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Golgi Apparatus and Endoplasmic Reticulum Dynamic during Meiotic Development in Canine Oocytes

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Content
The Golgi apparatus (GA) and endoplasmic reticulum (ER) play a central role in the events related to intracellular trafficking distribution. This work evaluated the dynamics and localization of the GA and ER in canine oocytes during meiotic development *in vitro*. Cumulus–oocytes complexes (COCs) from ovaries of adult bitches were incubated for IVM for 0, 48, 72 and 96 h. At each time, the nuclear status was determined using DAPI staining, and the GA was evaluated by immunofluorescence using two antibodies against Golgi proteins: GM130 and Giantin. ER was analysed with fluorescent lipid probes (ER-Tracker), for living cells. Golgi structures were homogeneous in the cytoplasm in non-matured oocytes, mainly in those GV-arrested oocytes. In contrast, at 48 h and from GVBD stage, the immunolocalization began to be subcortical, increasing at 72 h and 96 h. Meiotic development increased with time and the majority of oocytes at MI-MII stages showed cortical distribution of Golgi structure. Living ZP intact non-matured oocytes showed a reticular pattern of ER that covered oocyte cortex. Confocal microscopy showed that, in all levels cuts the fluorescence marks were located in the cortical region, irrespective of culture time. The changes and localization in these organelles during IVM might be related to meiotic development, but in a non-synchronous manner.

Introduction

Important changes occur in oocytes during maturation, as a consequence of endocrine, paracrine and autocrine stimulation. In dogs, these changes take place under preovulatory and post-ovulatory conditions, because maturation of dog oocytes occurs at both follicular and oviductal environments (Reynaud et al. 2005; De los Reyes et al. 2011). The processes conducting these changes are those involved in the resumption of meiosis to the state of second metaphase and those in the cytoplasm that promote fertilization and render the oocyte capable of supporting the development (Eppig 1996). These processes are referred to as nuclear and cytoplasmic maturation, respectively. In dog oocytes, the cytoplasmic process begins *in vivo* before ovulation (de Lesegno et al. 2008), while meiotic maturation is resumed in the oviduct reaching MII stage approximately, 50–70 h post-ovulation (Reynaud et al. 2005).

Cytoplasmic maturation is still unclear and involves both morphological and functional events. The changes in morphology and redistribution observed in cytoplasmic organelles during oocyte maturation are expected to be associated with the timing of nuclear maturation. The cytoplasmic movements and rearrangements of components within the cytoplasm include dynamic changes in the distribution and integrity of the Golgi apparatus (GA) and endoplasmic reticulum (ER) (Vinke et al. 2011). This GA-ER system plays an essential role in intracellular signalling and trafficking events distribution through a complex multi-step process, regulated by fusion and fission events (Moreno et al. 2002; Racedo et al. 2012). These trafficking events include a flux of newly synthesized proteins and lipids coming from the ER to the plasma membrane and the extracellular medium, as well as the synthesis of many lipid species (Vinke et al. 2011). Evidence suggests that the redistribution of the ER is important in preparing the oocyte for the generation of repetitive calcium transients, which trigger oocyte activation at fertilization (Harris et al. 2007). Data regarding the role of the Golgi complex during oocyte maturation and the subsequent events still remain controversial. The dynamics of Golgi membranes during meiosis in mammals are not entirely known. Further, these processes remain largely unexplained in dogs, and the distribution and modification of these vesicles in relation to meiotic resumption and their importance for canine oocyte maturation have not been fully examined. Thus, the aim of this work was to evaluate the dynamics and localization of the GA and ER in dog oocytes during meiotic development *in vitro*, using both GM130 and Giantin, Golgi membrane proteins as a Golgi markers and a vital labelling for ER detection.

Material and Methods

This study followed the guidelines for ethical conduct in the care and use of animals instituted by the Bioethics Commission of FONDECYT (National Foundation for Sciences and Technology, Chile).

Unless specified, reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Oocyte recovery and *in vitro* maturation

Ovaries were collected after ovariohysterectomy from adult healthy bitches at different stages of the oestrous cycle and transported to the laboratory in 0.9% (w/v) NaCl containing 100 IU/ml penicillin and 50 μg/ml streptomycin at 37°C. Within 2 h of surgery, the follicular oocytes were recovered by slicing the ovaries. Only oocytes possessing a compact cumulus mass and evenly granulated cytoplasm were selected for the experiments, as indicated in previous reports (De los Reyes et al. 2010). The oocyte–cumulus complexes (COCs) were washed twice with PBS and then incubated for IVM in the maturation medium TCM-199 (Earle's

salt, buffered with 25 mM Hepes; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS), 0.25 mM pyruvate, 10 IU/ml of hCG, 100 IU/ml penicillin, and 30 μg/ml streptomycin, at 38°C, 5% CO_2 in air and maximum humidity for 0, 48, 72 and 96 h (De los Reyes et al. 2011).

Immunofluorescence for Golgi proteins

After each time of culture, COCs were denuded from cumulus cells by passage through a narrow glass pipette and the zona pellucida was removed by a short incubation in acid Tyrode's medium with 0.1% pronase. Denuded oocytes were washed in buffer containing 1% Triton X-100 for 10 min and then rinsed with 2% BSA in PBS (pH 8.2) for 1 h at room temperature (22°C). Oocytes were then randomly allocated into two groups; one was assessed for immunofluorescence using a purified mouse anti-GM130 monoclonal antibody at 1:100 dilution (BD Transduction Laboratories, Becton Drive, Franklin Lakes, NJ, USA), and the other group with rabbit anti-Giantin antibody at 1:100 dilution (Scripps Institute, La Jolla, CA, USA). Both oocyte groups were incubated overnight at 4°C and then washed with 0.1% PBS-Tween 20 and incubated separately for 1 h at room temperature with the appropriate secondary antibodies: 1:1000 of Goat polyclonal anti-mouse antibody conjugated to Alexa Fluor® 488 (Molecular Probes, Eugene, OR, USA) that specifically recognizes GM130 or using 1:1000 Goat anti-rabbit antibody conjugated to Alexa Fluor® 488 (Molecular Probes), for Giantin group.

Endoplasmic reticulum detection

To imaging live bitch oocyte, we used the fluorescent lipid probes for living cells maintained ER-Tracker™ Green (BODIPY® FL Glibenclamide, Invitrogen) probe. ER-Tracker was prepared in a stock solution at 1 mM in DMSO and stored at 4°C until use. The working solution was prepared at 100 nM in TCM 199. After each culture period, live intact oocytes were incubated with the probe ER-Tracker for 2 h at room temperature, and then, they were washed three times. The oocytes were then mounted in medium alone and visualized in a Leica TCS-NT confocal microscope (Bensheim, Germany).

Statistical analysis

Relative distribution of endoplasmic reticulum and Golgi patterns in each group as well as oocyte nuclear maturation rates was analysed by chi-square (χ^2) test. For all analyses, $p \leq 0.05$ was considered significant.

Results

Distribution of Golgi apparatus during IVM of canine oocytes

A total of 381 oocytes throughout four experimental repetitions were evaluated for GA analysis. The distribution of GA in relation to meiotic progression is presented in Tables 1 (anti-GM130 Ab) and 2 (anti-Giantin Ab).

Table 1. Golgi apparatus distribution (%) detected by anti-GM130 polyclonal antibody in canine oocytes during IVM at different culture periods and meiotic stages

Oocyte maturing groups	Golgi distribution	GV n = 35	GVBD n = 72	MI n = 101	MII n = 19	Total n = 227
No IVM	H	44.1^{aA}	25.4^{bA}	15.3^{cA}	–	84.7
n = 59	C	5.1	5.1^{X}	5.1^{X}	–	15.3
IVM 48	H	8.8^{aB}	8.8^{aB}	14.7^{bA}	–	32.4
n = 68	C	–	27.9^{aY}	33.8^{aY}	5.9^{bX}	67.6
IVM 72	H	–	6.1^{aC}	16.3^{bA}	–	22.4
n = 49	C	–	22.4^{aYZ}	38.8^{bY}	16.3^{cY}	77.6
IVM 96	H	–	9.8^{C}	9.8^{B}	7.8	27.4
n = 51	C	–	19.6^{aZ}	47.1^{bZ}	5.9^{cX}	72.6

Golgi apparatus distribution: H, homogeneous throughout the cytoplasm; C, cortical. Nuclear stages: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, first metaphase; MII, second metaphase.
[a–c]Within a row and Golgi distribution, numbers without a common superscript differed ($p < 0.05$).
[A–C]Within a column and homogeneous distribution, numbers without a common superscript differed ($p < 0.05$).
[X–Z]Within a column and cortical distribution, numbers without a common superscript differed ($p < 0.05$).

In non-matured oocytes, Golgi structures, as visualized using the Giantin and GM130 Ab, were homogeneous in the cytoplasm (Fig. 1a). This distribution was mainly observed in GV-arrested oocytes. In contrast, during the initial 48 h of IVM, the immunolocalization from GVBD stage began to be subcortical (Fig. 1b). This cortical distribution increased throughout time using GM130 and Giantin Ab.

At the GVBD stage, the Golgi fragments spread more evenly throughout the oocyte, labelling for both GM130 and Giantin, but were mainly in the cortex when using GM130 antibody. At 48 h, the majority of oocytes at MI-MII stages showed cortical distribution of Golgi structure, and as meiotic maturation progressed, during 72–96 h, GM130 and Giantin staining in the oocyte was progressively observed in cortical distribution.

Distribution of the endoplasmic reticulum during IVM of canine oocytes

The ER-Tracker probe is highly sensitive and specific for the endoplasmic reticulum and it allowed us imaging *in vivo* ER after each culture time of oocyte maturation without removing the zona pellucida. Therefore, in 87 living ZP intact oocytes, we were able to visualize the ER. Results from confocal microscopy showed that in all sections, the fluorescence marks were observed only in the cortical region of the oocyte and did not penetrate into the interior (Fig. 2).

Non-matured oocytes showed a reticular pattern that apparently covered the entire oocyte cortex (observed in 14/16). At GV stage, the ER was arranged as a network of small accumulations in the cortex. In the high magnification photomicrographs, it is possible to observe that this network is not homogeneous, but is

Table 2. Golgi apparatus distribution (%) detected by anti-Giantin monoclonal antibody in canine oocytes during IVM at different culture periods and meiotic stages

Oocytes	Golgi apparatus distribution	GV n = 32	GVBD n = 44	MI n = 69	MII n = 9	Total n = 154 (%)
matNo IVM	H	54^{aA}	32^{bA}	6^{cA}	–	92
n = 50	C	–	8^{X}	–	–	8
IVM 48	H	13.9^{aB}	19.4^{bB}	22.2^{bB}	–	55.6
n = 36	C	–	19.4^{Y}	25^{X}	–	44.4
IVM 72	H	–	10^{aC}	28^{bB}	6^{a}	44
n = 50	C	–	6^{aX}	38^{bY}	12^{c}	56
IVM 96	H	–	–	11.1^{A}	–	11.1
n = 18	C	–	11.1^{aX}	77.7^{bZ}	–	88.9

Golgi apparatus distribution: H, homogeneous throughout the cytoplasm; C, cortical. Nuclear stages: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, first metaphase; MII, second metaphase.
$^{a–c}$Within a row and Golgi distribution, numbers with differing superscripts differed ($p < 0.05$).
$^{A–C}$Within a column and homogeneous distribution, numbers without a common superscript differed ($p < 0.05$).
$^{X–Z}$Within a column and cortical distribution, numbers without a common superscript differed ($p < 0.05$).

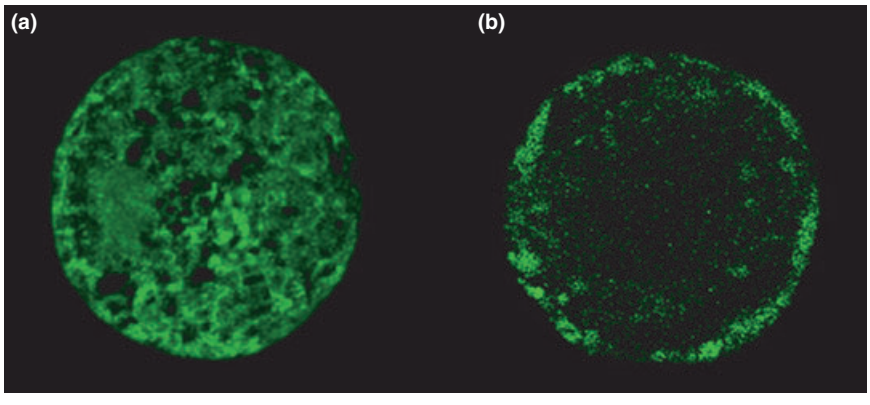

Fig. 1. Imaging of the Golgi apparatus in *in vitro* matured bitch oocytes. The figures show non-matured oocyte (a) with homogeneous distribution and cultured for 96 h with cortical distribution pattern (b). Oocytes were fixed in paraformaldehyde, and then, the Golgi apparatus was visualized using an antibody again GM130 (×200).

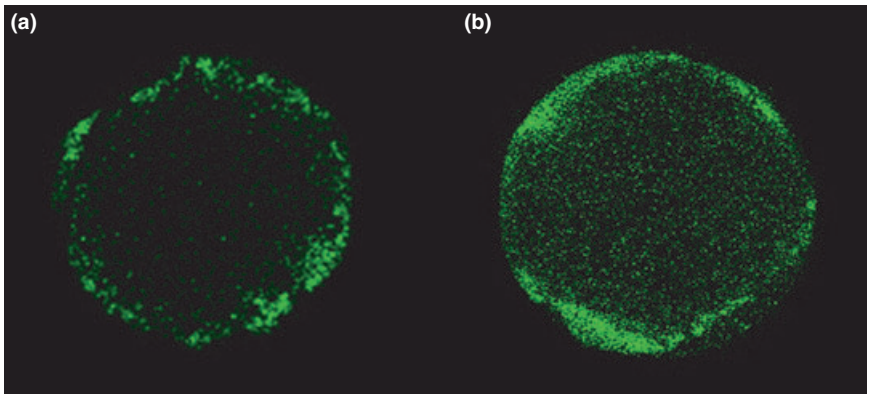

Fig. 2. Confocal microscopy image of the endoplasmic reticulum in immature bitch oocytes by ER-Tracker. The figures show non-matured bitch oocyte (a) and *in vitro* matured oocyte (72 h) (b). Most of the labels were cortical (×200).

characterized by an ER cluster near the surface of the oocyte (Fig. 2a). At 48 h (22/25), the ER extends in a more reticular manner following GVBD stage.

In the oocytes matured for 72 and 96 h (Fig. 2b), we observed this cortical localization of the ER, as assessed by the probe ER-Tracker (this distribution was observed in 26/28 and 14/18, for oocytes matured for 72 and 96 h, respectively). The results showed that, in general, the pattern of distribution of the endoplasmic reticulum is maintained after 72 and 96 h of IVM. Fluorescence marks were still cortical, but more diffuse than in the non-mature GV oocyte.

Discussion

Many changes in the number, morphology and distribution of cytoplasmic organelles occur during oocyte growth; here, we showed that during *in vitro* maturation of canine oocytes, ER and the GA are developed and have specific positions that can be visualized dynamically by using ER-Tracker and by immunofluorescence with antibodies against the Golgi network proteins as GM130 and Giantin.

The reorganization of calcium stores and the sensitivity of the oocyte to its release increased during

maturation (Zhang et al. 2010), and the pattern of proteins synthesized by oocytes during maturation undergoes changes related to the dynamics of these organelles. Immunofluorescence detection of the Golgi apparatus was consistent with previous reports of ultrastructural characteristics of canine oocytes assessed by transmission electron microscopy (TEM) (de Lesegno et al. 2008). The immunolabelling pattern of Golgi detected with both, GA130 and Giantin antibodies, was related with the duration of the period to which dog oocytes were incubated; the majority of non-matured oocytes showed GM130 dispersed throughout the cytoplasm. This pattern observed mainly in GV-arrested oocytes was similar to the Golgi distribution observed with Giantin Ab and also to those Golgi structures previously detected and characterized in rhesus monkey GV oocytes (Moreno et al. 2002). Although the functional significance of this localization is not completely clear, it appears to be an important part of the secretory pathway necessary for overall metabolism, because mitochondria also displayed the same distribution in GV canine oocytes (De los Reyes et al. 2011).

During culture, from GVBD stage, we found a fundamental change in the Golgi distribution, which later dispersed and migrated to the oocyte periphery. These changes are possibly essential for preparing normal fertilization and embryonic development, because interestingly, this localization is related to cortical granule migration. This suggests that Golgi proteins may also associate with cortical granules, membrane-bound organelles produced by the participation of both, the Golgi complex and the rough ER (Selman and Anderson 1975). The major cortical Golgi distribution found here with either GM130 or Giantin antibodies after GVBD agrees well with our previous results in bitch oocytes that show, by labelling with *Lens culinaris agglutinin* – FITC-conjugated staining, migration of cortical granules during IVM close to the cell surface at 72 h or 96 h of culture, mainly in MI-MII stages (De los Reyes et al. 2010). In the same way, analyses of canine oocytes by transmission electron microscopy have shown that at LH surge, Golgi bodies are present in many layers, which concentrated around trans zona pellucida projections with cortical granules that increase after ovulation (de Lesegno et al. 2008). The dispersion, and reorganization of the Golgi apparatus observed here during incubation between GV and MI-MII stages and the close relationship with ER, raises the possibility of a reorganization of calcium stores prior to oocyte activation and cortical granule exocytosis.

Although this cytoplasmic maturation, evaluated by GA reorganization, could occur apparently successfully during culture, the acquisition of meiotic maturation was much less efficient than Golgi changes, because a low percentage of oocytes reached the MII stage. The apparent capacity of some GVBD-arrested oocytes to develop Golgi movements in the absence of complete nuclear maturation suggests that *in vitro,* some structural feature of cytoplasmic maturation can be uncoupled from the nuclear status. However, aggregation of organelles during maturation might be initiated at GVBD stage, as a result of the mixing of the nucleus contents with the cytoplasm.

The organization of the ER structure is also of particular interest as this organelle releases calcium at fertilization mediating oocyte activation, and this ability to release calcium develops during oocyte maturation (Shiraishi et al. 1995; Eppig 1996). Previous works using different probes such as antibodies against IP3 receptors, calreticulin (Zhang et al. 2010) and also ultrastructral organelle analysis (de Lesegno et al. 2008) have shown that the ER is uniformly distributed throughout the cytoplasm in GV oocyte. Here, we used a new methodology with the ER probe, a cell permeable compound that binds to the sulphonylurea receptors of ATP-sensitive K^+ channels, which are prominent on ER. In the course of our investigation, we identified the ER surrounding the cortex irrespective of culture time or meiotic configuration, because this probe labelled only a cortical network of non-matured oocytes and those incubated for up to 96 h for IVF. This difference in results could be due to the fact that bitch oocytes do not expressed high levels of sulphonylurea receptors ATP-sensitive K^+ channels, which are important in the probe uptake. In addition, because this method is based upon the uptake of the probe and then its incorporation into the ER, it is possible that these oocytes may have low or complete absence of retrograde flow, which could be reflected in the labelling of the cortical area and not in the whole ER. This suggests a deficiency in the *in vitro* conditions or in the canine oocyte itself. Studies with oocytes matured *in vivo* may help to clarify these aspects of cytoplasmic maturation found here.

In conclusion, the dynamic changes of GA and localization of ER are observed during IVM of canine oocyte, which are related to meiotic development; however, it is clear that during IVM, cytoplasmic maturation and nuclear maturation occur in a non-synchronous manner.

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Conflicts of interest

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

Author contributions

MDLR designed the study, supervised the experiments and drafted the manuscript; SJ and AV collected ovaries and processed the oocytes sampled; JP participated in preparation of antibodies against GA and performed the statistical analysis: RM involved in microscopy protocols and analysed the data. All authors read, revised and approved the final manuscript.

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