Ultrastructural cytoplasmic characteristics of domestic cat (*Felis catus*) oocytes according to ovarian status and *in vitro* maturation

Lílian Rigatto Martins¹, Rayf Roberto Tirloni¹, Fernanda da Cruz Landim-Alvarenga² and Maria Denise Lopes²

¹Animal Reproduction Laboratory, UFMT, University of Mato Grosso, Campus Sinop, Av. Alexandre Ferronato, 1200, 78556-266, Sinop-MT, Brazil
²Animal Reproduction and Veterinary Radiology Department, UNESP, University of the State of Sao Paulo “Júlio Mesquita Filho”, Distrito de Rubiao Jr., s/n, 18618-000, Botucatu-SP, Brazil

The present study describes ultrastructural cytoplasmic characteristics of non-matured oocytes (NM) and *in vitro* matured oocytes (IVM) recovered from queen ovaries of three distinct status - follicular, luteal or inactive – using transmission electronic microscopy (TEM). Oocytes harvested from ovaries of this species present some particular characteristics whose knowledge may be fundamental to improve some aspects of reproduction technologies. Cytoplasmic characteristics described includes mitochondrial distribution, lipid droplet size, Golgi complex development, cortical granules localization, the occurrence of gap junctions between cumulus cells and oocyte and the presence of microvilli. Our findings demonstrated that ultrastructural analysis of non-matured and *in vitro*-matured oocytes of three different ovarian status is a valuable tool to evaluate oocyte cytoplasmic maturation, an important pre-requisite to the success of *in vitro* maturation protocol.

**Keywords** ultrastructure; oocyte; cat

1. **Introduction**

The ability to grow and fertilize immature oocytes is useful for producing large numbers of embryos for developmental biology, cryopreservation and genetic studies, as well as for live animal production [1].

Nuclear maturation mainly involves chromosomal segregation, whereas cytoplasmic maturation involves organelle reorganization and storage of mRNAs, proteins and transcription factors that act in the overall maturation process, fertilization and early embryogenesis [2].

Domestic cat oocytes matured *in vitro* exhibit alterations in nuclear and cytoplasmic maturation that may affect developmental competence, particularly after cryopreservation [3].

Our understanding of such mechanisms is important for constantly improving therapy for human and animal reproductive disorders as well as for understanding the process of nuclear reprogramming during cloning procedure or stem cell generation [4].

Thus, ultrastructural analysis of oocytes can represent a potentially valuable tool for understanding physiological aspects of oocyte maturation demonstrated by a change in cytoplasmic organelle distribution and by the lack of contact between follicular cells and the oocyte [5].

2. **Materials and Methods**

All chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise mentioned.

2.1 Oocyte collection and selection

Ovaries were recovered after ovariohysterectomy performed at a local veterinary clinic and transported in Dulbecco phosphate buffered saline (DPBS; Nutricell®, Brazil) containing 1% antibiotic-antimicotic solution at 4°C using ice packs in a thermal box. Ovaries were processed for gamete collection within 4 hours of gonadectomy. Ovaries were dissected and then sliced into a plastic Petri dish (60 x 15 mm, TPP®, Brazil) containing 5 ml de DPBS (Nutricell®, Brazil) at 38°C using a scalpel blade to release *cumulus oophorus* complexes (COC). Under stereomicroscope (MZ 125, Leica®, Germany) COC were selected and classified in grade I, II and III. To perform this experiment, only COC grade I, defined by a uniform, dark cytoplasm surrounded by at least five layers of cumulus was selected. Grade I COC were washed three times in Hepes-buffered Minimum Essential Medium (H-MEM; Gibco®, USA) supplemented with 3 mg/ml bovine serum albumin (BSA), 2.0 mM glutamine, 1.0 mM pyruvate, 1.2 mM cysteine, 100 mg/ml streptomycin and 100 UI/ml penicillin (Gibco®, USA). Non-matured oocytes were stored in glutaraldehyde at 4°C until the performance of transmission electronic microscopy (TEM).
2.2 Oocyte in vitro maturation

Cumulus oophorus complexes from in vitro matured groups (36 hours) were cultured before TEM. Those selected for maturation were incubated (20 to 30/400µl) in a four-well dish (Nunc®, Denmark or Ingámed®, Brazil), containing 400µL DMEM (Dulbecco’s Modified Essential Medium, Gibco®, USA) supplemented with 3 mg/ml BSA, 2.0 mM glutamine, 1.0 mM pyruvate, 1.2 mM cysteine, 100 mg/ml streptomycin and 100 UI/ml of penicillin (Gibco®, USA), 10µg/mL bovine FSH (Folltropin®-V, Bioniche® Animal Health, Canada), 1µg/mL LH (Lutropin®-V, Bioniche® Animal Health, Canada), 1µg/ml estradiol, 20 ng/ml insulin-like growth factor I (IGF-I) e 10 ng/ml basic fibroblast growth factor (bFGF) for 12 and 24 hours at 38°C in humified environment of 5% de O₂, 5% CO₂ e 90% N₂. After the maturation time, oocytes were stored in glutaraldehyde at 4°C until TEM performance. Specimens were divided into six groups: non-matured oocytes from inactive ovaries (NMI); in vitro-matured oocytes from inactive ovaries (IVMI); non-matured oocytes from luteal ovaries (NML); in vitro-matured oocytes from luteal ovaries (IVML); non-matured oocytes from inactive ovaries (NMI); in vitro-matured oocytes from inactive ovaries (IVMI); non-matured oocytes from follicular ovaries (NMF) and in vitro-matured oocytes from follicular ovaries (IVMF).

a. Assessment of cytoplasmic maturation

Cytoplasmic maturation of the oocytes was assessed by transmission electron microscopy. COC were randomly collected immediately after slicing the ovaries and they were not matured. In vitro-matured oocytes were cultured for 36 hours and then analyzed. The distribution of cortical granules and mitochondria, and the occurrence of gap junctions between cumulus cells and the oocyte, were used to estimate oocyte maturity. The ultrastructural analysis was done at Electron Microscopy Center of Biological Institute at UNESP, Botucatu. Six oocytes per replicate for each group were randomly selected before and after in vitro maturation. The oocytes were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, for 24 h and then post-fixed in 1% osmium tetroxide in the same buffer. After dehydration in an increasing series of acetone, the oocytes were included in Epon. Semithin sections (1-2 m thick) were stained with toluidine blue to select those showing oocyte. Ultrathin sections were obtained with a diamond knife, mounted on cooper grids and stained with uranyl acetate and lead citrate. The samples were examined with a Philips CM 100 transmission electron microscope.

3. Results

NMI and NML oocytes presented mitochondrial clusters in the cortical zone of the ooplasm whereas, but greater clusters could also be observed in other regions of the cytoplasm. The peripheral region occupied by these clusters was narrower in NML than in NMI and NMF. IVMI mitochondrias were distributed in the ooplasm; lipid droplets and vesicles were evenly distributed in the ooplasm except for the cortical zone and cortical granules were present only in the peripheral area of the cytoplasm (Fig. 1).

NMI and NMF perivitelline spaces were not well developed and their surface contained many erect microvilli extending into the zona pellucida (Fig. 2).

NMF were characterized by evenly distributed mitochondria within the ooplasm. Lipid droplets were smaller than those observed in NMI and NML (Fig. 3).

The density of cortical granules in NMF was higher in the peripheral zone, although they could also be observed in other regions of the ooplasm (Fig. 4a). Cortical granules were present only in the peripheral area of the cytoplasm of NML and their density was smaller compared to NMI and NMF (Fig. 4b).

NML microvilli were not so developed as in NMI and NMF. Cumulus cell projections penetrated ZP and formed gap junctions (GJ) with the oolemma and well developed Golgi complexes were observed peripherally in NMF, NML and NMI (Fig. 5). The number of microvilli of IVMI was similar to NMI. This finding could be applied to IVML and NML.

Mitochondrial clusters of IVM oocytes were no more observed; they migrated centrally and cortical granules were present in small number in the peripheral region of IVMI, IVMF and IVML, although they could be observed in central region of the ooplasm of IVMF. The perivitelline space was smaller in NM oocytes than in IVM oocytes (Fig. 6).

Lipid droplets and vesicles of IVMI were not present at the peripheral region of the ooplasm as it could be observed in IVMF and IVML. IVMF presented numerous small vesicles in the cortical region and presented the largest perivitelline space compared to IVMI and IVML. Microvilli and granulosa cell projections were almost absent and gap junctions were not observed.

IVM oocytes presented Golgi complexes that were observed peripherally, but they were not so developed as in NM oocytes (Fig. 7).

A structure called nuage, represented by an electrondense material of nuclear origin surrounded by mitochondrias could be observed in a non-matured oocyte from an inactive ovary (Fig. 8).
Fig. 1 Electron micrograph showing morphological aspects of a non-matured (a) and from an *in vitro*-matured oocyte (b) from an inactive ovary of domestic cat (NMI). Mitochondrial clusters may be observed in the cortical zone of the ooplasm (a) whereas IVMI mitochondrias were distributed in the ooplasm (b). Magnification 3306 x (a) and 2470 x (b).

Fig. 2 Electron micrograph showing morphological aspects of non-matured oocytes from an inactive (a) and a follicular (b) ovary of domestic cat (NMI and NMF). NMI and NMF perivitelline spaces (PVS) were not well developed and their surface contained many erect microvilli (MV) extending into the zona pellucida (ZP). Magnification 10070 x (a) and 7410 x (b).

Fig. 3 Electron micrograph showing morphological aspects of non-matured oocytes from a follicular (a) and a luteal (b) ovary of domestic cat (NMI and NMF). NMF (a) were characterized by evenly distributed mitochondria within the ooplasm and lipid droplets were smaller than those observed in NML. Magnification 3306 x (a) and 2470 x (b).
**Fig. 4** Electron micrograph showing morphological aspects of non-matured oocytes from a follicular (a) and luteal (b) ovary of domestic cat (NMF and NML). Observe that the density of cortical granules in NMF is higher in the peripheral zone, although they could also be observed in other regions of the ooplasm (a). Cortical granules were present only in the peripheral area of the cytoplasm of NML and their density was smaller compared to NMF (b). Magnification 1862 x (a) and 17480 x (b).

**Fig. 5** Electron micrograph showing morphological aspects of non-matured oocytes from a follicular (a), luteal (b) and inactive (c) ovary of domestic cat (NMF, NML and NMI). Cumulus cell projections penetrated ZP and formed gap junctions (GJ) with the oolemma. Magnification 29400x (a), 31920x (b) and 43700x (c).
Fig. 6 Electron micrograph showing morphological aspects of in-vitro matured oocytes from a follicular (a), a luteal (b) and an inactive (c) ovary of domestic cat (IVMF, IVMI and IVML). Mitochondrial clusters of IVM oocytes were no more observed; they migrated centrally and cortical granules were present in small number in the peripheral region of IVMI, IVMF and IVML, although they could be observed in central region of the ooplasm of IVMF. Magnification 2470 x (a), 2470 x (b) and 3306 x (c).

Fig. 7 Electron micrograph showing morphological aspects of non-matured oocyte from an inactive ovary (a) and in vitro-matured oocyte from a luteal (b) ovary of domestic cat (NMI and IVML). Golgi complexes that were observed peripherally in IVML, but they were not so developed as in NMI oocytes. Magnification 23940 x (a) and 58900 x (b).
4. Discussion

Non-matured oocytes presented clusters of mitochondria in the cortical area, cortical granules in the cortical region and well-developed Golgi Complex; these are classical signs of cytoplasmic immaturity. In mammalian oocytes, migration of cortical granules has been used as an important criterion to evaluate cytoplasmic maturation [6]. Additionally, ultrastructural analysis shows that mitochondria, ribosomes, endoplasmic reticulum, cortical granules and the Golgi complex assume different positions during the transition from the germinal vesicle stage to metaphase II [2]. This information reinforces our knowledge that the establishment of a well-controlled environment during in vitro maturation is a relevant step during the whole process of in vitro embryo production, once it is known that, in domestic cat, there is a population of approximately 65% of atretic follicles [7].

Also, non-matured oocytes from inactive and follicular ovaries presented on their surface many erect microvilli extending into the zona pellucida. These microvilli were less evident in vitro matured oocytes from luteal, inactive and follicular ovaries. The presence of microvilli is also an indicative of immaturity [8].

Lipid droplets were smaller in non-matured oocytes from follicular ovaries compared to those observed in non-matured oocytes from luteal and inactive ovaries although in the three situations the lipid droplets were closely associated with mitochondrial clusters. This association was not observed in vitro oocytes from three different ovary status, suggesting that there was no longer any lipid metabolization when oocytes reaches metaphase II [9]. Considering this characteristic isolated, we can suggest that non-matured oocytes from follicular ovaries are closer to a maturity state than non-matured oocytes from luteal and inactive ovaries.

In non-matured oocytes, cortical granules were seen only in the peripheral area of the cytoplasm, although, in non-matured oocytes from follicular ovaries they could be observed in other regions of the oolemma. In vitro-matured oocytes presented cortical granules evenly distributed in the cytoplasm, independently of the ovarian status. Some authors observed that as the maturation of the oocyte proceeds, there is progressive centripetal migration of the cortical granules from the periphery [6].

Gap junctions formations were seen in non-matured oocytes from three distinct ovarian status as result of corona radiate cells projections that crossed zona pellucida. This connection indicates the existence of an intimate connection between the oocyte and the corona radiate cells [10], and its termination is apparently important for the metabolic changes characteristic of the final maturation and the preparation of the oocyte for the fertilization [11]. Thus, gap junctions were not observed in vitro-matured oocytes, as its presence is no more necessary.

Golgi complexes were seen at the periphery of the cytoplasm of non-matured and in vitro-matured oocytes from three ovarian status, although in immature oocytes, the Golgi complexes were more developed than in vitro-matured oocytes suggesting a continuous process of metabolization which decreases as maturation is achieved.
An electrondense material of nuclear origin which aggregates mitochondrias is called *nuage* and it was observed in an electron micrograph of a non-matured oocyte from an inactive ovary (NMI). This material is found in gametes of many vertebrates [12]. In the primary growth stage, material is frequently found in the oocytes and it seems to be transferred in its entirety to the cytoplasm [13]. Later, it associates with mitochondria and disperses in the cytoplasm at the beginning of vitellogenesis [14]. Its appearance in a non-matured oocyte from an inactive ovary is consistent with those described in the literature for early-developed oocytes.

In summary, we conclude that ultrastructural analysis of oocytes matured *in vitro* is an important instrument for estimating oocyte cytoplasmatic maturation. Our results also indicate that this IVM is efficient in inducing some gradual morphological changes necessary for cytoplasmatic maturation of cat oocytes.

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**References**


