Osuchowska, 2006). The same author reported that the creation of ovarian follicles starts on day 56 p.c., while follicles encircled by a single layer of somatic cells are present on day 106 p.c. During oestrus cycle, porcine females express only one wave of follicular activity (Ratky and Brussow, 1998). During the early phases of gestation, follicles are already present in the bovine fetus (Erickson et al., 1966).

Process of folliculogenesis in different animals has been investigated *in vivo* and *in vitro* in many laboratories. Through this process, an ovarian follicle passes through several stages: primordial-resting, primary, secondary-preantral, tertiary-antral, and the preovulatory-Graafian follicle stage. Rüsse (1983) reported first appearance of primordial, primary, secondary and early antral follicles in bovine fetus. Several million primordial follicles are present in the ovaries during the fetal period containing an oocyte surrounded by layers of somatic granulosa and theca cells (Knight and Glister, 2006; Krisher, 2013). The primordial follicles go through consequential growth and development forming preovulatory follicle containing a mature oocyte (Volarcik et al., 1998). The preantral phase is characterized by zona pellucida formation, granulosa cell proliferation, the recruitment of thecal cells to the follicular basal lamina and a dramatic increase in oocyte volume (Pedersen, 1969). Antral follicle is characterized by the existence of antrum in the granulosa and theca externa and a fibrous layer around theca interna. In the preovulatory follicle, the fully grown oocyte has collected nutrient stores, mRNA, proteins and organelles, also great number of mitochondria (Dunning et al., 2014). Immature mammalian oocytes liberated from ovarian follicles proceed in meiosis and complete *in vitro* maturation.

The beginning phase of folliculogenesis starts independently of gonadotrophic hormones (Roche, 1996). However, the oocytes resume meiosis throughout progress from preantral to antral stage and after stimulation by pituitary gonadotropins FSH and LH, the mature oocyte is removed from the follicle and transfer into the oviduct (Uhlenhaut and Treier, 2011). Findings of Tanaka et al. (2001) demonstrated that in bovine fetal ovary, the serum concentration of FSH may play an important role as an initiator of early follicular development. After formation of follicular antrum, which roughly corresponds to the final stage of oocyte growth, granulosa cells differentiate into the mural granulosa cells which have a steroidogenic role and cumulus cells which create a close relatedness with the oocyte (Gilchrist et al., 2008).

In mammals, folliculogenesis is a greatly selective process that includes steps of differentiation and proliferation of somatic and germ cells (Hernandez-Medrano et al., 2012). During prospering from the preantral to the antral stage, the follicle is structurally changed to three separate populations of somatic cells: theca cells, granulosa cells and cumulus oophorus (Piotrowska et al., 2013). After activation of growth, the granulosa cells start to proliferate, at the same time the oocyte initiates the growth stage. After this processes of initiation, development of follicle consists of proliferation and differentiation of the granulosa layers and the oocyte development. Roche (1996) reported that inhibins, activin, insulin-like growth factor I (IGF-I) and their binding proteins have direct and indirect effects on granulosa and theca cells that can modulate follicular development and steroidogenesis. During oocyte growth and maturation, the cumulus cells are metabolically linked with the oocyte and these cells are involved in the process of ovulation and fertilization (Tanghe et al., 2002).

The quality achievement of oocytes, their maturation, fertilization and development are complex processes and the size of follicles from which oocytes are obtained have a significant influence on their maintenance. Size of the follicle from which the oocyte is derived affects the oocytes ability to resume meiosis and reach optimum maturation during IVM (Hyttel et al., 1997). The meiotic and developmental capability of oocytes is achieved gradually, during development of follicles (Eppig et al., 1994; Schramm and Bavister, 1995). According to Marchal et al. (2002), developmental competence rises simultaneously with the size of follicles. The porcine oocytes acquire capability to complete meiotic maturation when they accomplish their full size in antral follicles of approximately 2 mm of diameter and at the same time, the transcriptional activity of oocytes decreases (Motlik et al., 1984).

In vitro embryo production (IVP) represents a method for enhancing the population of genetically valuable animals (Pfeifer et al., 2008). The examination of folliculogenesis is

significant for improving of IVP techniques. Machatkova et al. (2004) reported that interaction among follicle size and the phase of follicular wave has an impact on embryo production. The production of embryos was primarily affected by the number of oocytes collected from medium follicles and the lower developmental competence of oocytes from small follicles. The production of embryos *in vitro* is usually diminished, which implies that all oocytes are not capable of successful *in vitro* maturation and fertilization. The quality of oocytes and *in vitro* conditions are the primary elements which characterize development of embryo and production of normal offspring.

3. 5 Mitochondria

Mitochondria represent evolutionary fragment of bacteria that intruded our ancestral cells about a billion years ago (Chappel, 2013). Mitochondria exist in the cytoplasm of almost all eukaryotic cells and represent important organelles that have been widely investigated by many authors. Mitochondria contain a double membrane, external membrane which separates the mitochondria from the cytoplasm of the cell, and internal membrane forming cristae (Spikings et al., 2006), which represent folds of the mitochondrial internal membrane that ensures an increase in the surface region for ATP production. Intensive research of mitochondria may contribute to better understanding of their function, behavior, dysfunction and their impact on infertility. In all living cells, mitochondria play an important role in metabolic activities. Mitochondria are organelles that represent storehouses in the cytoplasm and participate in redox and Ca^{2+} homeostasis, adaptive thermogenesis, ensure mediator metabolites and store proapoptotic factors (Dumollard et al., 2007; St. John et al., 2010; Koopman et al., 2012). Mitochondrial disorders are responsible for a lot pathophysiological processes, neurodegenerative diseases, diabetes, obesity, aging, and infertility (Chappel, 2013). The most important function of mitochondria is the production of ATP (Dumollard et al., 2007; Koopman et al., 2012). These organelles use high ability of oxidative phosphorylation process to provide ATP, as a necessary source of energy for the cell (Chappel, 2013; Spikings et al., 2006). ATP production via this mechanism is much more efficient compared to anaerobic glycolytic processes. The process of oxidative phosphorylation occurs within the electron transfer chain (ETC) and is highly dependent on the expression of proteins encoded by the mitochondrial DNA and chromosomally encoded genes (St. John et al., 2010).

Mitochondria and mitochondrial DNA (mtDNA) are inherited through the female germ line in mammals (Dumollard et al., 2007; Shoubridge and Wai, 2007; Chappel, 2013). These organelles are monitored through double genome model (Cummins, 2001). The circular DNA of mitochondrial genome is about 16.7 kbp and DNA material of mitochondria (mtDNA) does not contain introns (Chappel, 2013). This DNA encodes enzymes included in the aerobic process of oxidative phosphorylation. Anderson et al. (1981) reported that human mitochondrial genome consists of DNA encoding 13 mitochondrial proteins, 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). The expression of gene products is monitored by signals emitted from the nucleus (Chappel, 2013). Mature oocytes contain at least 100,000 copies of mtDNA, arranged at 1–2 copies per organelle (Dumollard et al., 2006; Shoubridge and Wai, 2007). Regulation of mitochondrial functions is enabled by specific transcription factors encoded in nuclear DNA (Rodley et al., 2012), which also encodes ADP and NADPH precusors necessary for ATP synthesis (Chappel, 2013). The changes of mitochondrial ultrastructure, increased mtDNA copy number, accompanied by diminished levels of adenosine 5´-triphosphate (ATP) and tricarboxylic acid (TCA) cycle metabolites were discovered to be related with deficient reproductive performance (Bentov and Casper, 2013). Aggregations of mutations and deletions of mitochondrial DNA (mtDNA) represent significant feature of the aging process (Mulder, 1982).

The great number of mitochondria and mtDNAs in the oocyte serve as genetic structure to provide their allocation to the gametes and somatic cells of the next generation (Shoubridge and Wai, 2007). According to Piko and Taylor (1987), in contrast to somatic cells, each mitochondrion in oocytes contains only a single copy of mitochondrial genome, this indicates that they represent mitochondrial number (Santos et al., 2006). However, the mitochondrial number represents a good indicator of cell activity. According to Chappel (2013) neurons, muscle cells, and mature oocytes contain more replicas of mtDNA in comparison with other somatic cells. The mitochondrial number enhances, during oogenesis and maturation of oocytes. Mitochondrial replication is initiated in the primordial germ cells. The primordial germ cells consist of approximately 10 mitochondria, in this stage mitochondrial numbers constantly increase (Shoubridge and Wai, 2007), while mature oocytes contain significantly larger numbers (Jansen and De Boer, 1998) which increase to 100,000 (Ramalho-Santos et al., 2009).

Mitochondria are an important marker that designates quality of mammalian oocytes. However, recent data suggest a central role for mitochondria in oocyte quality. In oocyte and early embryo, mitochondria represent the basic point of ATP supply. Mutations of

mitochondria and sequential decrease of ATP can advance follicular atresia and early ovarian defect. The study of Thouas et al. (2005) confirms that mitochondria have an important function in the maintenance of oocyte developmental competence with age via metabolic regulation of energy production and apoptosis. During development, mitochondria have special behavior. Several authors already described different models of mitochondrial allocation in bovine (Stojkovic et al., 2001), murine (Nishi et al., 2003), human (Liu et al., 2010) and canine (Turathum et al., 2010) oocytes. The fully grown oocytes remaining at the GV stage did not demonstrate mitochondrial redistribution, even after 24 h in culture conditions (Van Blerkom and Runner, 1984). The authors reported that at early germinal vesicle (GV) stage, mitochondria appeared uniformly dispersed, but the first redistribution *in vitro* occured after 4-4,5 hours of maturation in murine oocytes. Most of mitochondria were organized in a spherical composition around the metaphase I, and their accumulation in this area increased gradually in the next few hours. However, similar results were observed *in vivo*, after the injection of hCG. During formation of the first metaphase (MI) spindle, mitochondria translocate to the perinuclear area. Perinuclear mitochondrial organization is a special event related to prosperous meiotic maturation (Van Blerkom and Runner, 1984). Van Blerkom and Bell (1986) investigated cytoplasmic translocation of mitochondria during the resumption of arrested meiosis in laboratory mouse. They suggest that the prompt rearrangement of the mitochondria in the cytoplasm is linked to the requirements for the production of ATP. The results of the study of Kruip et al. (1983) on bovine oocytes were similar to the results on murine oocytes by Van Blerkom and Runner (1984). They consistently indicate that redistribution of mitochondria is involved in production of different levels of ATP for the oocyte requirements. Mitochondrial clustering and redistribution begins approximately 1.5-2 h after germinal vesicle breakdown (GVBD). After the process of GVBD, mitochondrial clusters form in the cortical cytoplasm. These mitochondrial clusters are combined with lipids and smooth endoplasmic reticulum to spherical units and move to the center of the oocyte. The second considerable allocation of mitochondria occured during abstriction of the first polar body between 10-12 h after resumed meiosis. Previously, Kruip et al. (1983) described and defined this structure as metabolic unit in bovine oocytes. During the period of maturation, the formation of metabolic units and movement to the central area might ensure required production of ATP. The maturation conditions may affect the oocyte quality and relocation of mitochondria (Stojkovic et al., 2001). The function and dispersion of mitochondria are necessary for maturation and fertilization of oocytes. In bovine oocytes, the major redistribution of mitochondria occurs during oocyte maturation and is associated with

the developmental competence acquired by the oocyte (Bavister and Squirrell, 2000). Before *in vitro* maturation of bovine oocytes, mitochondrial clusters were located in the periphery, but after maturation they concentrated more in the center (Stojkovic et al., 2001). The mitochondrial relocation in rhesus monkey occurs during fertilization, but not in all oocytes (Bavister and Squirrell, 2000). Different factors such as the temperature, production of reactive oxidative species, changes of pH and osmolarity changes may be the reasons for different behavior of mitochondria between *in vitro* and *in vivo* conditions (Nazmara et al., 2014).

Sustention of functional mitochondria is substantial for the early embryo. The type and scope of mitochondrial relocation could be meaningful for embryo development and the appearance of the mitochondrial organization pattern represents a reliable indicator of the developmental competence of the oocyte and embryo (Bavister and Squirrell, 2000). The localization of mitochondria during maturation and their segregation to blastomeres in the cleaving embryo are tightly controlled (Dumollard et al., 2007). In early hamster embryos, active mitochondria surround the pronuclei to create a form that persist during the early cleavage stage (Bavister and Squirrell, 2000). Mitochondrial function runs in a special way in which they can influence the further development of the embryo or disqualify them. Disturbed function of mitochondria is detrimental to the developmental progress and can activate apoptosis in the embryo. Each harmful influence on mitochondrial function will have a negative effect on embryo development before implantation. However, from these facts, it can be concluded that mitochondria play an important double role. They can sustain life and also participate in cell death.

Mitochondria represent considerable intracellular source and target sites of reactive oxygen species (ROS) that are constantly produced as derivates of aerobic metabolism in animal and human cells (Lee and Wei, 1997). The reactive oxygen species are byproducts of mitochondrial respiration, around 90 % of cellular ROS are produced by the mitochondria. The mitochondria liberate reactive oxygen species during the production of ATP (Dumollard et al., 2007). Reactive oxygen species must be detoxified, because they can cause oxidative detriment to mitochondrial DNA (mtDNA), such as mutations and deletions (Chappel, 2013). The consequences of accumulation of DNA mutations are reflected in a reduced ability to produce energy, capability for normal chromosome segregation and maintenance of regular cellular events. In the cell, the mitochondria are wonder of energy production, but they also have another less favorable heritage. Enhanced production of ROS has been associated with high oxygen tension, apoptosis, lack of enzymes that detract the activity of oxidative

phosphorylation, fatty acid oxidation and lower function of antioxidants (Balaban et al., 2005). The constant production of detriment reactive oxygen species is becoming a center of aging exploration. The process of aging has adverse effects on oocytes. Mitochondrial damage in oocytes may be caused during the process of aging or due to surrounding conditions (Kovacic et al., 2005). Oocytes from aging ovaries involve mitochondria with morphological and genetic defects. The oocytes that undergo aging are more susceptible to mitochondrial detriment in comparison with pubertal oocytes.

3. 6 Adenosine triphosphate (ATP)

Adenosine 5′-triphosphate (ATP) is a high nucleotide, contains three phosphate groups and an adenosine group (ribose and adenine). ATP is the energy compound of organisms and has important functions in bioenergetics, whereby its content is used to assess cell viability, proliferation, death, and energy transfer (Chida and Kido, 2014). Terada et al. (2012) described that the level of ATP determines the energy condition in living cells and tissues. The ATP is a substantial pool of energy and provides metabolic activity and synthesis of proteins (Brad et al., 2003) and represents a prominent point of supply of energy for muscle contraction (Terada et al., 2012). The ATP content determines the energy that exists in all living cells and tissues and primarily depends on mitochondrial activity. As is generally known, the levels of ATP represent appropriate marker of viability in cells and also in preimplantation embryos (Slotte et al., 1990).

In mammals, after ovulation and during early embryo development through the process of oxidative phosphorylation, mitochondria produce ATP for energy requests of cell (Dumollard et al., 2006). In this process, high energy electrons originated from oxidation are carried by NADH + H^+ and FADH2 to the inner mitochondrial membrane and transferred through cascades of electron transport chain that transform the electrons into ATP (Ramalho-Santos et al., 2009).

The carbohydrates (glucose), lipids (short, medium and long-chain fatty acids) and protein (amino acids) provide sources for the mitochondria to generate energy in the form of ATP. During early phase of maturation, the oocytes are linked to cumulus cells which ensure metabolic substrates in the form of ATP and pyruvate (Johnson et al., 2007). According to Dalton et al. (2014), the reason for higher levels of ATP in cumulus enclosed oocytes is that cumulus cells provide metabolic support for oocytes via gap junctions pathways.

Accumulation of ATP content during the period of maturation is required for the subsequent process of oocyte fertilization, completion of meiosis II and embryo development (Brad et al., 2003). The results of Nazmara et al. (2014) showed that ATP content of murine oocytes did not significantly change from GV to MII stage. They also reported that the level of ATP in matured oocytes was similar in both *in vitro* and *in vivo* conditions. Dalton et al. (2014) documented that ATP content undergoes dynamic alterations during different phases of maturation in murine oocytes. Using FRET probe they reported interesting results about regulation of ATP levels and its consumption during oocyte maturation. They revealed alterations in ATP content 1 h after the process of germinal vesicle breakdown and during polar body extrusion. However, the ATP utilization was higher in metaphase I and II than in germinal vesicle stage. In bovine oocytes, the ability to extrude polar body did not influence the ATP content, indicating that the metabolic activity is, at least in great part, independent on nuclear maturation (Stojkovic et al., 2001). After *in vitro* maturation of bovine oocytes, the ATP level significantly increases. During *in vitro* maturation, ATP levels significantly increase in porcine oocytes (Brevini et al., 2005). Brad et al. (2003) reported that ATP contents were not different between *in vitro* and *in vivo* matured porcine oocytes. The findings of Van Blerkom et al. (1995) suggest that human oocytes with more than 2 pmol of ATP may be associated with a higher developmental potential of embryos after fertilization and a good ability for their implantation. This suggests that a good quality oocyte consists of optimal mitochondrial numbers and adequate levels of ATP. The results of Thompson et al. (2000) indicate that partial downregulation of mitochondrial ATP production promotes development of *in vitro* produced bovine embryos during their blastulation stages. This is in accordance with the facts that hatching of bovine blastocysts is a mechanical process and is influenced by their energy regulation (Stojkovic et al., 1999). The matured oocytes or zygotes contain more ATP than embryos in blastocyst stage in mouse (Spielmann et al., 1984), cow (Stojkovic et al., 2001) and sheep (Rozell et al., 1992). During human early embryogenesis, the production of ATP can differ notably between oocytes and embryos (Van Blerkom et al., 1995).

Stojkovic et al. (2001) evaluated mitochondrial distribution and ATP content of immature and mature bovine oocytes. Their data indicate that mitochondrial distribution and ATP levels are different between morphologically good and poor oocytes and may affect their different developmental capacity after *in vitro* fertilization. According to Van Blerkom et al. (1995), the ATP content of human mature oocytes with normal morphological appearance was two fold higher than in abnormal oocytes. This is in line with results of Stojkovic et al. (2001) which indicate that immature oocytes with homogeneous cytoplasm and compact

multilayered cumulus oophorus contained two fold more ATP than oocytes with heterogeneous cytoplasm, completely or partially denuded. The ATP content of human expanded blastocysts was greater than in oocytes which failed to be fertilized (Slotte et al., 1990).

The level of ATP can be influenced by internal factors: age of donors, genetics, its production, and also by number and ultrastructure of mitochondria. Also, different contents of ATP in different studies may be due to external factors, such as variations among species, cultivation conditions and method of measuring of ATP content (Nazmara et al., 2014). In oocytes, the reason for the decreased metabolism in oocytes is associated with internal factors more than the conditions of cultivation (Chi et al., 1988).

The ATP is valuable factor which ensures energy indispensable for cellular processes such as resumption of meiosis, fertilization, embryonic development and synthesis of protein (Brad et al., 2003). The proliferation of granulosa cells and production of cellular matrix are coordinated processes which consume energy and demand sufficient production of ATP from intracellular energy sources (Dunning et al., 2014). The processes of chromosome migration and microtubule activity also need ATP for their regular function (Inoue and Salmon, 1995). Yu et al. (2010) examined several different enhancements of ATP levels in cytoplasm of murine oocytes, related to specific events during oocyte maturation. They also observed that alterations of ATP content area were associated with the movement of mitochondria to the perinuclear area and their clustering. During oxidative stress, defects of mitochondria decrease ATP levels and disarrange the meiotic spindle of mouse MII oocytes (Zhang et al., 2006). During oocyte maturation, concomitantly with mitochondrial clustering, cortical microfilaments are formed and their formation can be interrupted by cytochalasin B (Yu et al., 2010). Also ATP production is inhibited by cytochalasin B, which indicates dependence of mitochondria redistribution and thus ATP production on presence and function of cortical microfilaments.

3. 7 Lipid droplets (LDs)

The earliest research on these lipid structures was recorded in the 19th century. Altmann (1890) and Wilson (1896) characterized lipid droplets and contemplated about their ancestry (reviewed by Farese and Walther, 2009). In the early 1900s they were recognized as a

constituent of most cells and these organelles were named liposomes. Lipid droplets are comprehensive and widespread organelles, detected in most types of cells and tissues of vertebrates and invertebrates, also in plants, bacteria and yeast (Walther and Farese, 2009; Brasaemle and Wolins, 2012). Compared to the eukaryotes, only a small group of prokaryotes is known to accumulate lipids in the form of lipid particles for their energy supply. The mammalian and plant cells contain lipid particles with specific classes of polypeptides, mainly perilipins and oleosins. On the other hand, lipid particles of yeast contain a more complex set of enzymes which are involved in lipid biosynthesis. However, lipid particles of all types of cells have a common general arrangement (Zweytick et al., 2000). In comparison with other organelles, lipids bind a special supplement of proteins and lack an aqueous core (Brasaemle and Wolins, 2012). It is considered that the lipid droplets are derived from endoplasmic reticulum (ER), which contains lipid-biosynthesis enzymes, and their synthesis and size are regulated by particular proteins (Murphy and Vance, 1999; Yang et al., 2012). The lipid droplets contain a hydrophobic core of prevailing triacylglycerols (TAG) and sterol esters (SE), which are enclosed by a monolayer of phospholipids to divide the aqueous and hydrophobic phases within the cell (Murphy and Vance, 1999; Zweytick et al., 2000; Farese and Walther, 2009; Brasaemle and Wolins, 2012). These lipid components have significant importance. Phospholipids and cholesterol are necessary for membrane formation, which is needful during the cell division after fertilization, and triglycerides represent repository of stored energy and have a special role in cell functions and oocyte development (McEvoy et al., 2000). In oocytes, triglycerides are considerable constituents of intracellular lipid composition (Homa et al., 1986). Other observations indicate that indicators of reproductive performance of cows are influenced by the concentration of total lipids and cholesterol (Machal et al., 1999).

Mammalian species vary in abundance and exploitation of lipids. Depending on the type of mammalian cell, intracellular lipids are packed in lipid droplets (Zweytick et al., 2000). LDs vary in different types of cells, nevertheless in every organism and cell type, the lipid structure and organization is very conserved (Beller et al., 2010). However, LDs are dynamic and heterogenous in size, their volume, position and content of protein (Ducharme and Bickel, 2008; Watanabe et al., 2010; Prates et al., 2014). They are transported and stored as spherical lipid structures with the average size from 0.1 to 5 µm (Murphy and Vance, 1999). LDs size can range from 0.1 µm in yeast to above 100 µm in a white adipocyte (Walther and Farese, 2009). The cytoplasm of white adipose tissue contains one or a few large (10–100 µm diameter) triacylglycerol rich lipid droplets (Murphy and Vance, 1999). On the other hand,

hibernating animals and the mammalian fetuses/neonates aggregate brown adipose tissue, which consist of smaller (2–10 μm diameter) lipid droplets (Murphy and Vance, 1999). Yang et al. (2012) suggest that storage of neutral lipids and phospholipids may have an impact on LDs growth.

All mammalian oocytes tend to store endogenous lipids. In general, energy in cell is stored in the form of triacyglycerols and partitioned inside of lipid droplets. For the maintenance of normal cellular functions, the presence of lipids is essential. The main function of these organelles has been studied on many types of cells and described by many authors. Lipids are very dynamic structures and have a complex role. The activity of LDs means the storage and mobilization of fat and functional communication with other organelles, such as peroxisomes and mitochondria (Beller et al., 2010). They interact with other organelles and mutually undergo fission and fusion with other droplets (Walther and Farese, 2009). The most important basic functions of these organelles are forming of an energy reservoir, source of constituents necessary for biogenesis of membranes and also formation of lipophilic structures (Zweytick et al., 2000). Lipid droplets are metabolically active organelles that participate in lipid homeostasis, cell signaling and structure; they also modify the physical properties and metabolic functions of biological membranes and intracellular vesicle trafficking (Kim et al., 2001; Robenek et al., 2011; Beller et al., 2010). In mammalian cells, hormones and signaling pathways strongly controle depository and catabolism of LDs (Farese and Walther, 2009). The findings of Romek et al. (2011) indicate a significant role of lipids during porcine oocyte maturation and early embryonic development. In embryos, lipids play important role in sustaining of their viability and progress (Watanabe et al., 2010).

All mammalian oocytes contain an endogenous lipid stock (McEvoy et al., 2000). Many researchers have investigated lipid content in mammalian oocytes and embryos, their aggregation, impact on cell metabolism, structure and harmful effect during cryopreservation process. The intracellular lipids have been recognized by electron microscopy in oocytes of different species, including cow (Kruip et al., 1983), rabbit (Zamboni and Mastroianni, 1966), pig (Kikuchi et al., 2002), cat (Martins et al., 2009), and they were frequently united with mitochondria. Moreover, analysis of the lipid composition of immature and mature mammalian oocytes provides important information about the function of these dynamic organelles. During oocyte maturation, LDs are redistributed in the cytoplasm, this may result in coalescence of LDs and alteration of morphology (Prates et al., 2014). In equine matured oocytes, polar aggregation of LDs is associated with cumulus expansion and nuclear

maturation (Ambruosi et al., 2009). In mouse embryo, LDs form aggregates, while in other models LDs tend to fuse (Watanabe et al., 2010). The results of Fair et al. (1995) indicate that lipid content of bovine oocyte increases in parallel with its size. Hiraga et al. (2013) used stereomicroscope for identification of lipids structure after maturation. They showed that lipid droplets showed homogeneous distribution in the cytoplasm of immature porcine oocytes. Intracellular lipids go through dynamic modifications during the process of oocyte maturation. Cran (1985) reported that the structure and size of the lipid droplets altered during the maturational stage and also formed an enclosing sheath with the ER. Lipid droplets in the cytoplasm of porcine oocytes represent important marker for *in vitro* maturation (IVM) of oocytes with high developmental competence (Hiraga et al., 2013).

LDs are organelles that have substaintal functions in the regulation of metabolism and conditions of various disorders (Watanabe et al., 2010). Enormous storage of lipids can be harmful for the organism, and it is characteristic for the onset of disease such as atherosclerosis, diabetes and obesity (Beller et al., 2010). On the other hand, incapability to accumulate lipids leads to other diseases, e.g. lipodystrophy (Herranz et al., 2008). However, lipid droplets can be involved in different cellular mechanisms. Miyanari et al. (2007) revealed that lipid droplets are involved in the production of infectious particles of hepatitis C virus. They detected virus particles in the vicinity to lipid droplets. Dengue virus infection augments numbers of LDs which documents multiple functions of LDs in life cycle of viruses (Samsa et al., 2009). These results demonstrate the important biological functions of LDs in the presence of different negative factors.

Porcine oocytes are rich in lipids and lipid droplets, which changes their morphological appearance during folliculogenesis. However, it is reported that porcine oocytes contain high amount of lipid droplets and they are very sensitive to low temperatures. This specificity is an obstacle for cryopreservation process (Zhou and Li, 2009). McEvoy et al. (2000) determined that the porcine oocytes contain fatty acids, such as palmitic, stearic and oleic acid, as well as neutral fats and cholesterols. Oxidation of the fatty acid palmitate can produce 106 ATP molecules in contrast to glucose oxidation which generate 30 ATP molecules. The porcine oocytes contain two times higher content of fatty acids in comparison with ruminant oocytes (McEvoy et al., 2000). This is not in line with the study of Yahia Khandoker et al. (1997), who analyzed the fatty acid content of oocytes, follicular, uterine and oviductal fluids of pig and cow using gas chromatography. Their results indicate that the examined samples had similar amount of lipids. Using electron microscope, Cran (1985) revealed that the lipid droplets in porcine oocytes noticeably enhance their number from 30 h to 50 h after human

Chorionic Gonadotropin (hCG) injection, whereas the total amount of lipids in the oocytes remained unchanged from 0 h to 50 h after the injection. Niimura et al. (2002) detected Sudanophilic lipids in cultured porcine oocytes and discovered that lipid droplets reduced in size after 22 h of culture. In their study the number of Sudanophilic lipid droplets of different size was comparable between ovulated oocytes and those after *in vitro* maturation.

3. 8 Adipose differentiation-related protein (ADRP)

Lipid structures presumably originate from microdomains of the endomplasmic reticulum that include lipid-biosynthesis enzymes, while their synthesis and size are under the control of specific proteins (Murphy and Vance, 1999). Modern proteomic analysis enables the identification of novel proteins involved in oocyte development. The lipid surface is covered with a a large number of proteins controlling the biological processes of lipid droplets, which comprise synthesis and distribution of lipids and their communication with other organelles (Bartz et al., 2007; Yang et al., 2012). These interactions are considered to promote transmission of lipids and proteins to other organelles (Miyanari et al., 2007). The variety of the proteins which encircle lipids during adipocyte differentiation connotes a specific mechanism of lipids maturation and their distribution from peripheral location where the synthesis takes place to perinuclear location of lipids storage (Ducharme and Bickel, 2008). Lipid droplets are active organelles consisting of neutral lipids in the center encircled by a monolayer of phospholipids, cholesterol, particular PAT proteins and proteins which comprise in the movement of lipids and in the creation and transport of the droplets (Olofsson et al., 2009). Proteomic studies have described several groups of proteins that are related to the size of LDs. Bartz et al. (2007) reported that lipid droplets include different groups of proteins, such as structural proteins, proteins which cover biosynthesis and breakdown of lipids, and proteins that interfere membrane traffic. The categorisation of LDs according to their proteins indicates that they can participate in a variety of functions in cell. Proteomic investigations on isolated lipid droplets confirmed that proteins are included in membrane turnover, storaging and allocation of lipids in cell.

Members of PAT family proteins are considerable structural proteins which surround lipid droplets. The PAT family includes perilipin, adipholipin/adipose differentiation-related protein (ADRP), tail-interacting protein of 47 kilodaltons (TIP47), S3-12 (Brasaemle et al.,

2004; Ackerman et al., 2007), myocardial lipid droplet protein (MLDP), oxidative tissues-enriched PAT protein (OXPAT), lipid storage droplet protein 5 (LSDP5) (Ducharme and Bickel, 2008), which target to lipid droplets and have important functions in lipid structure and regulation of their metabolic processes. PAT proteins may be implanted to the lipid droplets via insertion of hydrophobic helices to the surface of the droplets (Walther and Farese, 2009). The accumulation and consumption of lipid droplets are controlled by the perilipin group proteins which surround lipids (Ducharme and Bickel, 2008).

Adipose differentiation-related protein (ADRP) coats intracellular lipid droplets (Imamura et al., 2002; Wei et al., 2005) and is expressed early during adipose differentiation (Jiang and Serrero, 1992; Brasaemle et al., 1997). Gao and Serrero (1999) detected ADRP as a 50-kDa protein cloned from a mouse 1246 adipocyte and reported that this protein participated in fatty acid uptake. ADRP is a surface-bound acylated protein. It is expressed simultaneously with lipid droplets creation (Murphy and Vance, 1999) and is involved in neutral lipid transport (Schultz et al., 2002). ADRP is broadly spreaded lipid droplets associated protein and has a function in the metabolism of lipids. The ADRP is the major PAT protein with a prominent homology to perilipin in the N-terminus (Brasaemle, 2007). The hydrophobic N-terminus of ADRP protein, linked with an obvious covalent bond with fatty acids, appears to be suitable for communication with lipid droplets (Heid et al., 1996). Role of ADRP protein is reflected in the balanced lipid droplets structure and regulation of lipid hydrolysis after the initial deposition of neutral lipids within lipid droplets (Brasaemle et al., 1997). The expression of ADRP is highly associated to the amount of neutral lipids in the cell (Heid et al., 1998). At the transcriptional level ADRP is regulated by peroxisome proliferator-activated receptor α (PPARα) (Edvardsson et al., 2006), and through post-translational degradation by the proteasome pathway (Xu et al., 2005).

Heid et al. (1998) described that ADRP was expressed in a wide range of lipid accumulated cells and the ADRP was a marker of lipid accumulation. Immunocytochemical analysis detected ADRP association with neutral lipid droplets in cultured murine 3T3-Ll adipocytes, murine MA-10 Leydig cells, Chinese hamster ovary (CHO) fibroblasts, and human HepG2 hepatoma cells (Brasaemle et al., 1997) and milk lipid globules from humans, cows and rats (Heid et al., 1996). ADRP has an impact on controlling lipid storage depots in different cells, and its locus represents tempting candidate gene for fat deposition characteristics (Kim et al., 2005). In intracellular neutral lipid droplets, this also improves the deposition of triacylglycerols. Imamura et al. (2002) reported that ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. Also Samsa et al. (2009)

determined association of ADRP with LDs. In their research, DENV (dengue virus) infected BHK (baby hamster kidney fibroblasts) cells were analyzed by immunofluorescence with anti-ADRP and anti C antibodies, and labeled with BODIPY. Data of Xu et al. (2005) indicate that ADRP can be degraded through the proteasome pathway. The exogenous oleic acid increases the accumulation of ADRP protein in chinese hamster ovary cells.

4 MATERIALS AND METHODS

Unless otherwise indicated, all the chemicals used in the experiments were purchased from Sigma (Sigma-Aldrich Co., Prague, Czech Republic).

4. 1 Oocyte donors

A total of 325 cycling pubertal gilts (age, 8.5–9 months; weight, 130–150 kg), Landrace × Czech Large White crossbred, were used as oocyte donors. Ovaries of each donor were collected at the local abattoir and transported to the laboratory at 35 °C. They were evaluated in terms of corpus luteum morphology and follicle population and those from the early luteal to the early follicular phase, i.e., before dominant follicle progression and subordinate follicle atresia, were used for oocyte collection.

4. 2 Oocyte collection

The oocytes were collected either from medium (MF; 6–9 mm) or small follicles (SF; 3–5 mm) by aspiration or cutting of the ovarian cortex. Only healthy cumulus oocyte complexes with dark, evenly granulated ooplasm and at least three cumulus layers were selected from each oocyte category and examined before (0 h) or after (44 h) maturation.

4. 3 Oocyte maturation

Both MF and SF oocytes were matured separately in TCM-199 medium (Earle's salts), supplemented with 0.20 mM sodium pyruvate, 0.57 mM cysteamin, 50 IU/ml penicillin, 50 µg/ml streptomycin, 10% NBCS (newborn calf serum) and gonadotropins (P.G.600 15 IU/ml; Intervet, Boxmeer, Holland) in four-well plates (Nunc, Intermed, Denmark) at 38.8 °C under 5% CO_2 in air. An adequate part of oocytes was used to check nuclear maturation and optimize confocal microscopy examination.

In all experiments the oocytes were manually denuded from cumulus cells in TCM-199 medium containing 1% NBCS and 0.1% (w/v) hyaluronidase before they were stained and examined.

4. 4 Experiment 1

4. 4. 1 Mitochondrial-lipid dual staining

The oocytes were incubated at first in phosphate buffered saline (PBS) supplemented with 0.4% BSA (bovine serum albumin) and 2 µM MitoTracker Green FM (Invitrogen, Molecular Probes, Oregon, USA) for 30 min at 38.8 °C to be stained for mitochondria. They were washed three times in PBS and fixed in 3.7% paraformaldehyde for 24 hours. After the oocytes were again washed in PBS, lipid droplets were stained in PBS with 0.4% BSA and 10 µM Nile red dye (Invitrogen, Molecular Probes, Oregon, USA) for 20 min at room temperature. They were washed three times in PBS and mounted on slides, without oocyte compression, using Vectashield Mounting Medium (Vector Laboratories., Burlingame, CA, USA) with 1 µM of DNA dye TO-PRO-3 (Invitrogen, Molecular Probes, Oregon, USA) for either GV or MII stage control. The slides were stored below 0 °C until oocyte examination.

4. 4. 2 Oocyte examination

The oocytes were examined for mitochondrial-lipid co-localization using a confocal microscope (Leica TCS SP2 AOBS; Heidelberg, Germany) equipped with Ar and HeNe lasers and an HXC PL APO CS objective (x 40). The 488 nm excitation band and 490–515 nm detector, the 561 nm excitation band and 565–600 nm detector and 638 nm excitation band and 640–710 nm detector were used for mitochondria, lipid visualization and chromatin control, respectively. The pinhole, offsets, gain and AOBS were adapted and kept throughout the whole experiment. The oocytes were scanned in equatorial optical sections, microphotographs were saved and processed using the NIS-Elements AR 3.00 software (Laboratory Imaging, Prague, Czech Republic).

Mitochondria and lipid droplet scans were fused using NIS-Elements software to visualize mitochondrial-lipid association. The lipid droplets encircled with mitochondria and forming the characteristic morphological structures similar to those described by Sturmey et al (2006) were evaluated as metabolic units. An oocyte in which ≥ 50% of the ooplasm in scanned section was taken up by a metabolic unit area was categorized as the oocyte with metabolic units.

4. 5 Experiment 2

4. 5. 1 ATP examination

The ATP amount in an oocyte was determined with a bioluminescent somatic cell assay kit (FLASC). Briefly, the oocytes were rinsed in PBS and transferred individually, with a 50 µl PBS, into 1.5 ml plastic tubes on ice. Then, 100 µl of ice-cold somatic cell reagent (FLSAR) was added to each tube and the oocytes were incubated on ice for 5 min. Subsequently, 100 µl of ice-cold assay mix (FLAAM assay mix and FLAAB dilution buffer; 1:25) was added to each tube. The tubes were kept for additional 5 min at room temperature in darkness. The intensity of chemiluminescence was measured using a Luminoskan (type 391A; Labsystems, Helsinki, Finland). Each sample was evaluated three-times. The ATP content per oocyte was calculated by the formula derived from a linear regression of the standard curve.

4. 6 Experiment 3

4. 6. 1 Lipid droplet staining

The oocytes were fixed in 3.7% paraformaldehyde solution for 60 min at room temperature, washed in PBS and permeabilized with 1% Triton X-100 for 1 h. The lipid droplets were stained in PBS supplemented with 0.4% BSA and 1 µM Nile red dye (Invitrogen, USA) at room temperature for 10 min. The oocytes were washed three times in PBS and mounted on slides, avoiding oocyte compression, using Vectashield Mounting Medium (Vector Laboratories, USA) with 1 µM of DNA dye TO-PRO-3 (Invitrogen, USA) for either GV or MII stage control. The slides were stored below 0 °C until oocyte examination.

4. 6. 2 Oocyte examination

The oocytes were examined under a confocal microscope (Leica TCS SP2 AOBS; Leica, Germany) equipped with Ar and HeNe lasers and a HXC PL APO CS objective (x 40). The

488 nm excitation band and 540–600 nm detector and the 638 nm excitation band and 640–710 nm detector were used for visualization of lipid droplets and chromatin configuration control, respectively. The pinhole, offsets, gain and AOBS were adapted and kept throughout the whole experiment. The oocytes were scanned in equatorial optical sections, microphotographs were saved and processed using the NIS-Elements AR 3.00 software (Laboratory Imaging, Czech Republic).

4. 7 Experiment 4

4. 7. 1 ADRP staining

The oocytes were fixed in 3.7% paraformaldehyde solution for 60 min at room temperature. They were washed in PBS, permeabilized with 1% Triton X-100 in PBS for 1 h, blocked using 5% rabbit serum in PBS-0.01% Tween 20 with 0.4% BSA, and incubated with primary goat polyclonal antibody against ADRP (C-20:sc-32450; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) diluted 1:50 at 4 °C overnight. Subsequently, oocytes were washed and incubated with secondary rabbit anti-goat IgG antibody conjugated with Cy5 (Jackson ImmunoResearch, Inc., Pennsylvania, USA) diluted 1:100 at room temperature for 1 h. The lipid droplets were stained as was described in Experiment 3. After that the oocytes were washed in PBS and mounted on glass slides, avoiding their compression, using Vectashield Mounting Medium (Vector Laboratories, USA) with 1 µM of DNA dye Sytox blue (Invitrogen, USA) for either GV or MII stage control. The slides were stored below 0 ºC until oocyte examination.

4. 7. 2 Oocyte examination

The lipid droplets and ADRP scans were fused using NIS-Elements software to visualize and check lipid droplets associated with ADRP.

4. 7. 3 ADRP identification by Western blotting

The oocytes were lysed in whole cell lysis buffer (WCL; 10 mM Tris at pH 7.4, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.1% SDS, 1% Triton X-100 and the protease inhibitor cocktail). For ADRP protein detection, 100 oocytes homogenized by sonication were used as a sample. They were heated for 2 min at 95 °C prior to electrophoresis. Proteins were separated in a 4–15% PreCAST polyacrylamide gel and wet blotted on polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK). Blots were blocked with 5% low fat milk at room temperature for 1 h. Subsequently, the blots were incubated with primary goat polyclonal antibody against ADRP (C-20:sc-32450; Santa Cruz Biotechnology, Inc., Germany) diluted 1:250 in washing buffer (100 mM NaCl, 20 mM Tris, 0.1% Tween) with 5% low fat milk at 4 °C overnight. The blots were rinsed with washing buffer. The secondary rabbit anti-goat IgG antibodies conjugated with HRP (Santa Cruz Biotechnology, Inc., Germany) diluted 1:1000 were added to the blots and incubated at room temperature for 1h. The detection of ADRP proteins were performed using chemiluminescence (ECL-detection kit, Santa Cruz Biotechnology, Inc., Germany) and x-ray films.

4. 8 Statistical analysis

At least three replicas were carried out for each experiment. The data were analyzed either with Student's t-test (Experiment 1) or Fisher's least significant difference (LSD) test (Experiment 2–4) using ANOVA SPSS version 11.5 for Windows (SPSS, Inc., Chicago, IL, USA). Differences at $P<0.05$ were considered statistically significant.

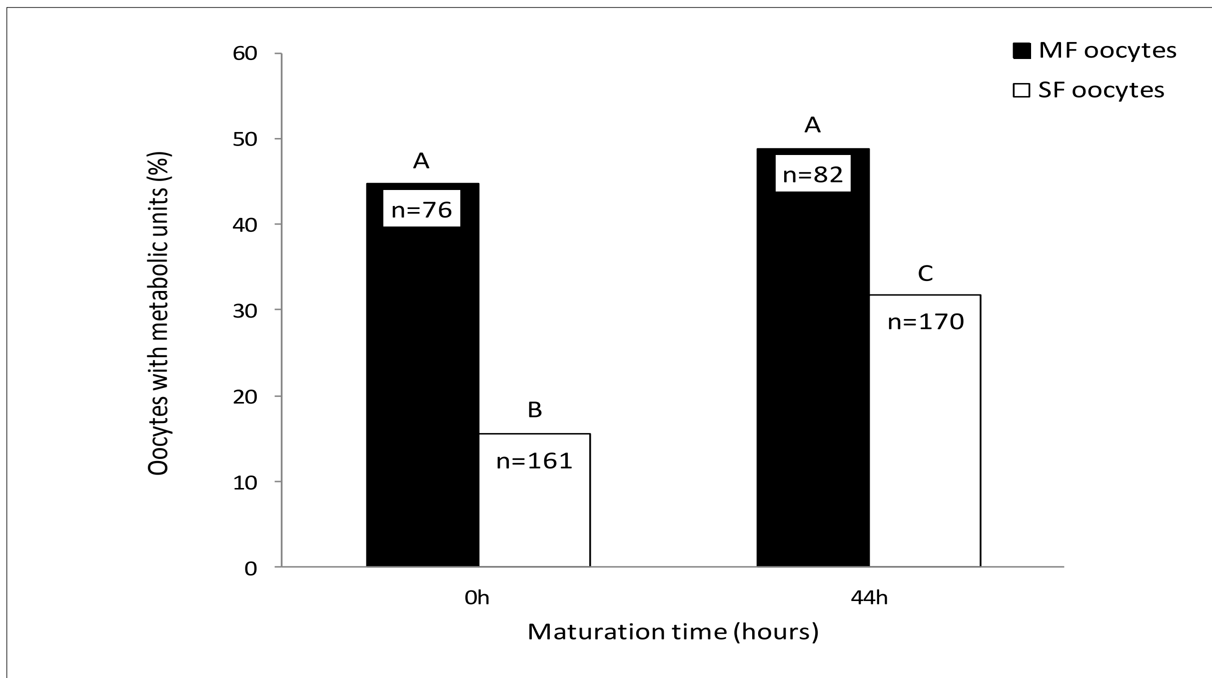
5 RESULTS AND DISCUSSION

5. 1 Evaluation of metabolic units

Experiment 1 was designed to identify changes in metabolic unit formation during oocyte maturation. A total of 489 oocytes derived from medium and small follicles matured for 0 h or 44 h were investigated for metabolic unit formation using confocal microscopy. The proportion of oocytes with metabolic units from examined oocytes was calculated for each oocyte category before and after maturation.

The mean proportions of oocytes with metabolic units derived from medium (MF) and small (SF) follicles before and after maturation are presented in Fig. 1. Significantly higher ($P < 0.05$) proportions of oocytes with metabolic units were detected both before and after maturation. While no significant difference in the proportion of oocytes with metabolic units was observed between oocytes derived from medium follicles before and those after maturation. However, in oocytes derived from small follicles, a significant increase in the number of oocytes with metabolic units was detected after maturation.

Figure 1: Metabolic units in MF and SF oocytes



Values with different superscripts are significantly different (A-C, $P < 0.05$)

Figure 1. Metabolic units (%) in MF and SF oocytes before (0 h) and after maturation (44 h).The oocytes with greater meiotic competence from medium follicles (MF) and those less competent from small follicles (SF) were examined by confocal microscopy either after collection or maturation in standard conditions.

5. 1. 1 Discussion

To elucidate the molecular structure of oocytes, scientists have used different techniques and approaches. In recent years, different experiments are oriented to clarify energy metabolism and they try to explain utilization of nutritive components inside cytoplasm of mammalian oocytes and embryos. Furthermore, a multitude of experiments which are designed to investigate the influence of lipids on oocytes and embryos, explain the mechanism and function of physiological processes by which these organelles affect oocyte quality and their capability for successful fertilization and subsequent embryo development. Activity of organelles may be associated with reliance on intracellular energy when oocyte misplaces contact with follicle cells (Ferguson and Leese, 1999).

Kruip et al. (1983) investigated ultrastructural changes of cytoplasmic organelles in bovine oocytes during *in vivo* maturation and they noticed the first changes 8 h after starting of LH surge. These alterations in cytoplasm are accompanied by germinal vesicle breakdown, disappearance of rough endoplasmic reticulum (RER) and the creation of mitochondrial clusters in correlation with lipid droplets and smooth endoplasmic reticulum (SER). At the same time with increasing of LH surge, formation of mitochondrial clusters in association with lipid droplets and components of SER, change of lipids, fusion of vesicles and the presence of ribosomes in the cytoplasm become much more intense. The lipid droplets and mitochondria are associated with endoplasmic reticulum (ER) in bovine (Kruip et al.,1983) and porcine oocytes (Cran, 1985). The distance between lipids and mitochondria is specifically important, because fatty acids positioned within lipid droplets are metabolized by β-oxidation process inside of mitochondrial matrix. The association among mitochondria, lipid droplets, smooth endoplasmic reticulum and vesicles was identified in cytoplasm of immature camel oocyte by both light and transmission electron microscopy (TEM) (Nili et al., 2004) and in bovine oocytes by electron microscopy (Hyttel et al., 1986).

Mitochondria start to surround lipid droplets forming special structures of metabolic units 9 to 12 hours after LH surge, in parallel with the process of germinal vesicle breakdown. In particular, during maturation lipids and mitochondria form metabolic units in bovine (Kruip et

al., 1983) and porcine (Sturmey et al., 2006) oocytes. Crocomo et al. (2013) observed presence of metabolic units and multiformity of mitochondria in ovine oocytes. They detected large amount of lipid droplets and some of them were in association with mitochondria constituting the metabolic units. The present study was designed to compare mitochondria and lipids co-localization and forming of metabolic units between meiotically more and less competent porcine oocytes and their changes during maturation since no relevant data in pigs are available. We used porcine oocytes with increasing meiotic competence recovered from different-size follicles of gilts at the early luteal to early follicular phase of the ovarian cycle, before and after maturation. We used confocal microscopy for estimation of mitochondrial and lipid co-localization in porcine oocytes and detected metabolic units similar to those described by Sturmey et al. (2006). In experiment 1 we discovered that before maturation, metabolic units were more iterative in meiotically more competent oocytes than in less competent oocytes. However, after maturation this distinction became smaller because less competent oocytes were more active in creation of metabolic units than more competent oocytes.

Interesting fact about mitochondria revealed that their relocation in different mammalian species is in proportion to their developmental capacity and it is frequently changed when development is disabled. However, during oocyte maturation, between 19 h after the LH peak level and ovulation, mitochondria dissipate, the most of organelles begin to locate in the middle of the oocyte and polar body is extruded (Kruip et al., 1983). These findings indicate that mitochondria change the position in the cytoplasm during maturation. The mitochondria are important organelles that are widely described and detected in variety of species with different methods. In mammalian oocytes mitochondria play an important role to supply ATP for fertilization and early development of embryos, in cellular apoptosis, aging, metabolism as well as in a lot of disorders (Wilding et al., 2001; Torner et al., 2004). Intracellular distribution of mitochondria has been evaluated and a significant differences were observed in their activity and relocation between species in human (Noto et al., 1993; Wilding et al., 2001; Liu et al., 2010), primate (Squirrell et al., 2003), porcine (Sun et al., 2001; Torner et al., 2004; Katayama et al., 2006; Egerszegi et al., 2010), bovine (Stojkovic et al., 2001; Tarazona et al., 2006), canine (Valentini et al., 2010), hamster (Suzuki et al., 2006; Bavister and Squirrell 2000) and mouse (Van Blerkom et al., 2002; Nagai et al., 2006; Nazmara et al., 2014). However, the timing and distribution of mitochondria varied between observed species. The activity and distribution of mitochondria represent a critical point for oocyte maturation

(Nazmara et al., 2014). Motta et al. (2000) estimated morphology of mitochondria and their association with other organelles in the human female germ cell during oogenesis, maturation and fertilization by electron microscopy. Generally, mitochondria demonstrate dynamic morphological alterations in oocytes, they also create complexes with another cell organelles in accordance with metabolic requirements of the cell during maturation and fertilization. Machatkova et al. (2012) reported specific differences in the mitochondrial patterns in bovine oocytes with different meiotic competence during maturation.

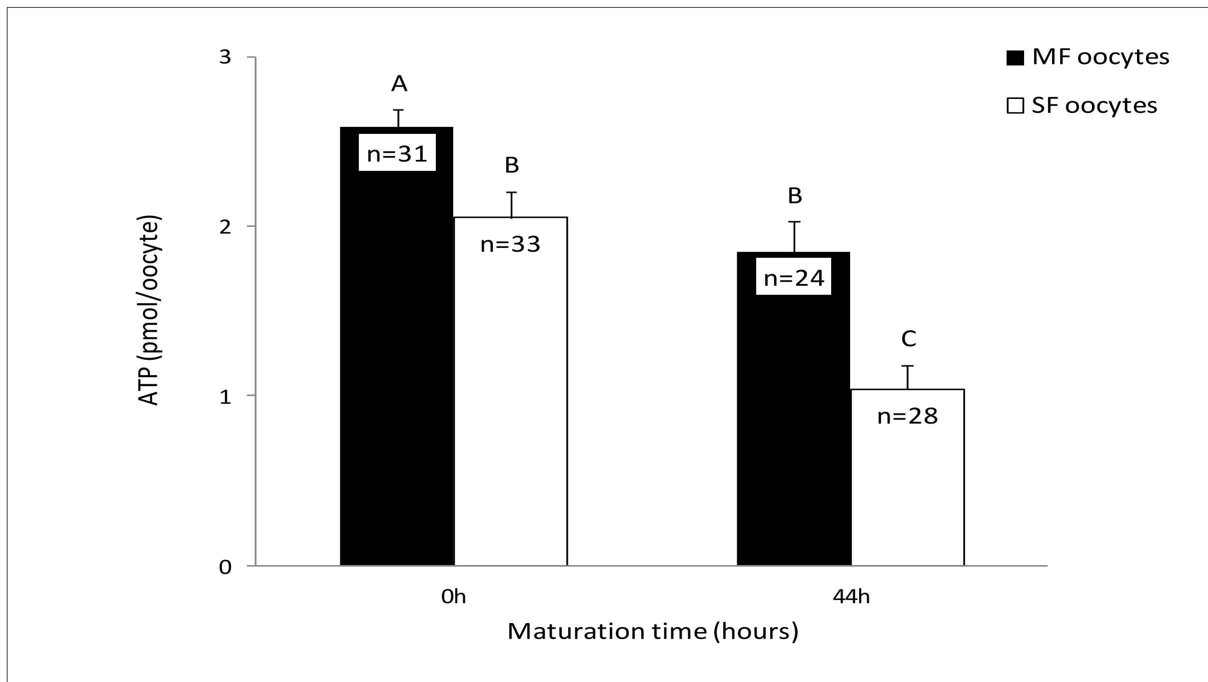
Sun et al. (2001) reported that distribution of mitochondria in the pronuclear region was interfered by microtubules but not by microfilaments in porcine oocytes. Brevini et al. (2005) noted a comprehensive remodelling of mitochondria and developed network of cytoplasmic microtubules merely in highly competent porcine oocytes. This finding parallels closely the observation that remodeling of active mitochondria is remarkably more iterative in the group of oocytes with high developmental competence. The results of Van Blerkom (1991) demonstrate that distribution of mitochondria is mediated by microtubules in murine oocytes. Microtubules in murine oocytes mediate aggregation of mitochondria during maturation *in vitro* for ensuring of higher ATP amount which is indispensable for nuclear maturation.

5. 2 Evaluation of ATP content

Experiment 2 was performed to determine ATP utilization during oocyte maturation. A total of 116 oocytes from medium and small follicles matured for 0 h or 44 h were examined for ATP content using a bioluminescent somatic cell assay. The mean (± S.E.M) ATP content per oocyte was calculated for each oocyte category before and after maturation.

The mean (± S.E.M.) ATP contents in oocytes derived from medium and small follicles before and after maturation are presented in Figure 2. The oocytes derived from medium follicles had a significantly higher ATP content than oocytes derived from small follicles, both before and after maturation ($P < 0.05$). A significant decline in ATP content was observed in both categories during maturation and was greater in oocytes derived from small follicles in comparison with oocytes derived from medium follicles.

Figure 2: ATP contents in oocytes



Values with different superscripts are significantly different (A-C, $P < 0.05$)

Figure 2. ATP contents (pmol) in oocytes derived from medium and small follicles before (0 h) and after maturation (44 h). The oocytes with greater meiotic competence from medium follicles (MF) and those less competent from small follicles (SF) were examinated using bioluminiscent assay kit either after collection or maturation in standard conditions.

5. 2. 1 Discussion

The quality of oocyte and optimal development of a healthy embryo depends on the metabolism of ATP (Dunning et al., 2014). Sufficient concentration of ATP is required for optimal development of oocytes (Van Blerkom et al., 1995). Among morphologically good and poor bovine oocytes, distribution of mitochondria and ATP content distinguish, which can be reflected to differences in their developmental potential, as a consequence of disruption in energy processes (Stojkovic et al., 2001). Dalton et al. (2014) reported that ATP passes through dynamic changes related to particular events of maturation and that oocytes during different stages of maturation manifested altered rates of ATP consumption. Denuded oocytes have lower levels of ATP than oocytes enclosed in cumulus during maturation, however this can be interrupted by inhibition of gap junctional contact among the oocytes and

cumulus cells. The ATP production is linked to number and ultrastructure of mitochondria (Nazmara et al., 2014). The generation of ATP subordinates on the activity of mitochondria existing in the cytoplasm which appears to be associated to developmental competence. However, with diminished mitochondrial activity, fertilization is unsuccessful and development of embryo is decreased. The favorable developmental capability of oocytes may be related to their capacity of producing ATP.

Dalton et al. (2014) applied recombinant FRET probe to explore ATP levels in mouse oocytes and consumption of ATP during maturation. They revealed that ATP levels rised as meiosis I resumed and subsequently declined about 1 h after germinal vesicle breakdown (GVBD) and that oocytes undergoing maturation from MI to MII stage consumed ATP faster than oocytes in GV stage. However, following GVBD, ATP levels increase progressively before exposing a peak during extrusion of the first polar body (PBE). These results are in line with our data of increased ATP consumption during maturation, because matured oocytes contain less ATP in comparison with oocytes in germinal vesicle stage (Figure 2). That indicates higher energy request for processes related to maturational progress. This altered rates of ATP decrease appeared as a consequence of different rates of ATP consumption. In the study of Van Blerkom et al. (1995) and Nazmara et al. (2014) the ATP content of murine oocytes at GV and MII did not significantly change. On the contrary, in other studies the ATP content was higher in matured bovine (Stojkovic et al., 2001) and porcine (Brevini et al., 2005) oocytes than before maturation. In our research ATP content of porcine oocytes declined during maturation independently of their meiotic competence. The authors discussed variations in ATP content in context with different animal species, genetics, age, culture conditions and the method of ATP evaluation. Stojkovic et al. (2001) and Van Blerkom (2011) described relationships among oocyte developmental competence, mitochondrial function and ATP content in bovine and human oocytes. Our results indicate that the formation of metabolic units was in agreement with ATP consumption regardless of meiotic competence of the oocytes. Although the ATP level was significantly higher both before and after maturation in the more competent oocytes, the less competent oocytes formed more metabolic units and consumed more ATP during maturation. However, the functional significance of ATP changes and mechanisms which regulate them still remain unclear. Dalton et al. (2014) speculated that the ATP changes during oocyte maturation might be an indicator of oocyte health and its ability to extrude polar body but no data to support this view have been provided.

5. 3 Evaluation of lipid content in porcine oocytes

5. 3. 1 Experiment A: The relative area of lipid droplets in oocytes derived from small and medium follicles from GV to MII stage as related to the phase

The results of this experiment provide the first detailed data on the changes in lipid content of porcine oocytes, during different phases of folliculogenesis, before and after maturation. The subpopulations of oocytes derived from small and medium follicles varied in the area of oocytes covered with lipid droplets during the folliculogenesis, from the early luteal to the early follicular phase, before and after maturation (Table 1). The relative area of oocytes covered with lipid droplets is lower in the late luteal phase in oocytes derived from small follicles than in the late luteal phase in oocyte derived from medium follicles, before and after maturation (Table 1). During the early follicular phase, area covered with lipid droplets in oocytes derived from medium follicles is higher than in oocytes derived from small follicles, before and after maturation (Table 1). The total area of oocytes covered with lipid droplets did not significantly differ throughout either the luteal or the follicular phase, before and after maturation.

Table 1: Relative area of lipid droplets from GV to MII stage

Follicle size	n	Phase	Stage	Relative area of lipid droplets (%)		Decline of relative lipid area during maturation (%)
				GV	MII	
Small	45	Luteal	early	29.3±7.2^{a}	27.6±7.0^{a}	5.81
	34		middle	29.2±5.5^{a}	28.9±6.6^{a}	1.03
	29		late	29.6±5.8^{a}	29.4±5.7^{a}	0.68
	29	Follicular	early	28.7±5.8^{a}	28.1±9.5^{a}	2.1
Medium	41	Luteal	late	31.1±6.7^{a}	30.9±6.9^{a}	0.65
	33	Follicular	early	29.6±7.3^{a}	29.1±9.4^{a}	1.69

Values with the same superscripts within the same column and inside the same phase are not significantly different (a, $P < 0.05$).

5. 3. 2 Experiment B: The relative number of lipid droplets in oocytes derived from small and medium follicles from GV to MII stage as related to the phase

In the early follicular phase, relative number of lipid droplets in oocytes derived from small follicles is significantly lower than in oocytes derived from medium follicles from late luteal and early follicular phase, before maturation (Table 2). The relative number of lipid droplets was significantly higher in the late luteal phase in oocyte derived from medium follicles in comparison with oocytes derived from small follicles in the middle and late luteal phases of folliculogenesis, before maturation.

After maturation, oocytes from small follicles in the early follicular stage have significantly lower number of lipid droplets in comparison with oocytes derived from small follicles in the early and middle stage of folliculogenesis and in oocytes in the late luteal and early follicular phase derived from medium follicles. In the early follicular phase, in oocytes obtained from small follicles, the biggest decline of relative number of lipid droplets during maturation was recorded (Table 2).

Table 2: Changes of relative number of lipid droplets from GV to MII stage

Follicle size	n	Phase	Stage	Relative number of lipid droplets		Decline of relative lipid number during maturation (%)
				GV	MII	
Small	45	Luteal	early	$350.6 \pm 83.1^{a,b,c}$	295.2 ± 96.8^{a}	15.8
	34		middle	$339.8 \pm 87.3^{a,c}$	308.5 ± 91.7^{a}	9.2
	29		late	$329.9 \pm 77.6^{a,c}$	$280.3 \pm 94.9^{a,b}$	15.0
	29	Follicular	early	327.4 ± 79.7^{c}	243.8 ± 105.7^{b}	25.5
Medium	41	Luteal	late	387.7 ± 98.8^{b}	292.2 ± 96.2^{a}	24.6
	33	Follicular	early	$373.4 \pm 92.1^{a,b}$	299.7 ± 119.5^{a}	19.7

Values with different superscripts within the same column and inside the same phase are significantly different (a–b, $P < 0.05$).

5. 3. 3 Experiment C: Relative number of lipid droplets in oocytes derived from small and medium follicles from GV to MII stage, as related to the phase

The number of small lipid droplets was higher in the late luteal and early follicular phase in oocytes derived from medium follicles in comparison with oocytes in all stages of luteal and follicular phases derived from small follicles, before maturation (Table 3). Correspondingly, small lipid droplets of the cytoplasm area was significantly higher in the late luteal phase in oocytes derived from medium follicles comparing to the middle and the late luteal phase of folliculogenesis in oocytes derived from small follicles, before maturation (Table 3). According to our data, the average number of large lipid droplets decreased consequently during the luteal and follicular phase in oocytes derived from small follicles, and increased in the late luteal phase in oocytes derived from medium follicles. The number of large lipid droplets was significantly lower in early follicular phase in comparison with oocytes from early luteal phase derived from small follicles, before maturation (Table 3). In oocytes derived from medium follicles before maturation, the number of large lipid droplets was significantly higher in the late luteal phase than in oocytes derived from small follicles in early follicular phase.

Table 3: The number of small and large lipid droplets in the luteal and the follicular phase in oocytes derived from small and medium follicles before maturation

Follicle size	Phase	Stage	n	Number of small lipid droplets	Number of large lipid droplets
				GV	
Small	Luteal	early	45	$182.3 \pm 59.2^{a,b}$	168.4 ± 52.4^{a}
		middle	34	172.9 ± 62.2^{a}	$166.9 \pm 46.4^{a,b}$
		late	29	172.8 ± 66^{a}	$157.1 \pm 42.4^{a,b}$
	Follicular	early	29	$183.4 \pm 62.6^{a,b}$	144.1 ± 38.2^{b}
Medium	Luteal	late	41	209.1 ± 79.1^{b}	178.6 ± 47.3^{a}
	Follicular	early	33	208.6 ± 73.0^{b}	$164.7 \pm 50.8^{a,b}$

Values with different superscripts within the same column and inside the same phase are significantly different (a–b-c-d, $P < 0.05$).

After maturation, the number of small lipid droplets was significantly higher in early luteal phase in comparison with early follicular phase in oocytes derived from small follicles. (Table 4). In the early follicular phase the number of large lipid droplets in oocytes derived from small follicles was significantly lower in comparison with other phases of folliculogenesis, except for the early stage of luteal phase in oocytes derived from small follicles (Table 4).

Table 4: The number of small and large lipid droplets in oocytes from small and medium follicles in the luteal and the follicular phase after maturation

Follicle size	Phase	Stage	n	Number of small lipid droplets	Number of large lipid droplets
				MII	
Small	Luteal	early	45	165.1±68.5[a]	130.0±45.2[a,b]
		middle	34	156.0±61.8[a,b]	152.5±48.8[a]
		late	29	131.6±52.4[a,b]	148.7±53.0[a]
	Follicular	early	29	125.9±67.2[b]	117.9±60.9[b]
Medium	Luteal	late	41	141.1±70.4[a,b]	151.1±48.1[a]
	Follicular	early	33	152.4±81.0[a,b]	147.3±71.2[a]

Values with different superscripts within the same column and inside the same phase are significantly different (a–b, $P < 0.05$).

Table 5: Ratio of small and large lipid droplets in oocytes in different stages of folliculogenesis before maturation

Follicle size	Phase	Stage	n	Number of small lipid droplets	Number of large lipid	Small/large
				GV (%)		
Small	Luteal	early	45	52.0±10.7	48.0±10.7	1.1±0.5
		middle	34	50.9±10.7	49.1±10.7	1.0±0.5
		late	29	52.4±10.9	47.6±10.9	1.1±0.6
	Follicular	early	29	56.0±9.4	44.0±9.4	1.3±0.5
Medium	Luteal	late	41	53.9±10.3	46.1±10.3	1.2±0.5
	Follicular	early	33	55.9±9.6	44.1±9.6	1.3±0.7

Before maturation, the ratio of small lipid droplets was higher in oocytes derived from small and medium follicles in early follicular phase, before maturation (Table 5). After maturation, the ratio of small droplets was higher in oocytes derived from small follicles in early luteal phase (Table 6).

Table 6: Ratio of small and large lipid droplets in oocytes in different stages of folliculogenesis after maturation

Follicle size	Phase	Stage	n	Number of small lipid droplets	Number of large lipid droplets	Small/large
				MII (%)		
Small	Luteal	early	45	55.9±10.1	44.1±10.1	1.3±0.6
		middle	34	50.6±10.1	49.4±10.1	1.0±0.5
		late	29	46.9±9.3	53.1±9.3	0.9±0.4
	Follicular	early	29	51.6±13.0	48.4±13.0	1.1±0.7
Medium	Luteal	late	41	48.3±12.6	51.7±12.6	0.9±0.6
	Follicular	early	33	50.9±14.6	49.1±14.6	1.0±0.4

5. 3. 4 Experiment D: Lipid droplets in oocytes derived from small and medium follicles regardless of the phase

Results in Table 7 represent observed parameters of lipids in oocytes derived from different sizes of follicles, before maturation. The relative area covered with lipid droplets was similar between the subpopulations of the oocytes derived from small and medium follicles, regardless of the phase. On the contrary, in oocytes with expanded cumulus after reaching germinal vesicle breakdown (GVBD) the relative area of lipid droplets was significantly higher ($P < 0.05$) in comparison with oocytes derived from small and medium follicles (Table 7).

The relative number of lipid droplets increased during follicular growth and was significantly higher ($P < 0.05$) in oocytes derived from medium follicles in comparison with oocytes derived from small follicles and oocytes with expanded cumulus after reaching of GVBD stage, regardless of the phase. The relative number of lipid droplets in oocytes with expanded cumulus decreased in GV and after reaching GVBD stage (Table 7). Results from

Table 7 show that the relative number of small lipid droplets increased during follicular growth, and was significantly higher in oocytes derived from medium follicles and with expanded cumulus in GV stage in comparison with oocytes derived from small follicles and oocytes after reaching GVBD stage. No significant differences in number of large lipid droplets were found among observed categories before maturation (Table 7).

Table 7: Characteristics of lipid parameters in oocytes derived from small and medium follicles regardless of the phase before maturation

Follicle size	n	Area of lipid droplets(%)	The relative number of lipid droplets (n)	Small lipid droplets(n)	Large lipid droplets(n)
		GV			
Small	137	29.2 ± 6.2^{a}	338.7 ± 82.0^{a}	178.2 ± 61.6^{a}	160.5 ± 46.6^{a}
Medium	74	30.5 ± 7.0^{a}	381.3 ± 95.5^{b}	208.9 ± 75.9^{b}	172.4 ± 49.1^{a}
Expanded COCs GV	25	$30.7\pm4.1^{a,b}$	$368.1\pm72.6^{a,b}$	211.1 ± 68.6^{b}	157.1 ± 29.2^{a}
Expanded COCs after GVBD	52	32.8 ± 6.7^{b}	340.1 ± 92.7^{a}	179.8 ± 62.5^{a}	160.4 ± 50.2^{a}

Data with different superscripts within the same column and inside the same group are significantly different (a–b, $P< 0.05$).

Results from Table 8 represent observed parameters of lipids in oocytes derived from follicles of different sizes after maturation. The relative area of lipid droplets increased with follicular growth, and it was similar between oocytes derived from small and medium follicles, after maturation. The relative area of lipid droplets in oocytes with expanded cumulus was significantly lower after 24 h of maturation, than in oocytes which were matured for 44 h. The oocytes with expanded cumulus after 44 h of maturation had significantly higher relative area of lipid droplets than oocytes from small follicles. The relative number of lipid droplets and number of small lipid droplets increased during follicular growth, and was significantly higher in oocytes with expanded cumulus after 24h of maturation in comparison with other observed categories. However, after 44 h of maturation, the number of lipid droplets and number of small lipid droplets started to decrease. The number of large lipid droplets was similar between observed categories during maturation and follicular growth.

Table 8: Characteristics of lipid parameters in oocytes derived from small and medium follicles regardless of the phase after maturation

Follicle size	n	Area of lipid droplets(%)	The relative number of lipid droplets	Small lipid droplets(n)	Large lipid droplets(n)
		MII			
Small	137	28.5±7,4[a]	281.7±99.7[a]	144.7±64.5[a]	137.1±53.9[a]
Medium	89	29.8±8.4[a,b]	296.6±109.9[a]	147.7±76.5[a]	148.9±62.4[a]
Expanded COCs 24h	46	27.8±6.3[a]	349.8±90.4[b]	210.1±66.1[b]	139.8±46.6[a]
Expanded COCs 44h	51	30.9±6.9[b]	286.4±82.8[a]	150.4±62.4[a]	136.1±36.3[a]

Data with different superscripts within the same column and inside the same group are significantly different (a–b, P < 0.05).

5. 3. 1 Discussion

The components of the oocyte cytoplasm ensure conditions for the production of indispensable energy for successful process of maturation, fertilization and early embryo development. The oocytes are well known to contain lipid droplets, however mechanism of their utilization during maturation is still not fully explained. Lipid droplets have a special role during oocyte maturation, fertilization and early embryo growth (Kikuchi et al., 2002). Lipid metabolism ensures effective source of energy during oocyte maturation and their pertinence in this processes is evident. During the process of β-oxidation, the metabolism of lipids in the COC before fertilization affects developmental potential of oocytes as well (Dunning et al., 2014). According to Jeong et al. (2009), the content of cytoplasmic lipids could be an indicator of the developmental competence because they coordinate functions of mitochondria which are indispensable for early development. The surplus of lipids may be used by mitochondria for enchancement of ATP production which is necessary for embryo development (Crosier et al., 2001).

Mammalian adipose tissue contains large number of lipid droplets, where triacylglycerols (TAGs) are deposited. The oocytes consist of triglycerides, phospholipids, cholesterol esters and free fatty acids. Important metabolic and regulatory function of fatty acids in the advancement of embryo progress have been broadly examined (McKeegan and Sturmey, 2011). During accumulation of triglycerides, unsaturated fatty acids have a protective role against lipotoxicity (Listenberger et al., 2003). Moreover, this can save cells from the

detrimental effects of lipid accumulation. The lipolytic activity centered in oocytes explains participation in lipid catabolism during maturation (Cetica et al., 2002). Using different methods such as fatty acid oxidation, inhibition of triglyceride oxidation, culture in the absence of exogenous substrates, cytoplasmic organization and delipidation, a lot of researchers have examined lipids in mammalian oocytes and revealed that triacylglycerol is the main component of lipids (Sturmey et al., 2009). The porcine oocytes contain a high amount of lipids and almost a half of the total fatty acids positioned in lipids is represented by triglycerides (Homa et al., 1986; McEvoy et al., 2000). Triglyceride content varied among species. Using a microfluorescence technique, Ferguson and Leese (1999) evaluated the triglyceride content in bovine oocytes and preimplantation embryos and their results indicate that triglyceride represents energy source during maturation and fertilization of bovine oocytes. Before maturation porcine oocytes contain 74 ng triglyceride of a total lipid content of 156 ng (McEvoy et al., 2000), bovine oocytes 59 ng (Ferguson and Leese, 1999), sheep oocytes 25 ng of a total lipid content of 89 ng (Coull et al., 1997). Youngs et al. (1994) reported that porcine embryo in 10 and 11 days contain 113 and 141 ng. The levels of triglycerides, phospholipids and cholesterol in porcine oocytes decrease during *in vivo* maturation. Ferguson and Leese (1999) reported that after maturation the triglyceride amount in bovine oocytes declined to 46 ng and noted decrease in triglyceride content during fertilization and the first cleavage. Their findings are in accordance with results of Kim et al. (2001), who reported that the triglyceride content of bovine oocytes decreased significantly during maturation. These reports demonstrate that triglycerides play an important metabolic role in the process of maturation, fertilization and early development of embryo.

Storage and trafficking of lipids occurs in the form of spheroidal droplets (Murphy and Vance, 1999). The quantification of lipids and the evaluation of the size and distribution of lipid droplets within oocytes and embryonic cells can provide necessary information about the metabolism of these cytoplasmic markers. Hulinska et al. (2011) reported that the oocytes derived from small follicles in the late luteal phase have positive results in process of their fertilization in comparison with other phases of folliculogenesis. Fielding et al. (1998) documented that for the process of reverse cholesterol transport, the small lipid droplets can be substantial. However, they used a different classification of lipid droplets size (small <2.5 μm, medium 2.5-4.9 μm and large >5 μm), in comparison to our study (small ≤5 μm and large ≥ 9 μm), in addition, they aspirated only oocytes from 3-6 mm follicles. In our research we also estimated lipid droplets in oocytes derived from larger follicles (6-9 mm). That

indicates that our study provides information about destiny of lipid droplets in oocytes derived from follicles of different sizes with different meiotic competence.

As is well known, the lipid content shows significant variations in different species. Dunning et al. (2014) performed staining of human, mouse, sheep, cow and pig oocytes with BODIPY 493/503 and compared lipid content among these species. Porcine oocytes contain a large number of lipids in comparison with human, bovine, equine and rodent oocytes. The cryopreservation methods of porcine embryos are more problematic than in many other mammals, because porcine oocytes contain large amounts of lipid droplets (Zhou and Li, 2009). Abe et al. (2002) investigated quantitative variations of lipid droplets positioned in cytoplasm of bovine embryos using electron microscopic method, their results demonstrate that bovine embryos with higher accumulation of lipid droplets in cytoplasm are more sensitive to process of cryopreservation. Homa et al. (1986) reported the first precise lipid analysis of immature porcine oocytes. In this study we detected intracellular lipid droplets by confocal microscopy using Nile Red staining. Several authors already applied the fluorescent probe Nile Red for identification of lipid droplets in mammalian oocytes (Genicot et al., 2005; Leroy et al., 2005; Romek et al., 2011). Genicot et al. (2005) also used Nile Red for quantification of lipids in a single mammalian oocytes and they assume that a greater lipid content in an oocyte causes a greater quantity of emitted fluorescent light. During evaluation of lipid droplets in bovine oocytes and embryos with Nile Red staining, Leroy et al. (2005) concluded that bovine oocytes with darker cytoplasm contain more lipid droplets. Romek et al. (2011) applied Nile Red staining and confocal microsopy to quantify the content of triglycerides, phospholipids and cholesterol in porcine oocytes and pre-implantation embryos.

In the present study we clarified variations in the relative area of lipid droplets in porcine oocytes during maturation. Our results revealed that before maturation the relative lipid area was higher in oocytes derived from medium follicles in comparison with oocytes derived from small follicles in observed phases of folliculogenesis. The lowest relative area of lipid droplets was found in the early follicular phase in oocytes derived from small follicles before and after maturation. The relative lipid area was higher in oocytes from the late luteal phase derived from medium follicles before and after maturation in comparison with other phases of folliculogenesis. However, relative lipid area declined from GV to MII stage. The greatest decline of relative lipid area was found in oocytes derived from small follicles in the early luteal phase (Table 1). Our findings that area of lipid droplets is greater in oocytes after GVBD (Table 7) also correspond to results of Niimura et al. (2002) who documented that

proces of germinal vesicle breakdown occurred in oocytes with higher numbers of lipid droplets. During maturation, the meiotically more competent oocytes utilized more lipid droplets to meet energy demands in comparison with the less competent oocytes. We assume that these oocytes favored more easily available ATP for energy production. The lipid composition in bovine oocytes were degraded during maturation, which indicates that lipids may be utilized as a source of the ATP necessary for synthesis of proteins (Kruip et al., 1983).

The second observed parameter the relative number of lipid droplets was significantly higher in oocytes in the luteal and the follicular phase derived from medium follicles in comparison with oocytes in the same phases derived from small follicles before maturation. On the contrary after maturation, the relative number of lipid droplets was lower in the observed phases of folliculogenesis. The largest decline was observed in the early follicular phase in oocytes derived from small follicles and the late luteal phase in oocytes derived from medium follicles. Considering the numbers of lipid droplets before maturation, we concluded that oocytes with greater meiotic competence had more lipid droplets available for energy production than those with lesser competence regardless of the results that the relative amount of lipids in cytoplasm was similar in both oocyte categories. During maturation in ovine oocytes, Crocomo et al. (2013) also observed decline in the number of lipid droplets. Cran (1985) described the existence of lipid droplets in porcine oocytes and characterized their number and size in immature and matured porcine oocytes. They reported that the number of lipid droplets increased and the volume fraction of large vesicles declined with the progression of maturation. In bovine oocytes, the number of lipid droplets was significantly increased after maturation, whereas the size of the lipid droplets remained similar (Aardema et al., 2011).

Different mammalian cells have smaller lipid droplets. It is assumed that they are an energy source needed for the synthesis of membranes (Listenberger and Brown, 2007). In the present investigation before maturation, the number of small lipid droplets was constantly higher than the number of large lipid droplets during all stages of luteal and folicular phases. But after maturation, the number of small lipid droplets was lower (0.9%) in the late luteal phase in oocytes derived from small and medium follicles. Niimura et al. (2002) described that lipid droplets increased in their number in the process of transformation of large lipid droplets into smaller and consequently the small lipid droplets were used as an energy source for future fertilization and early embryo development.

In immature porcine oocytes, lipid droplets are positioned on the periphery (Sturmey et al., 2006). Murine oocytes in germinal vesicle stage contain small centrally located lipid

structures, but after maturation large lipid structures are concentrated in the center (Wood et al., 2008; Yang et al., 2010). These findings indicate that lipid droplets in murine oocytes during *in vitro* maturation alter the structure. This is in the line with the results of Nili et al. (2004) who reported that small lipid droplets show central distribution in immature camel oocytes.

The coherence among cytoplasmic appearance and developmental competence was investigated in bovine oocytes (Nagano et al., 2006) and bovine embryos (Abe et al., 2002.). These studies propose that lipid composition in cytoplasm may affect the quality of bovine oocytes and embryos. Nagano et al. (2006) reported that developmental potential of bovine oocytes obtained from small antral follicles is reflected in the morphological appearance of the oocyte. A dark cytoplasm in bovine oocytes is specific for cumulation of lipids and represents a good developmental potential, although a light-coloured cytoplasm is specific for a low density of organelles and lower developmental potential. Jeong et al. (2009) examined connection among ultrastructural characteristics of lipids and developmental capacity in bovine embryos with cellular staining procedure. This study also confirmed that cytoplasmic lipids may have an impact on the successful development of embryos. Hiraga et al. (2013) reported that localization of lipid droplets in cytoplasm of porcine oocytes can correlate with developmental competence. In their study, the porcine oocytes with central localization of lipid droplets demonstrated a significantly higher rate of blastocyst development. Campagna et al. (2006) studied influence of organochlorine mixture on the ultrastructure of porcine oocytes. They revealed considerable changes in the different density of lipid droplets. They also did not detect declined number and volume of lipid droplets, and their results also suggest that the process of lipolysis occurs on the periphery of lipid droplets in interaction with smooth endoplasmic reticulum and mitochondria, because lipid droplets change in electron density but not in volume.

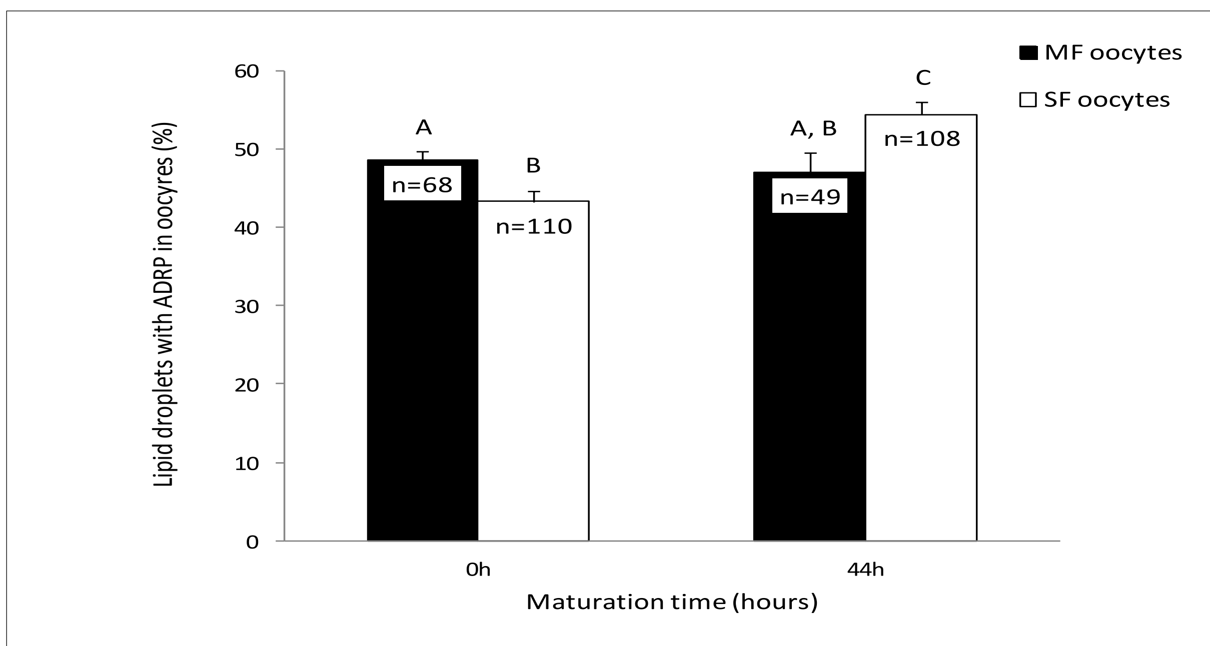
Generally, lipid composition can be changed depending on the maturation conditions of oocytes, especially on serum and lipid components in IVM medium. Kim et al. (2001) reported that triacylglycerol and cholesterol contents were significantly higher in bovine oocytes matured in medium supplemented with 10% fetal calf serum in comparison with oocytes matured in medium with polyvinyl alcohol. Also, murine oocytes cultured in 5% serum have higher content of neutral lipids (Yang et al., 2010). According to this information, we can assume that serum has an impact on the lipid structure during maturation.

5. 4 Detection of adipose differentiation-related protein-ADRP

Experiment 8 was performed to reveal changes in ADRP expression during oocyte maturation and proportion of lipid droplets with ADRP expression from total number of lipids. We used confocal microscopy and Western blot analysis for detection of lipid droplets with ADRP expression before and after maturation. In order to describe the differences between meioticaly greater and lesser competent oocytes used for this experiment, we obtained them from medium and small follicles. A total of 335 oocytes obtained from medium (MF) and small (SF) follicles matured for 0 h and 44 h were used for confocal microscopy scanning and 200 oocytes for Western blot analysis.

The relative number (± S.E.M.) of lipid droplets with ADRP expression was significantly higher in MF than in SF oocytes before maturation whereas after maturation, it was significantly higher in SF than in MF oocytes. While the relative number of lipid droplets with ADRP expression did not change in MF oocytes, it showed a significant increase in SF oocytes during maturation (Figure 3a). The 52 kDa ADRP was confirmed in both MF and SF oocytes by Western blotting (Figure 3b).

Figure 3a: Lipid droplets with ADRP in oocytes



Values with different superscripts are significantly different (A-C, $P < 0.05$)

Figure 3a. Lipid droplets with adipose differentiation-related protein (ADRP) in MF and SF oocytes before (0h) and after maturation (44 h). The oocytes with greater meiotic

competence from medium follicles (MF) and those with lesser competence from small follicles (SF) were examined by confocal microscopy either after collection or maturation in standard conditions.

Figure 3b: Expression of ADRP in oocytes

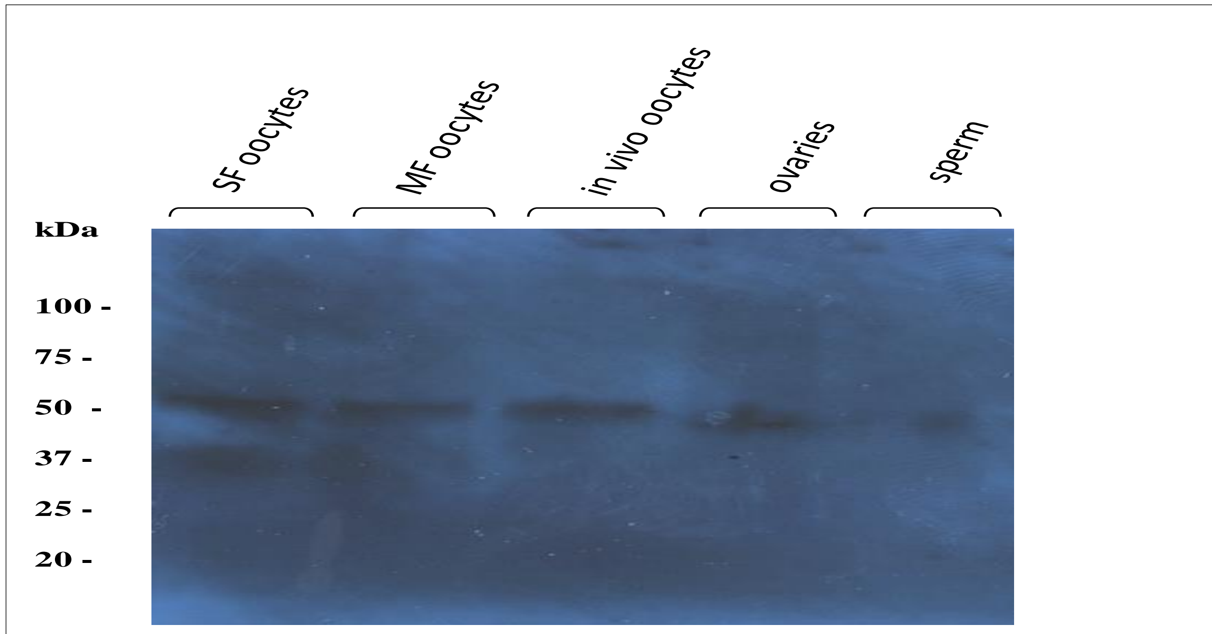


Figure 3b. Expression of ADRP in MF and SF oocytes identified by Western blotting. The oocytes with greater meiotic competence from medium follicles (MF) and those with lesser competence from small follicles (SF) were examined only after collection. Porcine oocytes matured *in vivo*, ovarian tissue and boar spermatozoa were used as controls.

5. 4. 1 Discussion

The lipid droplet-associated proteins such as perilipins are considerable constituents of lipid droplets in porcine oocytes (Zhang et al., 2014). Consistent with the work of Aardema et al. (2011), who detected ADRP protein on the surface of lipid droplets in porcine oocytes, we found that LDs of porcine oocytes were specifically associated with ADRP. In oocytes, ADRP is a lipid droplet protein and its induction in the COC concurrent with dynamic reorganisation of lipid droplets connotes considerable changes in lipid utilisation during oocyte maturation (Yang et al., 2010). We detected that porcine oocytes with greater meiotic competence showed more lipid droplets with ADRP expression than did those with lesser

competence, before maturation. However, during maturation the meiotically more competent oocytes did not increase ADRP expression while the less competent oocytes did. Zhang et al. (2014) detected PLIN2 or ADRP protein in neutral lipid droplets and showed identical distribution model and comprehensive colocalization in porcine oocytes, before and after maturation. However, PLIN2 expression was higher in oocytes before maturation in comparison with oocytes after maturation, while PLIN2 mRNA expression was similar in both groups.

Several authors already used specific neutral lipid stain BODIPY 493/503 for detection of lipid droplets and confirmed their specificity with the immunostaining method for visualisation of the lipid droplet-specific protein ADRP in mouse (Yang et al., 2010) and bovine oocytes (Aardema et al., 2011). Using immunofluorescence and electron-microscopic immunolocalization, Heid et al. (1998) detected specifically ADRP association with lipid droplets in different types of cells. ADRP was detected in lipid droplets of L-cell fibroblasts (Frolov et al., 2000). Gross et al. (2006) detected association of ADRP with lipid droplets in murine NIH-3T3 fibroblasts by indirect immunofluorescence. In addition, they assumed that upon lipolytic stimulation ADRP substitutes perilipin on the present lipid droplets. To examine the influence of ADRP protein on lipid droplets, Listenberger and Brown (2007) detected expression of this protein in human embryonic kidney (HEK 293) cells. They reported that ADRP surrounded lipid droplets and caused enhancement of cellular triacylglycerol (TAG) content that was stored in lipid droplets. Magnusson et al. (2006) demonstrate that higher expression of ADRP increases triglyceride aggregation and size of the cytoplasmic lipid droplets, on the contrary reduction of ADRP declines the storehouse of triglycerides. During adipocyte differentiation of white adipose tissue, small lipids are fused to create larger TAG droplets found in mature cells (Murphy and Vance, 1999). The fact that ADRP advance the storage of triglycerides may be explained by increasing of energy depletion, because the biosynthesis of triglycerides consumes energy during the activation of fatty acids (Magnusson et al., 2006).

During early differentiation of 3T3 adipocytes, ADRP was discovered on small and developing lipid droplets, until perilipin was lacking in these nascent lipid droplets. The favored presence of ADRP on small lipid droplets indicates its prevalent role in lipid droplets formation. After double staining with polyclonal antibodies against ADRP and perilipin, there is an evidence that perilipin and ADRP are localized on the same lipid droplets. Although ADRP was detected on small lipid droplets in 3T3-L1 preadipocytes and early differentiated adipocytes, it was lacking in maturing adipocytes (Brasaemle et al., 1997). Study on 3T3-L1

adipocytes revealed conversion of ADRP to the perilipins on the surface of lipid droplets 3 days after differentiation. At the same time with adipocyte differentiation, perilipins substitute ADRP as a prevalent protein on the surface of the lipid droplets (Murphy and Vance, 1999). This replacement of ADRP with perilipin in adipocytes indicates that ADRP cannot ensure complete function, and is replaced by perilipin that completes it. Additional investigation is necessary to determine whether ADRP is demanded for the coalescence of droplets to produce the characteristic large lipid storage droplets found in oocytes. In murine 3T3-L1 cells Franke et al. (1987) described the arrangement of vimentin intermediate filaments (IFs) and forming cages around small lipid droplets, which disenable their subsequent fusing. The number of vimetin fibres decline concurrently during cell maturation. This indicates their possible role in lipid droplets fusion (Murphy and Vance, 1999). On another hand Colucci-Guyon et al. (1994) reported absence of vimetin in transgenic mice, but they normally performed biogenesis of lipids. This information indicates that fusion of lipid droplets might be highly regulated. Study of Steiner et al. (1996) suggested a possible role among expression of ADRP in liver and lipid accumulation. They showed that treatment of rats with etomoxir causes the expression of the adipose differentiation-related protein in liver.

6 CONCLUSIONS

In porcine oocytes with defined meiotic competence, the energy status before and after maturation *in vitro* was assessed on the basis of selected cytoplasmic parameters. This cytoplasmic energy markers were identified during different phases of folliculogenesis. The oocytes with greater meiotic competence derived from medium follicles and those with lesser meiotic competence from small follicles were compared in mitochondrial-lipid patterns, ATP and lipid droplet consumption, and presence of lipid droplets with ADRP expression. The observed parameters in porcine oocytes showed different variations.

It is concluded that the more competent oocytes are advanced in terms of energy storage before maturation because they were found to be richer in metabolic units, ATP content and lipid droplets including those with ADRP expression. On the other hand, the less competent oocytes are deficient in energy stores and they became more active in metabolic unit formation and lipid droplet production with ADRP expression, and consumed more ATP during maturation.

The detailed analysis of the lipid content of immature and mature porcine oocytes during different stages of folliculogenesis revealed interesting facts. Our findings demonstrate that the relative area covered with lipid droplets did not show significant differences in immature oocytes among different stages of luteal and follicular phases of folliculogenesis. But we found that the lipid content declined in all stages of luteal and follicular phases during the maturation. The relative number of lipid droplets also showed a tendency to decline during maturation, when their contents between different stages of luteal and follicular phases of folliculogenesis were compared. From these facts, we can conclude that the lipid content decreased during the maturation in order to provide energy necessary for this complex process.

Before maturation, the number of small lipid droplets showed higher values than large lipid droplets during all observed phases. While after maturation, in oocytes in the late luteal phase derived from small and medium follicles, the numbers of large lipid droplets were higher in comparison with small lipid droplets. The decline of small lipid droplets can be substantial and indicates their importance in the process of maturation.

All observed lipid parameters were declined during maturation regardless of the phase of folliculogenesis.

Immature oocytes with expanded COCs showed a lower value of relative area of lipid droplets and higher value of relative number of lipid droplets than cells that have undergone the process of germinal vesicle breakdown. While the number of small lipid droplets declined more in comparison with large lipid droplets before the process of GVBD. These facts suggest that the consumption of small lipid granules was higher to reach the process of GVBD.

In conclusion, the present study indicates that oocytes after 24 h of maturation showed a lower area of lipid droplets and reduced relative number of lipid droplets and the numbers of small and large lipid droplets in comparison with oocytes after 44 h of maturation. This can be due to the use of small lipid droplets for energy purposes and their merging, which results in increasing number of large lipid granules. From this we can conclude that lipid metabolism is effective during the process of oocyte maturation.

The relationship between the energy status of oocytes and their meiotic competence found in this study has not, to our knowledge, been reported yet. The detailed examination of observed parameters and their mutual connection described in this thesis has indeed shown a strong significance for support of oocyte quality. These results can contribute to better understanding of energy metabolism in porcine oocytes and to improvements of their maturation and embryo development for reproductive biotechnology in pigs.

Towards these perspectives, it appears important to gain a better knowledge in oocyte energy metabolism regulation. We bring complementary information related to cytoplasmic parameters and their mutual correlations. These results confirmed interactions between folliculogenesis, the level of meiotic competence of oocytes and their ability to exploit their energy potential.

7 REFERENCES

AARDEMA H., VOS P.L.A.M., LOLICATO F., ROELEN B.A.J., KNIJN H.M., VAANDRAGER A.B., HELMS J.B., GADELLA B.M. 2011: Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biology of Reproduction*, 85(1): 62–69.

ABD-ALLAH S.M. 2010: Effects of storage conditions of dromedary camel ovaries on the morphology, viability and development of antral follicular oocytes. *Animal Reproduction*, 7(2): 65-69.

ABE H., YAMASHITA S., SATOH T., HOSHI H. 2002: Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Molecular Reproduction and Development*, 61(1): 57–66.

ABEYDEERA L.R., WANG W.H., CANTLEY T.C., RIEKE A., PRATHER R.S., DAY B.N. 1998: Presence of epidermal growth factor during in vitro maturation of pig oocytes and embryo culture can modulate blastocyst development after in vitro fertilization. *Molecular Reproduction and Development*, 51(4): 395-401.

ACKERMAN IV W.E., ROBINSON J.M., KNISS D.A. 2007: Association of PAT proteins with lipid storage droplets in term fetal membranes. *Placenta*, 28(5-6): 465–476.

ALTMANN R. 1890: Die Elementarorganisem und ihre Beziehungen zu den Zellen (Leipzig: Veit). In: FARESE R V., WALTHER T.C. 2009: Lipid droplets finally get a little R-E-S-P-E-C-T. *Cell*, 139(5): 855–860.

AMBRUOSI B., LACALANDRA G.M., IORGA A.I., DE SANTIS T., MUGNIER S., MATARRESE R., GOUDET G., DELL'AQUILA M.E. 2009: Cytoplasmic lipid droplets and mitochondrial distribution in equine oocytes: Implications on oocyte maturation, fertilization and developmental competence after ICSI. *Theriogenology*, 71(7): 1093–1104.

AMI D., MEREGHETTI P., NATALELLO A., DOGLIA S.M., ZANONI M., REDI C.A., MONTI M. 2011: FTIR spectral signatures of mouse antral oocytes: Molecular markers of oocyte maturation and developmental competence. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1813(6): 1220–1229.

ANDERSON S., BANKIER A.T., BARRELL B.G., DE BRUIJN M.H., COULSON A.R., DROUIN J., EPERON I.C., NIERLICH D.P., ROE B.A., SANGER F., SCHREIER P.H., SMITH A.J., STADEN R., YOUNG I.G. 1981: Sequence and organization of the human mitochondrial genome. *Nature*, 290(5806):457-65.

AUCLAIR S., UZBEKOV R., ELIS S., SANCHEZ L., KIREEV I., LARDIC L., DALBIES-TRAN R., UZBEKOVA S. 2013: Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. *American Journal of Physiology. Endocrinology and Metabolism*, 304(6): E599–613.

BALABAN R.S., NEMOTO S., FINKEL T. 2005: Mitochondria, oxidants, and aging. *Cell*, 120(4): 483–495.

BARTZ R., LI W-H., VENABLES B., ZEHMER J.K., ROTH M.R., WELTI R., ANDERSON R.G.W., LIU P., CHAPMAN K.D. 2007: Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. *Journal of Lipid Research*, 48(4): 837–847.

BAVISTER B.D., SQUIRRELL J.M. 2000: Mitochondrial distribution and function in oocytes and early embryos. *Human Reproduction*, 15(2): 189–198.

BELLER M., THIEL K., THUL P.J., JACKLE H. 2010: Lipid droplets: A dynamic organelle moves into focus. *FEBS Letters*, 584(11): 2176-2182.

BENTOV Y., CASPER R.F. 2013: The aging oocyte-can mitochondrial function be improved? *Fertility and Sterility*, 99(1): 18–22.

BIELANSKA-OSUCHOWSKA Z. 2006: Oogenesis in pig ovaries during the prenatal period: ultrastructure and morphometry. *Reproductive Biology*, 6(2): 161–193.

BRAD A.M., BORMANN C.L., SWAIN J.E., DURKIN R.E., JOHNSON A.E., CLIFFORD A.L., KRISHER R.L. 2003: Glutathione and adenosine triphosphate content of *in vivo* and *in vitro* matured porcine oocytes. *Molecular Reproduction and Development*, 64(4): 492–498.

BRASAEMLE D.L. 2007: Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *Journal of Lipid Research*, 48(12): 2547–2559.

BRASAEMLE D.L., BARBER T., WOLINS N.E., SERRERO G., BLANCHETTE-MACKIE E.J., LONDOS C. 1997: Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *Journal of Lipid Research*, 38(11): 2249–2263.

BRASAEMLE D.L., DOLIOS G., SHAPIRO L., WANG R. 2004: Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *Journal of Biological Chemistry*, 279(45): 46835–46842.

BRASAEMLE D.L., WOLINS N.E. 2012: Packaging of fat: An evolving model of lipid droplet assembly and expansion. *Journal of Biological Chemistry*, 287(4): 2273–2279.

BREVINI T.A.L., CILLO F., ANTONINI S., GANDOLFI F. 2007: Cytoplasmic remodelling and the acquisition of developmental competence in pig oocytes. *Animal Reproduction Science*, 98(1-2): 23–38.

BREVINI T.A.L., VASSENA R., FRANCISCI C., GANDOLFI F. 2005: Role of adenosine triphosphate, active mitochondria, and microtubules in the acquisition of developmental competence of parthenogenetically activated pig oocytes. *Biology of Reproduction*, 72(5): 1218–1223.

CAMPAGNA C., BAILEY J.L., SIRARD M.A., AYOTTE P., MADDOX-HYTTEL P. 2006: An environmentally-relevant mixture of organochlorines and its vehicle control, dimethylsulfoxide, induce ultrastructural alterations in porcine oocytes. *Molecular Reproduction and Development*, 73(1): 83-91.

CETICA P., PINTOS L., DALVIT G., BECONI M. 2002: Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. *Reproduction*, 124(5): 675–681.

CHAPPEL S. 2013: The role of mitochondria from mature oocyte to viable blastocyst. *Obstetrics and Gynecology International*, 2013: 183024.

CHAUBE S.K. 2001: Role of meiotic maturation regulatory factors in development competence of mammalian oocytes. *Health and Population*, 24(4): 218–231.

CHI M.M., MANCHESTER J.K., YANG V.C., CURATO A.D., STRICKLER R.C., LOWRY O.H. 1988: Contrast in levels of metabolic enzymes in human and mouse ova. *Biology of Reproduction*, 39(2): 295–307.

CHIDA J., KIDO H. 2014: Extraction and quantification of adenosine triphosphate in mammalian tissues and cells. *Methods in Molecular Biology*, 1098: 21-32.

COLUCCI-GUYON E., PORTIER M.M., DUNIA I., PAULIN D., POURNIN S., BABINET C. 1994: Mice lacking vimentin develop and reproduce without an obvious phenotype. *Cell*, 79(4): 679-94.

COULL G.D., SPEAKE B.K., STAINES M.E., BROADBENT P.J., MCEVOY T.G. 1997: Lipid and fatty acid composition of zona-intact sheep oocytes. *Theriogenology*, 49: 179. (Abstract).

CRAN D.G. 1985: Qualitative and quantitative structural changes during pig oocyte maturation. *Journal of Reproduction and Fertility*, 74(1): 237–245.

CROCOMO L.F., FILHO W.C.M., SUDANO M.J., PASCHOAL D.M., ALVARENGA F.D.C.L., BICUDO S.D. 2013: Effect of roscovitine and cycloheximide on ultrastructure of sheep oocytes. *Small Ruminant Research*, 109(2-3): 156–162.

CROSIER A.E., FARIN P.W., DYKSTRA M.J., ALEXANDER J.E., FARIN C.E. 2001: Ultrastructural morphometry of bovine blastocysts produced in vivo or in vitro. *Biology of Reproduction*, 64(5): 1375–1385.

CUMMINS J.M. 2001: Mitochondria: Potential roles in embryogenesis and nucleocytoplasmic transfer. *Human Reproduction Update*, 7(2): 217–228.

DALTON C.M., SZABADKAI G., CARROLL J. 2014: Measurement of ATP in single oocytes: Impact of maturation and cumulus cells on levels and consumption. *Journal of Cellular Physiology*, 229(3): 353–361.

DESAI N., LUDGIN J., SHARMA R., ANIRUDH R.K., AGARWAL A. 2013: Female and male gametogenesis. *In: FALCONE T., HURD W.W. Clinical reproductive medicine and surgery*.

DUCHARME N.A., BICKEL P.E. 2008: Minireview: Lipid droplets in lipogenesis and lipolysis. *Endocrinology*, 149(3): 942–949.

DUMOLLARD R., DUCHEN M., SARDET C. 2006: Calcium signals and mitochondria at fertilisation. *Seminars in Cell and Developmental Biology*. 17(2): 314–323.

DUMOLLARD R., WARD Z., CARROLL J., DUCHEN M.R. 2007: Regulation of redox metabolism in the mouse oocyte and embryo. *Development*, 134(3): 455–465.

DUNNING K.R., RUSSELL D.L., ROBKER R.L. 2014: Lipids and oocyte developmental competence: The role of fatty acids and β-oxidation. *Reproduction*, 148(1): R15-27.

DURANTHON V., RENARD J.P. 2001: The developmental competence of mammalian oocytes: a convenient but biologically fuzzy concept. *Theriogenology*, 55(6): 1277-89.

EDSON M. A., NAGARAJA A. K., MATZUK M. M. 2009: The mammalian ovary from genesis to revelation. *Endocrine Reviews*, 30(6): 624–712.

EDVARDSSON U., LJUNGBERG A., LINDÉN D., WILLIAM-OLSSON L., PEILOT-SJÖGREN H., AHNMARK A., OSCARSSON J. 2006: PPARalpha activation increases triglyceride mass and adipose differentiation-related protein in hepatocytes. *Journal of Lipid Research*, 47(2): 329–340.

EGERSZEGI I., ALM H., RÁTKY J., HELEIL B., BRÜSSOW K.P., TORNER H. 2010: Meiotic progression, mitochondrial features and fertilisation characteristics of porcine oocytes with different G6PDH activities. *Reproduction, Fertility and Development*, 22(5): 830–838.

EPPIG J.J. 1991: Intercommunication between mammalian oocytes and companion somatic cells. *BioEssays*, 13(11): 569-574

EPPIG J.J., SCHROEDER A. C. 1989: Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. *Biology of Reproduction*, 41(2): 268–276.

EPPIG J.J., SCHULTZ R.M., O'BRIEN M., CHESNEL F. 1994: Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. *Developmental Biology*, 164(1): 1–9.

ERICKSON B. H. 1966. Development and radio-response of the prenatal bovine ovary. *Journal of Reproduction and Fertility*, 11: 97-105.

FAIR T., HYTTEL P., GREVE T. 1995: Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Molecular Reproduction and Development*, 42(4): 437-442.

FARESE R V., WALTHER T.C. 2009: Lipid Droplets Finally Get a Little R-E-S-P-E-C-T. *Cell*, 139(5): 855–860.

FAUSER B.C.J.M., RUTHERFORD A.J., STRAUSS J.F., VAN STEIRTEGHEM A. 1999: (co-eds.) Molecular Biology in Reproductive Medicine. Parthenon Publishing, New York and Carnforth.

FERGUSON E.M., LEESE H.J. 1999: Triglyceride content of bovine oocytes and early embryos. *Journal of Reproduction and Fertility*, 116(2): 373-378.

FERREIRA E.M., VIREQUE A. A., ADONA P.R., MEIRELLES F.V., FERRIANI R A., NAVARRO P.A.A. S. 2009: Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. *Theriogenology*, 71(5): 836–848.

FIELDING C.J., BIST A., FIELDING P.E. 1998: Selective Uptake of lipoprotein free cholesterol and its intracellular transport — Role of Caveolin. *In CHANG T.Y. and FREEMAN D.A. Intracellular cholesterol trafficking*. 273-288.

FRANKE W.W., HERGT M., GRUND C. 1987: Rearrangement of the vimentin cytoskeleton during adipose conversion: formation of an intermediate filament cage around lipid globules. *Cell*, 49(1): 131-41.

FROLOV A., PETRESCU A., ATSHAVES B.P., SO P.T.C., GRATTON E., SERRERO G., SCHROEDER F. 2000: High density lipoprotein-mediated cholesterol uptake and targeting to lipid droplets in intact L-cell fibroblasts. A single- and multiphoton fluorescence approach. *Journal of Biological Chemistry*, 275(17): 12769–12780.

FULKA J. Jr., FIRST N.L., MOOR R.M. 1998: Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. *Molecular Human Reproduction*, 4(1): 41–49.

GAO J., SERRERO G. 1999: Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. *Journal of Biological Chemistry*, 274(24): 16825–16830.

GENICOT G., LEROY J.L.M.R., VAN SOOM A., DONNAY I. 2005: The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. *Theriogenology*, 63(4): 1181–1194.

GILCHRIST R.B., LANE M., THOMPSON J.G. 2008: Oocyte-secreted factors: Regulators of cumulus cell function and oocyte quality. *Human Reproduction Update*, 14(2): 159–177.

GONZALES-FIGUEROA H., GONZALES-MOLFINO H.M. 2005: Maturation of pig oocytes in vitro in a medium with pyruvate. *Brazilian Journal of Medical and Biological Research*, 38: 869–872.

GOTO T., ADJAYE J., RODECK C.H., MONK M. 1999: Identification of genes expressed in human primordial germ cells at the time of entry of the female germ line into meiosis. *Molecular Human Reproduction*, 5(9): 851–60.

GROSS D.N., MIYOSHI H., HOSAKA T., ZHANG H-H., PINO E.C., SOUZA S., OBIN M., GREENBERG A.S., PILCH P.F. 2006: Dynamics of lipid droplet-associated proteins during hormonally stimulated lipolysis in engineered adipocytes: stabilization and lipid droplet binding of adipocyte differentiation-related protein/adipophilin. *Molecular Endocrinology*, 20(2): 459–466.

GURAYA S. S., & ebrary Academic Complete., 2008. Cellular and molecular biology of human oogenesis, ovulation and early embryogenesis: Fundamentals, biomedical and clinical implications in relation to infant disorder. New Delhi: New Age International (P) Ltd., Publishers. ISBN (10): 81-224-2249-7. ISBN (13) : 978-81-224-2249-8. Available at: http://www.bjcancer.org/Sites_OldFiles/_Library/UserFiles/pdf/Cellular%20and%20Molecular%20Biology%20of%20Human%20Oogenesis,%20Ovulation,%20and%20Early%20Embryogenesis.pdf

HEID H.W., MOLL R., SCHWETLICK I., RACKWITZ H.R., KEENAN T.W. 1998: Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. *Cell and Tissue Research*, 294(2): 309–321.

HEID H.W., SCHNÖLZER M., KEENAN T.W. 1996: Adipocyte differentiation-related protein is secreted into milk as a constituent of milk lipid globule membrane. *Biochemical Journal*, 320: 1025–1030.

HERNANDEZ-MEDRANO J.H., CAMPBELL B.K., WEBB R. 2012: Nutritional influences on folliculogenesis. *Reproduction in Domestic Animals*, 47(4): 274–282.

HERRANZ P., DE LUCAS R., PEREZ-ESPANA L., MAYOR M. 2008: Lipodystrophy syndromes. *Dermatologic Clinics*, 26(4): 569–578.

HILLENSJO T., CHANNING C.P. 1980: Gonadotropin stimulation of steroidogenesis and cellular dispersion in cultured porcine cumuli oophori. *Gamete Research*, 3(3): 233-240.

HINRICHS K., SCHMIDT A.L, FRIEDMAN P.P., SELGRATH J.P., MARTIN M.G. 1993: In vitro maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy. *Biology of Reproduction*. 48: 363–370.

HIRAGA K., HOSHINO Y., TANEMURA K., SATO E. 2013: Selection of in vitro-matured porcine oocytes based on localization patterns of lipid droplets to evaluate developmental Competence. *Journal of Reproduction and Development*, 59(4): 405-408.

HOMA S.T., RACOWSKY C., MCGAUGHEY R.W. 1986: Lipid analysis of immature pig oocytes. *Journal of Reproduction and Fertility*, 77(2): 425–434.

HULINSKA P., MARTECIKOVA S., JESETA ., MACHATKOVA M. 2011: Efficiency of in vitro fertilization is influenced by the meiotic competence of porcine oocytes and time of their maturation. *Animal Reproduction Science*, 124(1-2): 112-7.

HYTTEL P., CALLESEN H., GREVE T. 1986: Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. *Journal of Reproduction and Fertility*, 76(2): 645–656.

HYTTEL P., FAIR T., CALLESEN H., GREVE T. 1997: Oocyte growth, capacitation and final maturation in cattle. *Theriogeneology*, 47(1): 23-32.

IMAMURA M., INOGUCHI T., IKUYAMA S., TANIGUCHI S., KOBAYASHI K., NAKASHIMA N., NAWATA H. 2002: ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *American Journal of Physiology. Endocrinology and metabolism*, 283(4): E775–E783.

INOUE S., SALMON E.D. 1995: Force generation by microtubule assembly/disassembly in mitosis and related movements. *Molecular Biology of the Cell*, 6(12): 1619–1640.

JANSEN R.P.S., DE BOER K. 1998: The bottleneck: Mitochondrial imperatives in oogenesis and ovarian follicular fate. *Molecular and Cellular Endocrinology*, 145(1-2): 81–88.

JEONG W.J., CHO S.J., LEE H.S., DEB G.K., LEE Y.S., KWON T.H., KONG I.K. 2009: Effect of cytoplasmic lipid content on in vitro developmental efficiency of bovine IVP embryos. *Theriogenology*, 72(4): 584–589.

JIANG H.P., SERRERO G. 1992: Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. *Proceedings of the National Academy of Sciences of the United States of America*, 89(17): 7856–7860.

JOHNSON M.T., FREEMAN E.A., GARDNER D.K., HUNT P.A. 2007: Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo. *Biology of Reproduction*, 77(1): 2–8.

JOSEFSBERG L.B., GALIANI D., DANTES A., AMSTERDAM A., DEKEL N. 2000: The proteasome is involved in the first metaphase-to-anaphase transition of meiosis in rat oocytes. *Biology of Reproduction*, 62(5): 1270–7.

KANAYAMA N., MIYANO T., LEE J. 2002: Acquisition of meiotic competence in growing pig oocytes correlates with their ability to activate Cdc2 kinase and MAP kinase. *Zygote*, 10(3): 261–270.

KANITZ W., BRUSSOW K.P., BECKER F., TORNER H., SCHNEIDER F., KUBELKA M., TOMEK W. 2001: Comparative aspects of follicular development, follicular and oocyte maturation and ovulation in cattle and pigs. *Archiv Tierzucht*, 44: 9–23.

KATAYAMA M., ZHONG Z., LAI L., SUTOVSKY P., PRATHER R.S., SCHATTEN H. 2006: Mitochondrial distribution and microtubule organization in fertilized and cloned

porcine embryos: Implications for developmental potential. *Developmental Biology*, 299(1): 206–220.

KHATIR H., CAROLAN C., LONERGAN P., MERMILLOD P. 1997: Characterization of calf follicular fluid and its ability to support cytoplasmic maturation of cow and calf oocytes. *Journal of Reproduction and Fertility*, 111(2): 267–275.

KIKUCHI K., EKWALL H., TIENTHAI P., KAWAI Y., NOGUCHI J., KANEKO H., RODRIGUEZ-MARTINEZ H. 2002: Morphological features of lipid droplet transition during porcine oocyte fertilisation and early embryonic development to blastocyst in vivo and in vitro. *Zygote*, 10(44): 355-366.

KIM T.H., CHOI B.H., CHANG G.W., LEE K.T., LEE H.Y., LEE J.H., KIM K.S., PARK C.K., MORAN C. 2005: Molecular characterization and chromosomal mapping of porcine adipose differentiation-related protein (ADRP). *Journal of Animal Breeding and Genetics Zeitschrift fur Tierzuchtung und Zuchtungsbiologie*, 122(4): 240–246.

KIM N.H., FUNAHASHI H., PRATHER R.S., SCHATTEN G., DAY B.N. 1996: Microtubule and microfilament dynamics in porcine oocytes during meiotic maturation. *Molecular Reproduction and Development*, 43(2): 248-55.

KIM J., KIM J.S., JEON Y.J., KIM D.W., YANG T.H., SOH Y., Lee H.K., CHOI N.J., PARK S.B., SEO K.S., CHUNG H.M., LEE D.S., CHAE J. 2011: Identification of maturation and protein synthesis related proteins from porcine oocytes during in vitro maturation. *Proteome Science*, 9(1): 28

KIM J.Y., KINOSHITA M., OHNISHI M., FUKUI Y. 2001: Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. *Reproduction*, 122(1): 131–138. ISSN 14701626.

KNIGHT P.G., GLISTER C. 2006: TGF-beta superfamily members and ovarian follicle development. *Reproduction*, 132(2): 191-206.

KOENIG J.L., STORMSHAK F. 1993: Cytogenetic evaluation of ova from pubertal and third-estrous gilts. *Biology of Reproduction*, 49(6): 1158–1162.

KOOPMAN W.J.H., WILLEMS P.H.G.M., SMEITINK J.A.M. 2012: Monogenic Mitochondrial Disorders. *The New England Journal of Medicine*, 366: 1132-1141.

KOVACIC P., POZOS R.S., SOMANATHAN R., SHANGARI N., O'BRIEN P.J. 2005: Mechanism of mitochondrial uncouplers, inhibitors, and toxins: focus on electron transfer, free radicals, and structure-activity relationships. *Current Medicinal Chemistry*, 12(22): 2601-2623.

KRISHER R.L. 2013: In Vivo and In Vitro Environmental Effects on Mammalian Oocyte Quality. *Annual Review of Animal Biosciences*, 1(1): 393–417.

KRISHER R.L., BAVISTER B.D. 1998: Responses of oocytes and embryos to the culture environment. *Theriogenology*, 49(97): 103–114.

KRUIP T. A. M., CRAN D. G., VAN BENEDEN T. H., DIELEMAN S. J. 1983: Structural changes in bovine oocytes during final maturation in vivo. *Gamete Research*, 8(1): 29–47.

LEE H-C., WEI Y-H. 1997: Role of mitochondria in human aging. *Journal of Biomedical Science*, 4(6): 319–326. ISSN 1021-7770.

LEROY J.L.M.R., GENICOT G., DONNAY I., VAN SOOM A. 2005: Evaluation of the Lipid Content in Bovine Oocytes and Embryos with Nile Red: a Practical Approach. *Reproduction in domestic animals Zuchthygiene*, 40(1): 76–78.

LIAN H-Y., JIAO G-Z., WANG H-L., TAN X-W., WANG T-Y., ZHENG L-L., KONG Q-Q., TAN J-H. 2014: Role of Cytoskeleton in Regulating Fusion of Nucleoli: a Study Using the Activated Oocyte Model. *Biology of Reproduction*, 91: 1–8.

LISTENBERGER L.L., BROWN D.A. 2007: Fluorescent detection of lipid droplets and associated proteins. *Current protocols in cell biology* / editorial board, Juan S. Bonifacino et al. Chapter 24: Unit 24.2.

LISTENBERGER L.L., HAN X., LEWIS S.E., CASES S., FARESE R.V., ORY D.S., SCHAFFER J.E. 2003: Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proceedings of the National Academy of Sciences of the United States of America*, 100(6): 3077–3082.

LIU S., LI Y., GAO X., YAN J-H., CHEN Z-J. 2010: Changes in the distribution of mitochondria before and after in vitro maturation of human oocytes and the effect of in vitro maturation on mitochondria distribution. *Fertility and sterility*, 93(5): 1550–1555.

LUNA H.S., FERRARI I., RUMPF R. 2001: Influence of stage of maturation of bovine oocytes at time of vitrification on the incidence of diploid metaphase II at completion of maturation. *Animal Reproduction Science*, 68(1-2): 23-28.

MACHAL L., CHLADEK G., ZIZLAVSKY J. 1999: Relation among blood plasma total lipids, cholesterol, glucose and urea concentrations, and reproductive performance in Bohemian Spotted cows. *Journal of Animal and Feed Sciences*, 8: 209 – 221.

MACHATKOVA M., JESETA M., HULINSKA P., KNITLOVA D., NEMCOVA L., KANKA J. 2012: Characteristics of bovine oocytes with different meiotic competence in terms of their mitochondrial status and expression of nuclear-encoded factors. *Reproduction in Domestic Animals*, 47(5): 806–814.

MACHATKOVA M., KRAUSOVA K., JOKESOVA E., TOMANEK M. 2004: Developmental competence of bovine oocytes: effects of follicle size and the phase of follicular wave on in vitro embryo production. *Theriogenology*, 61(2-3): 329-335.

MAGNUSSON B., ASP L., BOSTRÖM P., RUIZ M., STILLEMARK-BILLTON P., LINDÉN D., BORÉN J., OLOFSSON S.O. 2006: Adipocyte differentiation-related protein promotes fatty acid storage in cytosolic triglycerides and inhibits secretion of very low-density lipoproteins. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 26(7): 1566–1571.

MANDELBAUM J. (2000). Oocytes. *Human Reproduction*, 15(4): 11–18.

MARCHAL R., VIGNERON C., PERREAU C., BALI-PAPP A., MERMILLOD P. 2002: Effect of follicular size on meiotic and developmental competence of porcine oocytes. *Theriogenology*, 57(5): 1523–1532.

MARTEIL G.., RICHARD-PARPAILLON L, KUBIAK J.Z. 2009: Role of oocyte quality in meiotic maturation and embryonic development. *Reproductive Biology*, 9(3): 203–224.

MARTINS L.R., FERNANDES C.B., MINTO B.W., LANDIM-ALVARENGA F.C., LOPES M.D. 2009: Ultrastructural characteristics of non-matured and in vitro matured oocytes collected from pre-pubertal and adult domestic cat ovaries. *Reproduction in Domestic Animals*, 44(2): 251-4.

MATTIOLI M., BACCI M.L., GALEATI G., SEREN E. 1989: Developmental competence of pig oocytes matured and fertilized in vitro. *Theriogenology*, 31(6): 1201-7.

MCEVOY T.G., COULL G.D., BROADBENT P.J., HUTCHINSON J.S., SPEAKE B.K. 2000: Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *Journal of Reproduction and Fertility*, 118(1): 163–170.

MCKEEGAN P.J., STURMEY R.G. 2011: The role of fatty acids in oocyte and early embryo development. *Reproduction, Fertility and Development*, 24(1): 59-67.

MEHLMANN L.M. 2005: Stops and starts in mammalian oocytes: Recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction*, 130(6): 791–799.

MENINO A.R.Jr., ARCHIBONG A.E., LI J.R., STORMSHAK F., ENGLAND D.C. 1989: Comparison of in vitro development of embryos collected from the same gilts at first and third estrus. *Journal of Animal Science*, 67(5): 1387-1393.

MERMILLOD P., LE BOURHIS D., LONERGAN P., KHATIR H., HEYMAN Y. 1998: Assessment of cytoplasmic competence of prepubertal calf oocytes by use of nuclear transfer. *Theriogenology*. 49(1): 187–187.

MERMILLOD P., OUSSAID B., COGNIE Y. 1999: Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *Journal of Reproduction and Fertility*, 54: 449-460.

MIYANARI Y., ATSUZAWA K., USUDA N., WATASHI K., HISHIKI T., ZAYAS M., BARTENSCHLAGER R., WAKITA T., HIJIKATA M., SHIMOTOHNO K. 2007: The lipid droplet is an important organelle for hepatitis C virus production. *Nature Cell Biology*, 9(9): 1089 – 1097.

MOTLIK J., CROZET N., FULKA J. 1984: Meiotic competence in vitro of pig oocytes isolated from early antral follicles. *Journal of Reproduction and Fertility*, 72(2): 323–328.

MOTLIK J., FULKA J. 1986: Factors affecting meiotic competence in pig oocytes. *Theriogenology*, 25(1): 87-96.

MOTTA P.M., NOTTOLA S.A., MAKABE S., HEYN R. 2000: Mitochondrial morphology in human fetal and adult female germ cells. *Human Reproduction*, 15(2): 129–147.

MULDER D.W. 1982: Clinical limits of amyotrophic lateral sclerosis. *Advances in Neurology*, 36: 15–22.

MURPHY D.J., VANCE J. 1999: Mechanisms of lipid-body formation. *Trends in Biochemical Sciences*, 24(3): 109–115.

NAGAI S., MABUCHI T., HIRATA S., SHODA T., KASAI T., YOKOTA S., SHITARA H., YONEKAWA H., HOSHI K. 2006: Correlation of abnormal mitochondrial distribution in mouse oocytes with reduced developmental competence. *The Tohoku Journal of Experimental Medicine*, 210(2): 137–144.

NAGANO M., KATAGIRI S., TAKAHASHI Y. 2006: Relationship between bovine oocyte morphology and in vitro developmental potential. *Zygote*, 14(1): 53–61.

NAZMARA Z., SALEHNIA M., HOSSEINKHANI S. 2014: Mitochondrial Distribution and ATP Content of Vitrified, In vitro Matured Mouse Oocytes. *Avicenna Journal of Medical Biotechnology*, 6(4): 210 - 217.

NIIMURA S., TAKANO H., ONISHI A., HOSOE M. 2002: Changes in the amount of proteins, glycogen and lipids in porcine oocytes during in vitro meiotic maturation. *Animal Science Journal*, 73(5): 327–332.

NILI H., MESBAH F., KAFI M., NASR ESFAHANI M.H. 2004: Light and transmission electron microscopy of immature camelus dromedarius oocyte. *Anatomia, Histologia, Embryologia*, 33(4): 196–199.

NISHI Y., TAKESHITA T., SATO K., ARAKI T. 2003: Change of the mitochondrial distribution in mouse ooplasm during in vitro maturation. *Journal of Nihon Medical School = Nihon Ika Daigaku zasshi*, 70(5): 408–415.

NOTO V., CAMPO R., ROZIERS P., SWINNEN K., VERCRUYSSEN M., GORDTS S. 1993: Mitochondrial distribution after fast embryo freezing. *Human Reproduction*, 8(12): 2115–2118.

OLOFSSON S.O., BOSTRÖM P., ANDERSSON L., RUTBERG M., PERMAN J., BORÉN J. 2009: Lipid droplets as dynamic organelles connecting storage and efflux of lipids. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1791(6): 448–458.

PEDERSEN T. 1969: Follicle growth in the immature mouse ovary. *Acta Endocrinologica (Copenh)*, 62: 117–132.

PELLINIEMI LJ., LAUTEALA L. 1981: Development of sexual dimorphism in the embryonic gonad. *Human Genetics*, 58(1): 64–67.

PFEIFER L.F.M., SCHNEIDER A., CORREA M.N. 2008: Factors that affect the in vitro production of bovine embryos : A review. *Revista Colombiana de Ciencias Pecuarias*, 21(53): 109–120.

PIKO L., TAYLOR K.D. 1987: Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Developmental Biology*, 123(2): 364-374.

PINCUS G., ENZMANN E.V. 1935: The comparative behaviour of mammalian eggs in vivo and in vitro. *The Journal of Experimental Medicine*, 62(5): 665–675.

PIOTROWSKA H., KEMPISTY B., SOSINSKA P., CIESIOLKA S., BUKOWSKA D., ANTOSIK P., RYBSKA M., BRUSSOW K.P., NOWICKI M., ZABEL M. 2013: The role of TGF superfamily gene expression in the regulation of folliculogenesis and oogenesis in mammals: A review. *Veterinarni Medicina*, 58(10): 505–515.

PRATES E.G., NUNES J.T., PEREIRA R.M. 2014: A role of lipid metabolism during cumulus-oocyte complex maturation: Impact of lipid modulators to improve embryo production. *Mediators of Inflammation*, 2014: 11.

PRATHER S., HAWLEY R. J., CARTER D. B., LAI L., GREENSTEIN J. L. 2003. Transgenic swine for biomedicine and agriculture. *Theriogeneology*, 59(1): 115-23.

PRESICCE G.A., JIANG S., SIMKIN M., ZHANG L., LOONEY C.R., GODKE R.A., YANG X. 1997: Age and hormonal dependence of acquisition of oocyte competence for embryogenesis in prepubertal calves. *Biology of Reproduction*, 56(2): 386–392.

RAHMAN A.N.M.A., ABDULLAH R.B., WAN-KHADIJAH W.E. 2008: Gametogenesis, Fertilization and Early Embryogenesis in Mammals with Special Reference to Goat: A Review. *Journal of Biological Sciences*, 8(7): 1115-1128.

RAMALHO-SANTOS J., VARUM S., AMARAL S., MOTA P.C., SOUSA A.P., AMARAL A. 2009: Mitochondrial functionality in reproduction: From gonads and gametes to embryos and embryonic stem cells. *Human Reproduction Update*, 15(5): 553–572.

RATKY J., BRUSSOW K.P. 1998: Ovarian activity in gilts including some characteristics of a native breed. *Reproduction in Domestic Animals*, 33(3-4): 219–222.

ROBENEK H., BUERS I., ROBENEK M.J., HOFNAGEL O., RUEBEL A., TROYER D., SEVERS N.J. 2011: Topography of lipid droplet-associated proteins: Insights from freeze-fracture replica immunogold labeling. *Journal of Lipids*, 2011: 1–10.

ROBERTS R., FRANKS S., HARDY K. 2002: Culture environment modulates maturation and metabolism of human oocytes. *Human Reproduction*, 17(11): 2950–6.

ROCHE J.F. 1996: Control and regulation of folliculogenesis--a symposium in perspective. *Reviews of Reproduction*, 1(1): 19–27.

RODLEY C.D.M., GRAND R.S., GEHLEN L.R., GREYLING G., JONES M.B., SULLIVAN J.M. 2012: Mitochondrial-nuclear DNA interactions contribute to the regulation of nuclear transcript levels as part of the inter-organelle communication system. *PLoS One*, 7(1): 1–11.

RODRIGUEZ K. F., FARIN C.E. 2004: Gene transcription and regulation of oocyte maturation. *Reproduction, Fertility and Development*, 16(1-2): 55-67.

ROMEK M., GAJDA B., KRZYSZTOFOWICZ E., KEPCZYNSKI M., SMORAG Z. 2011: New technique to quantify the lipid composition of lipid droplets in porcine oocytes and pre-implantation embryos using Nile Red fluorescent probe. *Theriogenology*, 75(1): 42–54.

ROZELL M.D., WILLIAMS J.E., BUTLER J.E. 1992: Changes in concentration of adenosine triphosphate and adenosine diphosphate in individual preimplantation sheep embryos. *Biology of Reproduction*, 47: 866-870.

RÜSSE I. 1983. Oogenesis in cattle and sheep. *Bibliotheca Anatomica*, 24: 77–92.

SAMSA M.M., MONDOTTE J.A., IGLESIAS N.G., ASSUNÇÃO-MIRANDA I., BARBOSA-LIMA G., DA POIAN A.T., BOZZA P.T., GAMARNIK A.V. 2009: Dengue virus capsid protein usurps lipid droplets for viral particle formation. *PLoS Pathogens*, 5(10): e1000632.

SANCHEZ F., SMITZ J. 2012: Molecular control of oogenesis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. 1822(12): 1896–1912.

SANTOS T.A., EL SHOURBAGY S., ST. JOHN J.C. 2006: Mitochondrial content reflects oocyte variability and fertilization outcome. *Fertility and Sterilility*, 85(3): 584–591.

SCHRAMM R.D., BAVISTER B.D. 1995: Effects of granulosa cells and gonadotrophins on meiotic and developmental competence of oocytes in vitro in non-stimulated rhesus monkeys. *Human Reproduction*, 10(4): 887–895.

SCHUH M., ELLENBERG J. 2008: A new model for asymmetric spindle positioning in mouse oocytes. *Current Biology : CB*, 18(24): 1986–92.

SCHULTZ C.J., TORRES E., LONDOS C., TORDAY J.S. 2002: Role of adipocyte differentiation-related protein in surfactant phospholipid synthesis by type II cells. American journal of physiology. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 283(2): L288–L296.

SHOUBRIDGE E.A., WAI T. 2007: Mitochondrial DNA and the mammalian oocyte. *Current Topics in Developmental Biology*, 77: 87-111.

SIRARD M.A. 2001: Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology*, 55(6): 1241-54.

SIRARD M.A., FLORMAN H.M., LEIBFRIED-RUTLEDGE M.L., BARNES F.L., SIMS M.L., FIRST N.L. 1989: Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biology of Reproduction*, 40: 1257-1263.

SIRARD M.A., RICHARD F., BLONDIN P., ROBERT C. 2006: Contribution of the oocyte to embryo quality. *Theriogenology*, 65(1): 126–136.

SLOTTE H., GUSTAFSON O., NYLUND L., POUSETTE A. 1990: ATP and ADP in human pre-embryos. *Human Reproduction*, 5(3): 319–322.

SMILJAKOVIC T., TOMEK W. 2006: Meiotic maturation and in vitro maturation of bovine oocytes. *Biotechnology in Animal Husbandry*, 22(1-2): 29–34.

SPIELMANN H., JACOB-MUELLER U., SCHULZ P., SCHIMMEL A. 1984: Changes of the adenine ribonucleotide content during preimplantation development of mouse embryos in vivo and in vitro. *Journal of Reproduction and Fertility*, 71(2): 467–473.

SPIKINGS E.C., ALDERSON J., ST. JOHN J.C. 2006: Transmission of mitochondrial DNA following assisted reproduction and nuclear transfer. *Human Reproduction Update*, 12(4): 401–415.

SQUIRRELL J.M., SCHRAMM R.D., PAPROCKI A.M., WOKOSIN D.L., BAVISTER B.D. 2003: Imaging mitochondrial organization in living primate oocytes and embryos using multiphoton microscopy. *Microscopy and Microanalysis*, 9(3): 190–201.

ST. JOHN J.C., FACUCHO-OLIVEIRA J., JIANG Y., KELLY R., SALAH R. 2010: Mitochondrial DNA transmission, replication and inheritance: A journey from the gamete through the embryo and into offspring and embryonic stem cells. *Human Reproduction Update*, 16(5): 488–509.

STEINER S., WAHL D., MANGOLD B.L.K, ROBISON R., RAYMACKERS J., MEHEUS L., ANDERSON N.L., CORDIER A. 1996: Induction of the adipose differentiation-related protein in liver of etomoxir-treated rats. *Biochemical and Biophysical Research Communications*, 218(3): 777–782.

STOJKOVIC M., MACHADO S.A., STOJKOVIC P., ZAKHARTCHENKO V., HUTZLER P., GONÇALVES P.B., WOLF E. 2001: Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biology of Reproduction*, 64(3): 904–909.

STOJKOVIC M., WESTESEN K., ZAKHARTCHENKO V., STOJKOVIC P., BOXHAMMER K., WOLF E. 1999: Coenzyme Q(10) in submicron-sized dispersion

improves development, hatching, cell proliferation, and adenosine triphosphate content of in vitro-produced bovine embryos. *Biology of Reproduction*, 61(2): 541–547.

STURMEY R.G., O'TOOLE P.J., LEESE H.J. 2006: Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. *Reproduction*, 132(6): 829–837.

STURMEY R.G., REIS A., LEESE H.J., MCEVOY T.G. 2009: Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reproduction in Domestic Animals*, 44(3): 50–58.

SUN Q.Y., NAGAI T., 2003: Molecular mechanisms underlying pig oocyte maturation and fertilization. *Journal of Reproduction and Development*, 49 (5): 347– 359.

SUN Q.-Y., SCHATTEN H. 2006: Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction*, 131(2): 193–205.

SUN Q.Y., WU G.M., LAI L., PARK K.W., CABOT R., CHEONG H.T., DAY B.N., PRATHER R.S., SCHATTEN H. 2001: Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. *Reproduction*, 122(1): 155–163.

SUZUKI H., SATOH M., KABASHIMA K. 2006: Distributions of mitochondria and the cytoskeleton in hamster embryos developed in vivo and in vitro. *Journal of Mammalian Ova Research*, 23(3): 128-134.

TANAKA Y., NAKADA K., MORIYOSHI M., SAWAMUKAI Y. 2001: Appearance and number of follicles and change in the concentration of serum FSH in female bovine fetuses. *Reproduction*, 121(5): 777–782.

TANGHE S., VAN SOOM A., NAUWYNCK H., CORYN M., DE KRUIF A. 2002: Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Molecular Reproduction and Development*, 61(3): 414–424.

TARAZONA A.M., RODRÍGUEZ J.I., RESTREPO L.F., OLIVERA-ANGEL M. 2006: Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced in vitro. *Reproduction in Domestic Animals*, 41(1): 5–11.

TELFORD N A., WATSON A.J., SCHULTZ G.A. 1990: Transition from maternal to embryonic control in early mammalian development: A comparison of several species. *Molecular Reproduction and Development*, 26(1): 90–100.

TERADA N., SAITOH Y., SAITOH S., OHNO N., FUJISHITA K., KOIZUMI S., OHNO S. 2012: Visualization of ATP with luciferin-luciferase reaction in mouse skeletal muscles using an “*In vivo* cryotechnique.” *Microscopy and Microanalysis*, 18(05): 1030–1036. ISSN 1431-9276.

THOMPSON J.G., MCNAUGHTON C., GASPARRINI B., MCGOWAN L.T., TERVIT H.R. 2000: Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. *Journal of Reproduction and Fertility*, 118(1): 47–55.

THOUAS G.A., TROUNSON A.O., JONES G.M. 2005: Effect of female age on mouse oocyte developmental competence following mitochondrial injury. *Biology of Reproduction*, 73(2): 366–373.

TOMEK W., TORNER H., KANITZ W. 2002: Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes in vitro. *Reproduction in Domestic Animals*, 37(2): 86–91.

TORNER H., BRÜSSOW K.P., ALM H., RATKY J., PÖHLAND R., TUCHSCHERER A., KANITZ W. 2004: Mitochondrial aggregation patterns and activity in porcine oocytes and apoptosis in surrounding cumulus cells depends on the stage of pre-ovulatory maturation. *Theriogenology*, 61(9): 1675–1689.

TURATHUM B., SAIKHUN K., SANGSUWAN P., KITIYANANT Y. 2010: Effects of vitrification on nuclear maturation, ultrastructural changes and gene expression of canine oocytes. *Reproductive Biology and Endocrinology : RB&E*, 8: 70.

UHLENHAUT N.H., TREIER M. 2011: Forkhead transcription factors in ovarian function. *Reproduction*, 142(4): 489–495.

VALENTINI L., IORGA A.I., DE SANTIS T., AMBRUOSI B., REYNAUD K., CHASTANT-MAILLARD S., GUARICCI A.C., CAIRA M., DELL'AQUILA M.E. 2010: Mitochondrial distribution patterns in canine oocytes as related to the reproductive cycle stage. *Animal Reproduction Science*, 117(1-2): 166–177.

VAN BLERKOM J. 1991: Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 88(11): 5031–5035.

VAN BLERKOM J. 2011: Mitochondrial function in the human oocyte and embryo and their role in developmental competence. *Mitochondrion*, 11(5): 797–813.

VAN BLERKOM J., BELL H. 1986: Regulation of development in the fully grown mouse oocyte: chromosome-mediated temporal and spatial differentiation of the cytoplasm and plasma membrane. *Journal of Embryology and Experimental Morphology*, 93: 213–238.

VAN BLERKOM J., DAVIS P.W., LEE J. 1995: ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Human Reproduction*, 10(2): 415–424.

VAN BLERKOM J., DAVIS P., MATHWIG V., ALEXANDER S. 2002: Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos. *Human Reproduction*, 17(2): 393–406.

VAN BLERKOM J., RUNNER M.N. 1984: Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. *The American Journal of Anatomy*, 171(3): 335–355.

VOLARCIK K., SHEEAN L., GOLDFARB J., WOODS L., ABDUL-KARIM F.W., HUNT P. 1998: The meiotic competence of in-vitro matured human oocytes is influenced by donor age: Evidence that folliculogenesis is compromised in the reproductively aged ovary. *Human Reproduction*, 13(1): 154–160.

VORONINA E., WESSEL G. M. 2003: The regulation of oocyte maturation. *Current Topics in Developmental Biology*, 58: 53–110.

WALTHER T.C., FARESE R V. 2009: The life of lipid droplets. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1791(6): 459–466.

WASSARMAN P. M., ALBERTINI D. F. 1994: The mammalian ovum. In: *Knobil E., Neill J.D. (eds.), The Physiology of Reproduction*, 1: 79–122.

WATANABE T., THAYIL A., JESACHER A., GRIEVE K., DEBARRE D., WILSON T., BOOTH M., SRINIVAS S. 2010: Characterisation of the dynamic behaviour of lipid droplets in the early mouse embryo using adaptive harmonic generation microscopy. *BMC Cell Biology*, 11: 38.

WEI P., TANIGUCHI S., SAKAI Y., IMAMURA M., INOGUCHI T., NAWATA H., ODA S., NAKABEPPU Y., NISHIMURA J., IKUYAMA S. 2005: Expression of adipose differentiation-related protein (ADRP) is conjointly regulated by PU.1 and AP-1 in macrophages. *Journal of Biochemistry*, 138(4): 399–412.

WILDING M., DALE B., MARINO M., DI MATTEO L., ALVIGGI C., PISATURO M.L., LOMBARDI L., DE PLACIDO G. 2001: Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Human Reproduction*, 16(5): 909–917.

WILSON E. 1896: The Cell in Development and Inheritance (New York: Macmillan). DOI http://dx.doi.org/10.5962/bhl.title.46211. In: FARESE R V., WALTHER T.C. 2009: Lipid Droplets Finally Get a Little R-E-S-P-E-C-T. *Cell*, 139(5): 855–860.

WOOD B.R., CHERNENKO T., MATTHÄUS C., DIEM M., CHONG C., BERNHARD U., JENE C., BRANDLI A.A., MCNAUGHTON D., TOBIN M.J., TROUNSON A., LACHAM-KAPLAN O. 2008: Shedding new light on the molecular architecture of oocytes using a combination of synchrotron fourier transform-infrared and raman spectroscopic mapping. *Analytical Chemistry*, 80(23): 9065–9072.

XU G., SZTALRYD C., LU X., TANSEY J.T., GAN J., DORWARD H., KIMMEL A.R., LONDOS C. 2005: Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway. *Journal of Biological Chemistry*, 280(5): 42841–42847.

YAHIA KHANDOKER M.A.M., TSUJII H., KARASAWA D. 1997: Fatty acid compositions of oocytes, follicular, oviductal and uterine fluids of pig and cow. *Asian-Australasian Journal of Animal Sciences*, 10(5): 523-527.

YANG X., DUNNING K.R., WU L.L.Y., HICKEY T.E., NORMAN R.J., RUSSELL D.L., LIANG X., ROBKER R.L. 2010: Identification of Perilipin-2 as a lipid droplet protein regulated in oocytes during maturation. *Reproduction, Fertility and Development*, 22(8): 1262–1271.

YANG H., GALEA A., SYTNYK V., CROSSLEY M. 2012: Controlling the size of lipid droplets: Lipid and protein factors. *Current Opinion in Cell Biology*, 24(4): 509–516.

YOUNGS C.R., KNIGHT T.J., BATT S.M., BEITZ D.C. 1994: Phospholipid, cholesterol, triacylgylcerol and fatty acid composition of porcine blastocysts. *Theriogenology*, 41343. (Abstract).

YU Y., DUMOLLARD R., ROSSBACH A., LAI F.A., SWANN K. 2010: Redistribution of mitochondria leads to bursts of ATP production during spontaneous mouse oocyte maturation. *Journal of Cellular Physiology*, 224(3): 672–680.

ZAMBONI L., MASTROIANNI L. Jr. 1966: Electron microscopic studies on rabbit ova. I. The follicular oocyte. *Journal of Ultrastructure Research*, 14: 95-117.

ZHANG R., FU X., JIA B., LIU C., CHENG K., ZHU S. 2014: Expression of perilipin 2 (PLIN2) in porcine oocytes during maturation. *Reproduction in Domestic Animals*, 49(5): 875–880.

ZHANG X., WU X.Q., LU S., GUO Y.L., MA X. 2006: Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles. *Cell Research*, 16(10): 841–850.

ZHOU G. B., LI N. 2009: Cryopreservation of porcine oocytes: Recent advances. *Molecular Human Reproduction*, 15(5): 279–285.

ZWEYTICK D., ATHENSTAEDT K., DAUM G. 2000: Intracellular lipid particles of eukaryotic cells. *Biochimica et Biophysica Acta - Reviews on Biomembranes*, 1469(2): 101–120.

8 LIST OF FIGURES

Figure 1. Metabolic units (%) in MF and SF oocytes before (0 h) and after maturation (44 h).The oocytes with greater meiotic competence from medium follicles (MF) and those less competent from small follicles (SF) were examined by confocal microscopy either after collection or maturation in standard conditions.

Figure 2. ATP contents (pmol) in oocytes derived from medium and small follicles before (0 h) and after maturation (44 h). The oocytes with greater meiotic competence from medium follicles (MF) and those less competent from small follicles (SF) were examined using bioluminiscent assay kit either after collection or maturation in standard conditions.

Figure 3a. Lipid droplets with adipose differentiation-related protein (ADRP) in MF and SF oocytes before (0h) and after maturation (44 h). The oocytes with greater meiotic competence from medium follicles (MF) and those with lesser competence from small follicles (SF) were examined by confocal microscopy either after collection or maturation in standard conditions.

Figure 3b. Expression of ADRP in MF and SF oocytes identified by Western blotting. The oocytes with greater meiotic competence from medium follicles (MF) and those with lesser competence from small follicles (SF) were examined only after collection. Porcine oocytes matured *in vivo*, ovarian tissue and boar spermatozoa were used as controls.

9 LIST OF TABLES

Table 1 Relative area of lipid droplets from GV to MII stage

Table 2 Changes of relative number of lipid droplets from GV to MII stage

Table 3 The number of small and large lipid droplets in the luteal and the follicular phase in oocytes derived from small and medium follicles before maturation

Table 4 The number of small and large lipid droplets in oocytes from small and medium follicles in the luteal and the follicular phase after maturation

Table 5 Ratio of small and large lipid droplets in oocytes in different stages of folliculogenesis before maturation

Table 6 Ratio of small and large lipid droplets in oocytes in different stages of folliculogenesis after maturation

Table 7 Characteristics of lipid parameters in oocytes derived from small and medium follicles regardless of the phase before maturation

Table 8 Characteristics of lipid parameters in oocytes derived from small and medium follicles regardless of the phase after maturation

10 LIST OF ABBERAVIATIONS

3T3-LI 1	Adipocytes cell line from mouse
ADP	Adenosine diphosphate
ADRP	Adipose differentiation-related protein
Ar	Argon laser
ATP	Adenosine triphosphate
BHK	Baby hamster kidney fibroblasts
BSA	Bovine serum albumin
Ca^{2+}	Calcium, ionized
cAMP	Cyclic adenosine monophosphate
CAMP-PDE	Cyclic adenosine monophosphate-phosphodiesterase
CENP-E	Centromere protein E
CHO	Chinese hamster ovary cells
COCs	Cumulus oocyte complexes
DENV	Dengue virus
DNA	Deoxyribonucleic acid
dpc	days post coitum
ER	Endoplasmic reticulum
ETC	Electron transfer chain
FADH2	Flavin adenine dinucleotide
FRET	Fluorescence resonance evergy transfer
FSH	Follicle stimulating hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotropin
HEK 293	Human Embryonic Kidney 293 cells
HeNe	A helium-neon laser
HepG2	Human hepato cellular liver carcinoma cell line
IFs	Intermediate filaments
IGF – 1	Insulin-like growth factor 1
IVM	*In vitro* maturation

IVP	*In vitro* embryo production
kbp	Kilobase pair
kDa	Kilodalton
LDs	Lipid droplets
LH	Luteinizing hormone
LSDP5	Lipid storage droplet protein 5
dpc	Days post coitum
MI	Metaphase I
MII	Metaphase II
MA-10	Tumor Leydig cells
MAP-K	Mitogen-activated protein kinase
mtDNA	Mitochondrial DNA
MEK/MAP kinase/$p90^{rsk}$	Methyl-ethyl ketone/Mitogen-activated protein kinase/p90 ribosomal S6 kinase
MF	Medium follicles
MLDP	Myocardial lipid droplet protein
MPF	Maturation promoting factor
mRNA	Mitochondrial RNA
mRNP	Message ribonucleoprotein
NADH + H^+	Nicotinamide-adenine dinucleotide, reduced nicotinamide-adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NBCS	Newborn calf serum
NIS-Elements	Nikon imaging software
OXPAT	Oxidative tissue-enriched PAT protein
PAT	Family of lipid droplet proteins
PBE	First polar body
PBS	Phosphate-buffered saline
pH	Pondus hydrogenii
pmol	Picomole
PLIN2	Perilipin 2
p.c.	Post coitum
PI 3-kinase	Phosphoinositide 3-kinase

PGCs	Primordial germ cells
PK-C	Protein kinase C
Plk1	Polo-like kinase 1
PPARα	Peroxisome proliferator-activated receptor α
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RER	Rough endoplasmic reticulum
ROS	Reactive oxygen species
S3-12	Adipocyte protein
SE	Sterol esters
SER	Smooth endoplasmic reticulum
SF	Small follicles
TAG	Triacylglycerols
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TIP47	Tail-interacting protein
tRNA	Transfer RNA

11 LIST OF ATTACHMENTS

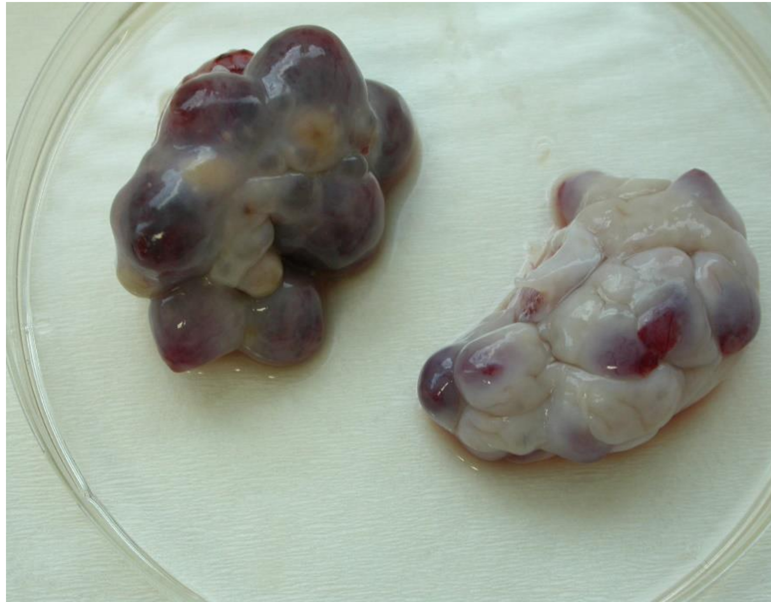


Figure 4 Representative image of the pig ovaries (Early luteal phase)

Figure 5 Representative image of the pig ovaries (Late luteal phase)

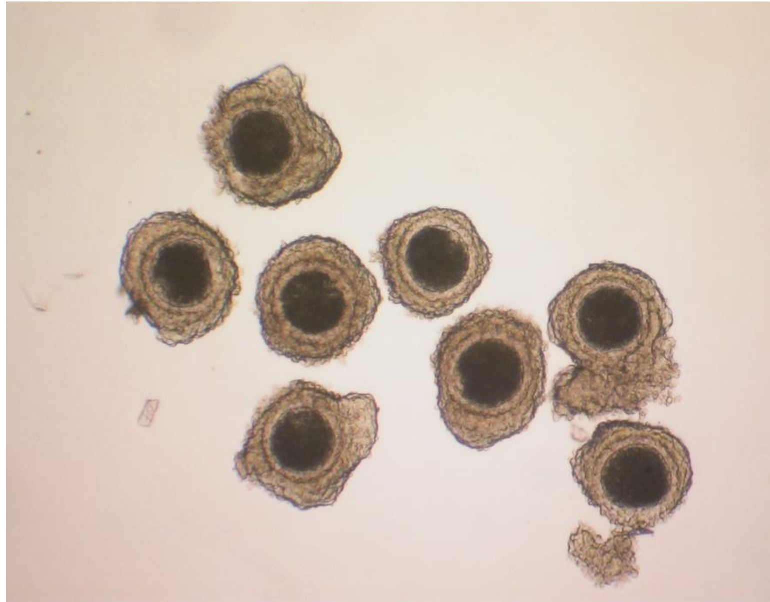


Figure 6 Oocytes after isolation derived from small follicles in the early luteal phase of the ovarian cycle (Native magnification 50x)

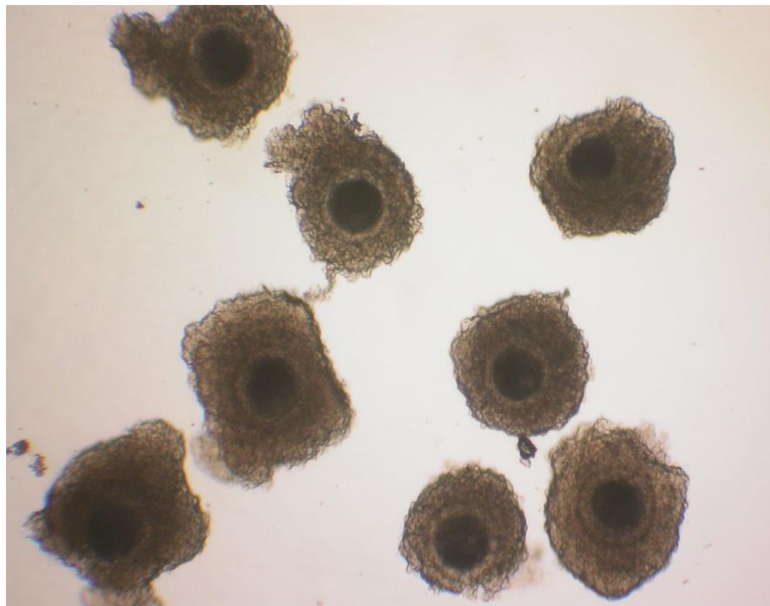


Figure 7 Oocytes after isolation derived from small follicles in the late luteal phase of the ovarian cycle (Native magnification 50x)

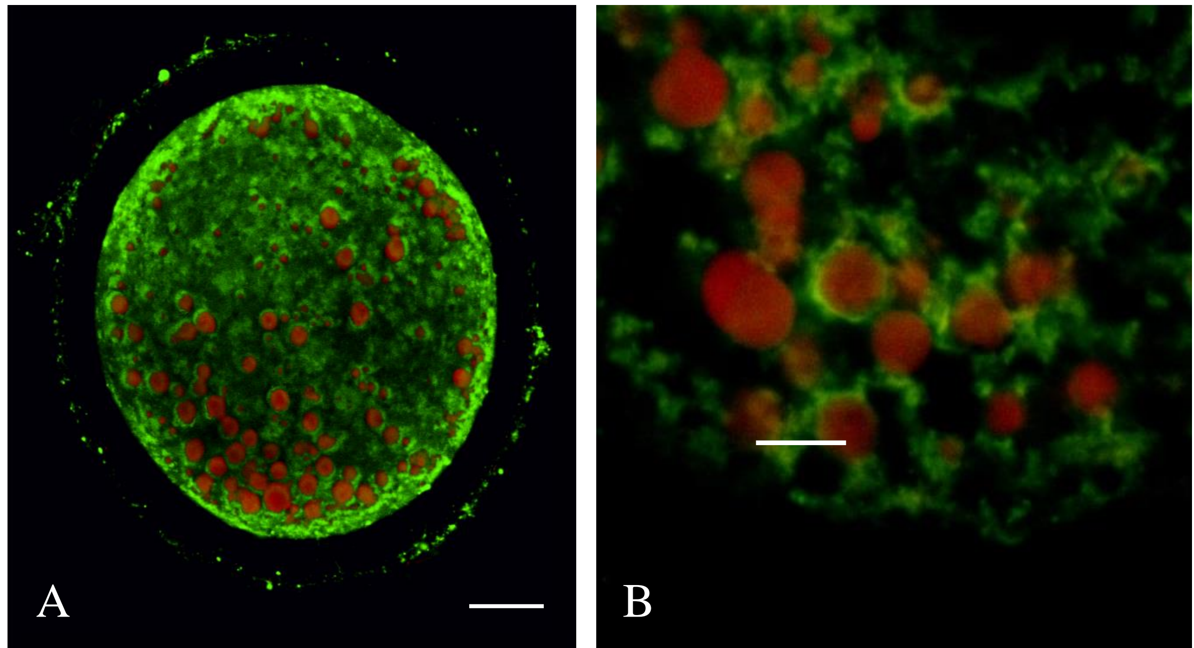


Figure 8 Representative images of mitochondria (green) and lipid droplet (red) localization (A), and mitochondrial-lipid droplet co-localization in metabolic units (B) visualized by dual-staining (MitoTracker Green and Nile red) of porcine oocytes. The scale bar represents 20 μm.

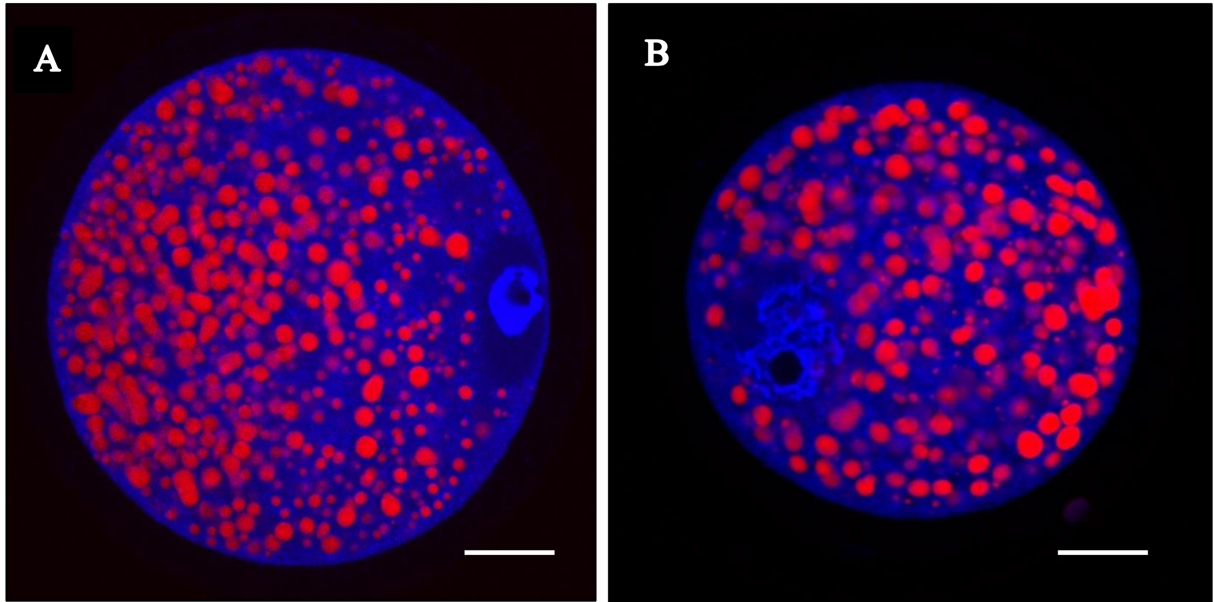


Figure 8 Representative images of lipid droplets (stained red) and chromatin (stained blue) localization in porcine oocytes after isolation (GV stage) visualized by dual staining (Nile red and TO-PRO-3). The scale bar represents 20 µm. (A and B).

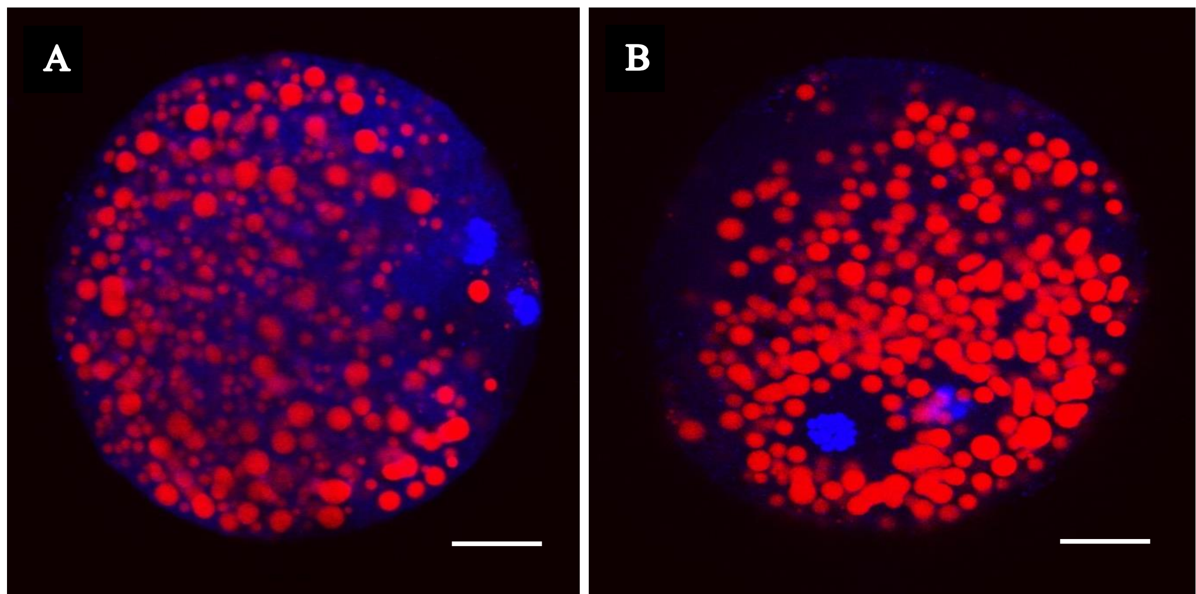


Figure 9 Representative images of lipid droplets (stained red) and chromatin (stained blue) localization in porcine oocytes after 44 h *in vitro* maturation (MII stage) visualized by dual staining (Nile red and TO-PRO-3). The scale bar represents 20 µm. (A and B).

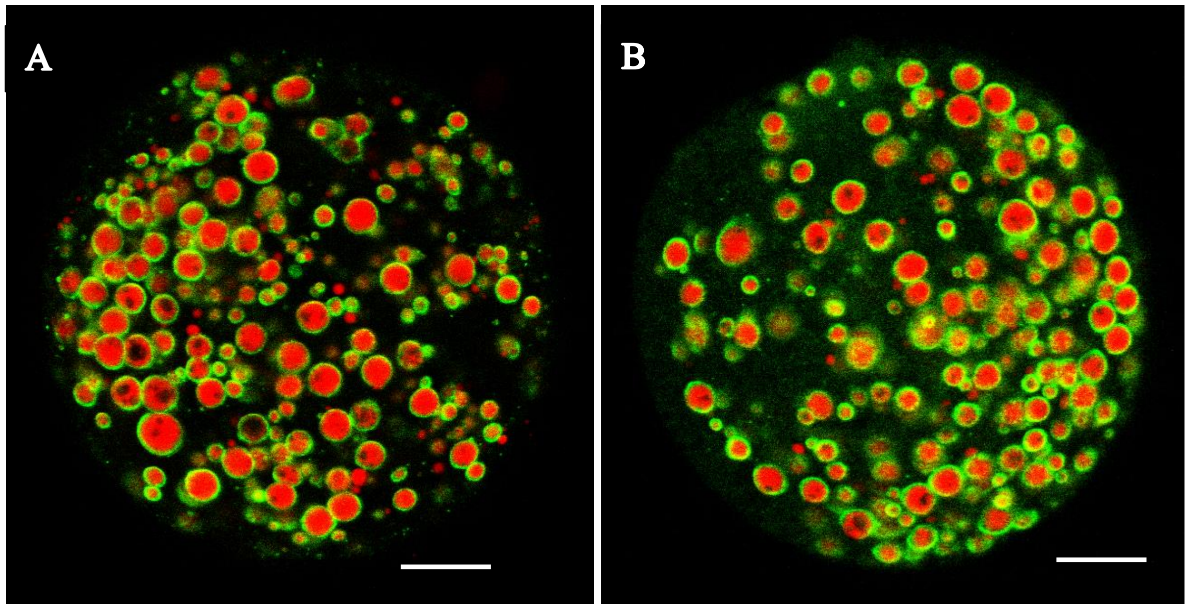


Figure 10 Representative images of lipid droplets (stained red, Nile red) associated with adipose differentiation-related protein (ADRP) (green, double immunostaining, conjugated by Cy5) in porcine oocytes after isolation (GV stage). The scale bar represents 20 µm. (A and B).