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Mitochondrial distribution and meiotic progression in canine oocytes during *in vivo* and *in vitro* maturation

M. De los Reyes[a,*], J. Palomino[a], V.H. Parraguez[a], M. Hidalgo[b], P. Saffie[a]

[a] *Laboratory of Animal Reproduction, Faculty of Veterinary Sciences, University of Chile*
[b] *Reproduction Unit, Faculty of Veterinary, University of Cordoba, Spain, Casilla 2 Correo 15, Santiago, Chile*

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Abstract

The objective was to evaluate mitochondrial distribution, and its relationship to meiotic development, in canine oocytes during *in vitro* maturation (IVM) at 48, 72, and 96 h, compared to those that were non-matured or *in vivo* matured (ovulated). The distribution of active mitochondria during canine oocyte maturation (both *in vitro* and *in vivo*) was assessed with fluorescence and confocal microscopy using MitoTracker Red (MT-Red), whereas chromatin configuration was concurrently evaluated with fluorescence microscopy and DAPI staining. During IVM, oocytes exhibited changes in mitochondrial organization, ranging from a fine uniform distribution (pattern A), to increasing clustering spread throughout the cytoplasm (pattern B), and to a more perinuclear and cortical distribution (pattern C). Pattern A was mainly observed in germinal vesicle (GV) oocytes (96.4%), primarily in the non-matured group ($P < 0.05$). Pattern B was seen in all ovulated oocytes which were fully in second metaphase (MII), whereas in IVM oocytes, ~64% were pattern B, irrespective of duration of culture or stage of nuclear development ($P > 0.05$). Pattern C was detected in a minor percentage ($P < 0.05$) of oocytes (mainly those in first metaphase, MI) cultured for 72 or 96 h. *In vitro* matured oocytes had a minor percentage of pattern B ($P < 0.05$) and smaller mitochondrial clusters in IVM oocytes than ovulated oocytes, reaching only 4, 11, and 17% of MII at 48, 72, and 96 h, respectively. Thus, although IVM canine oocytes rearranged mitochondria, which could be related to nuclear maturation, they did not consistently proceed to MII, perhaps due to incomplete IVM, confirming that oocytes matured *in vitro* were less likely to be competent than those matured *in vivo*.

Keywords: Mitochondria; Meiotic development; Canine oocyte; *In vitro* maturation; Dog

1. Introduction

Efforts have recently intensified to successfully mature canine oocytes *in vitro*; however, the developmental capacity of *in vitro* matured (IVM) oocytes was much lower than *in vivo* matured oocytes. Presumably, *in vitro* conditions were not completely adequate to support normal maturation, thereby limiting development of reproductive biotechnologies for research, clinical purposes, and preservation of endangered species.

Oocyte maturation is a complex and well-regulated process involving nuclear and cytoplasmic changes. It is noteworthy that oocyte maturation is one of the many aspects in which canine reproductive physiology differs from that of most domestic animals. In the majority of mammals, nuclear and cytoplasmic maturation occur within the follicle before ovulation [1]. However, in canids, oocytes are ovulated in the first meiotic process prophase [2,3], which is morphologically identified by a germinal vesicle (GV) stage. Therefore, meiotic

* Corresponding author. Tel.: +56-2-6785534; fax: +56-2-6785611.
E-mail address: mdlreyes@uchile.cl (M. De los Reyes).

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maturation occurs in the oviduct when the oocytes are >110 μm in diameter [4], requiring 2 to 5 d for completion [5]. Cytoplasmic maturation, evaluated through morphological redistribution of some organelles [6,7], occurs *in vivo* mainly between the onset of proestrus and 5 d after ovulation [8]. Changes within the oocyte during follicular and oviduct periods are essential for coordination of nuclear and cytoplasmic maturation [9]. Furthermore, the latter is critical for the establishment of normal embryonic development [10,11], which *in vitro* has been largely unsuccessful in canines. Thus, it is critical to know factors affecting developmental potential of IVM oocytes, as well as mechanisms which regulate them.

Developmental competence of an oocyte is related to its metabolic profile [12,13]. *In vitro* oocyte metabolism varies according to culture conditions and differs from *in vivo* metabolism [14]. Mitochondria play important roles in cellular energy metabolism for most reactions occurring in oocytes [15–17]. Mitochondria produce most of the ATP by oxidative phosphorylation [18,19]; in addition, they are important in regulating apoptosis [20], calcium signaling [21], reactive oxygen species, and production of intermediary metabolites [22]. The distribution of mitochondria and their metabolic activity undergo changes during oocyte maturation in many species [23–26], including dogs [27]; however, there appeared to be differences among species in the timing of activity and in distribution of mitochondria during oocyte maturation. Based on ultrastructural analysis, mitochondria assume specific positions during the transition from the GV stage to the MII stage [8,28]. Alterations of these changes have been reported to be determinants of oocyte competence in humans [24] and mice [29]. That nuclear maturation takes longer in canids than in other mammalian species studied, and in the absence of reports on the precise timing in mitochondrial localization in relation to nuclear maturation in canine oocytes, the aim of the present study was to evaluate changes in mitochondria distribution in relation to meiotic development, comparing *in vivo* and *in vitro* matured canine oocytes after various intervals of culture.

2. Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The protocol used in the present study was approved by the Chilean Bioethics Committee of the National Commission for Scientific and Technological Research (FONDECYT).

2.1. Oocyte processing

Oocytes for all experiments were obtained from healthy bitches, aged 1 to 6 y, of various breeds, following ovariohysterectomy. For the evaluation of non-mature oocytes (negative control) and for IVM experiments, ovaries were collected from bitches (n = 45) at random stages of the estrous cycle. Ovulated oocytes (positive control) were obtained from bitches (n = 11) ~72 h after ovulation. The time of ovulation was estimated before surgery by vaginal smears and blood serum progesterone concentrations, assessed by ELISA semi-quantitative commercial kit (Bio Veto Test; Ovulation® Test La Seyne-Sur-Mer, France) and by solid phase radioimmunoassay (RIA; Coat-a-count, Siemens, Health Care Diagnostics, LA, California, USA).

Ovaries, oviducts and the tip of uterine horns were collected and immediately transported to the laboratory in physiological saline solution (0.9% NaCl) at 37 °C. At the laboratory, the ovaries were placed in phosphate-buffered saline (PBS) supplemented with 50 μg/mL streptomycin and 100 IU penicillin at room temperature (21 °C). Cumulus oocytes complex (COCs) were released by slicing the ovarian cortex; those with a dark uniform ooplasm and a compact cumulus cell mass [30] were selected for use. After two washes in TCM-199 (Earle's salt, buffered with 25 mM Hepes; Invitrogen Carlsbad, CA, USA), the COCs were processed immediately as non-matured oocytes, or were incubated for IVM in the same TCM-199 medium 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 38.5 °C and 5% CO_2 for 48, 72, or 96 h [31]. *In vivo* matured oocytes (ovulated) were collected by flushing the oviducts with 15 mL of TCM 199 supplemented with 10% FCS (warmed prior to use).

2.2. Oocyte staining

Ovulated, non-matured and IVM oocytes recovered at each time period, were processed to evaluate mitochondrial patterns, as well as their nuclear configuration. After mechanical removal of cumulus cells by passage through a narrow glass pipette, oocytes were incubated in PBS buffer with 3% BSA and 180 nM MitoTracker Red CMX Ros (Molecular Probes Inc., Eugene, OR, USA) at 38.5 °C in 5% CO_2 and 95% air at high humidity for 30 min. This is a red-fluorescent dye that stains active mitochondria in live cells; its accumulation is dependent upon membrane potential. Thereafter, oocytes were washed three times in PBS and fixed for 15 min at 38 °C using 2% paraformaldehyde in Hank's balanced salt solution (HBSS; 1.3 mM

$CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.3 mM $NaHPO_4$, and 5.6 mM D-glucose). The nuclear status of the oocytes was determined using 4'-6-Diamidino-2-phenylindole (DAPI) staining (Thermo Fisher Scientific Inc., Rockford, IL, USA), which forms fluorescent complexes with natural double-stranded DNA; oocytes were incubated with 1 μg/mL DAPI for 15 min at room temperature. Thereafter, oocytes were washed three times in PBS, mounted on microscope slides with Vectashield (Vector Laboratories Inc, Burlingame, CA, USA) under coverslips supported with vaseline/paraffin dots and stored at 4 °C in the dark prior to evaluation.

2.3. Chromatin and mitochondrial evaluation

All morphologically normal oocytes were evaluated with an inverted epifluorescence microscope Olympus IX71 (UV emission 480 nm), equipped with an IX2-RFA lamp and a ProgRes-Capture Pro camera (Tokyo, Japan). Chromatin configurations were classified as follows [31]: immature or germinal vesicle (GV), when the vesicle was clearly visible; resumption of meiosis or germinal vesicle break down (GVBD), when the chromatin was dispersed and initiating condensation; first metaphase (MI), when chromosomes were condensed and present in equatorial view; and mature or second metaphase (MII), manifested by the presence of chromosomes in the second metaphase plate, with the first polar body (PB) extruded. Oocytes with irregular chromatin distribution or abnormal chromatin were considered degenerate and were discarded.

For mitochondrial evaluation, samples were also observed at 200× magnification with a Zeiss 410-Axiovert 100 Laser Scanning Confocal Microscope (Jena, Germany). A laser ray 543 nm and the G-2 A filter were used to detect MitoTracker Red CMXRos, and each oocyte were examined along the z-axis by means of 12 serial confocal planes (each 5 μm thick). Acquisition, storage and analysis of data were made with Carl Zeiss Microscope System LSM software 3.92-β. Considering that the fluorescent dye accumulates only in active mitochondria, mitochondrial distribution was assessed based on the cytoplasmic location of red fluorescence.

2.5. Statistical analysis

More than 138 oocytes in each group (non-matured and IVM for 48, 72, or 96 h) were analyzed throughout eight repeated experiments or replicates, except for the positive control group of *in vivo* matured oocytes (n = 28). Qualitative mitochondrial distribution pattern in each group and also oocyte nuclear maturation rates were compared statistically with a Chi-square (X^2) test, using the InfoStat Professional Program, Version 2004 (developed by Statistics Department, National University of Córdoba, Argentina). For all analyses, $P < 0.05$ was considered significant.

3. Results

Mitochondrial distribution patterns were classified into three main groups (Fig. 1): 1) Pattern A: Homogeneous fine, with small granulations spread throughout the cytoplasm (Fig. 1a); 2) Pattern B: Homogeneous granular, with large granulations spread throughout the cytoplasm, observed in IVM oocytes (Fig. 1b); and in ovulated oocytes (Fig. 1c); and 3) Pattern C: Heterogeneous with peripherical and central granular aggregations of mitochondria (Fig. 1d). These criteria were adapted from previous studies in dogs and other species [20,25].

Type A distribution was mainly (69.5%) observed in oocytes which were not cultured for IVM (non-matured oocytes) and less frequently in oocytes incubated for 48 (19.3%) or 72 h (11.1%; $P < 0.01$). The homogeneous granular distribution (Type B), was found in a small percentage ($P < 0.05$) in the non-mature group, but in IVM oocytes, pattern B was present in ~65% at each culture time (48, 72, and 96 h), as well as in the entire group (100%) of *in vivo* matured oocytes ($P < 0.05$). However, in the latter group, granulations were bigger than those observed in IVM oocytes. Type C mitochondrial distributions were only detected in IVM oocytes after 72 h (26.2%) or 96 h (36.4%) of culture (Table 1).

The contemporaneous staining of oocytes for mitochondria distribution and DNA enabled characterization of relocation of mitochondria with respect to nuclear development (Fig. 2; Table 1). In this regard, most oocytes displaying pattern A were at the GV stage (96.3%), and the majority of oocytes at the GV stage were pattern A (69.2%); In addition, some mitochondria were distributed in large granulations throughout the cytoplasm (type B; 30.8%). Oocytes at the GV stage mostly corresponded to non-matured oocytes (67.5%); this nuclear stage decreased significantly during *in vitro* culture (18, 11.5, and 3% at 48, 72, and 96 h, respectively). In the GVBD stage, IVM oocytes were mostly pattern B (76.9%), whereas after 72 or 96 h of culture, a minor proportion (5.3 and 10.6%; $P < 0.05$), also displayed pattern C. Metaphase I (MI) and MII stages were observed in IVM oocytes, displaying mainly ($P < 0.05$) pattern B (61.8 and 68.4% respectively). The

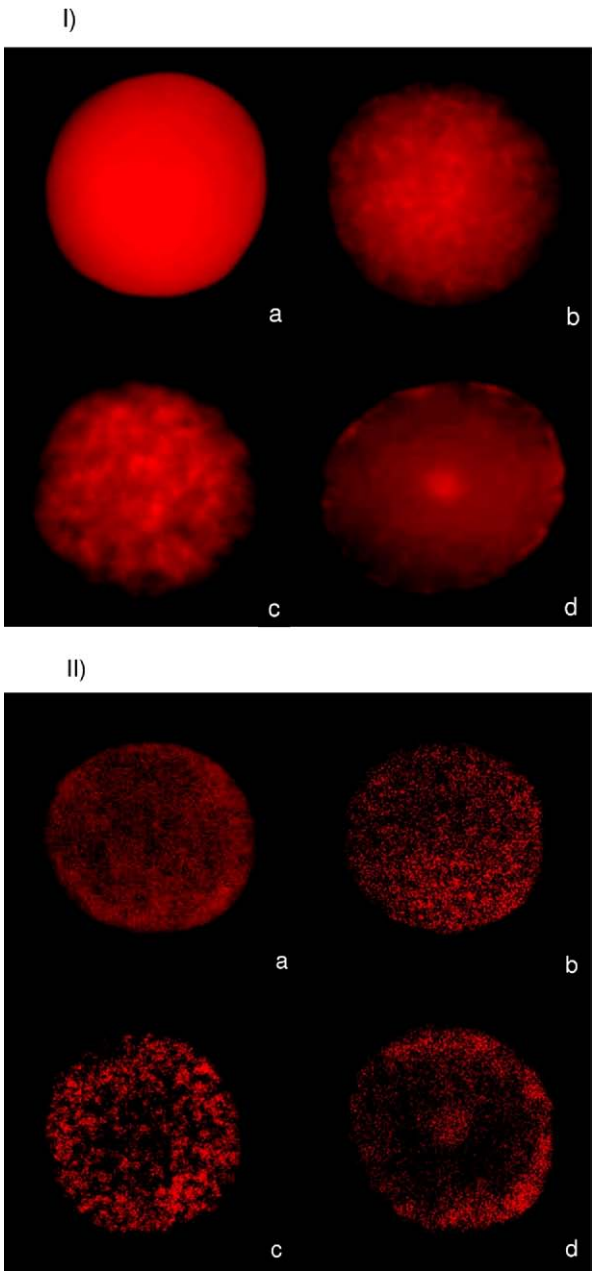

Fig. 1. Mitochondrial patterns in canine oocytes stained by MT-Tracker RED and visualized under: I Fluorescence microscopy (top images); and II Laser Scanning Confocal microscopy (bottom images). (a) Pattern A: Homogeneous fine, with small granulations distributed evenly throughout the cytoplasm; (b) Pattern B: Homogeneous granular, large granulations of red mitochondria distributed evenly throughout the cytoplasm (observed in IVM oocytes); (c) Pattern B: Homogeneous granular distribution present in ovulated oocytes, with larger granulations in comparison to pattern B of IVM oocytes, spread throughout the cytoplasm; (d) Pattern C: Heterogeneous with peripheral and central granular aggregation of mitochondria. (Magnification, × 200).

proportion of IVM oocytes that reached these meiosis stages increased significantly with time, reaching 1.4, 10.5, and 16.2% of MII after 48, 72, and 96 h respectively. This essentially corresponded to oocytes with pattern B (2.2, 11.2, and 17% of MII). After 72 or 96 h, some oocytes were pattern C (38.2% at MI and 31.6% at MII).

All ovulated or *in vivo* matured oocytes at the MII stage had only pattern B.

4. Discussion

Changes in mitochondrial organization are believed to be a reliable indicator of oocyte capacity to sustain cytoplasmic maturation and thus, further development. Based on the present study, it was clear that this organization changed in canine oocytes during *in vitro* maturation. In addition, there was an association between these changes in mitochondrial distribution and meiotic progression.

Based on examinations with fluorescence and confocal microscopy, IVM of canine oocytes lead to the translocation of mitochondria. In that regard, three mitochondrial patterns were detected throughout the IVM process, which were compared to non-matured and *in vivo* matured oocytes as markers of immaturity or maturation, respectively. There was a transition from fine homogeneous distribution (pattern A) of active mitochondria to increasing clustering spread throughout the cytoplasm (pattern B), and to perinuclear and cortical distribution (pattern C); however, as maturation proceeded, pattern B predominated in maturing oocytes. That structural rearrangements of the cytoplasm occur during maturation [11,32], these distributions might reflect changes in energy production and necessity during oocyte maturation in dogs, as in other species [23,33].

Organelle movements during maturation of porcine oocytes were coordinated with nuclear changes to ensure normal development [34]. In the current study, most oocytes with pattern A of mitochondrial localization were in the GV stage and although pattern B was observed at various stages of nuclear development, the majority of oocytes that reached MII displayed a pattern B, whereas pattern C was mostly observed in oocytes at intermediate stages of meiosis (MI), indicating that mitochondrial distribution could be associated with nuclear maturation, although the mitochondrial process might not be in parallel with chromatin changes.

In a recent report, the majority of GV canine oocytes collected at late diestrus and anestrus had their mito-

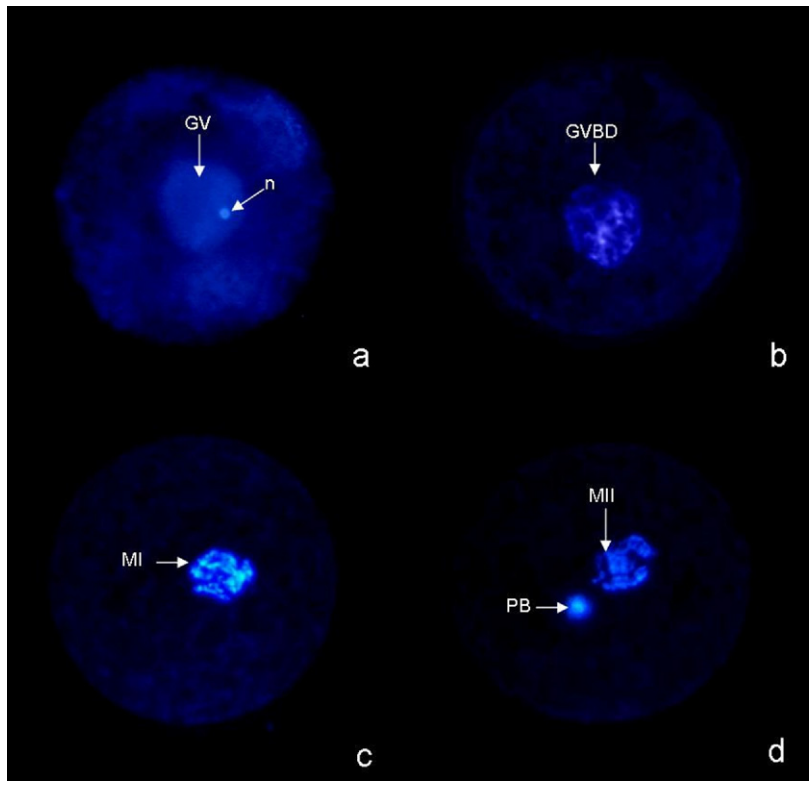

Fig. 2. Chromatin configurations in canine oocytes stained with DNA-specific binding dye, DAPI (4'-6-diamidino-2-phenylindole) and visualized under UV light. (a) Germinal vesicle (GV) and nucleolus (n); (b) Germinal vesicle breakdown (GVBD) with condensed chromatin; (c) First metaphase (MI); (d) Second metaphase (MII) with polar body (PB). Magnification is ×200.

chondrial complement in small aggregates uniformly distributed throughout the cytoplasm, whereas oocytes recovered in the follicular or early luteal phases, were characterized mainly with diffused tubular mitochondria network, but also spread throughout the cytoplasm [27]. In the present study, pattern A was the most common in GV oocytes, and a much lower percentage had pattern B, which seemed similar to the tubular presentation described by Valentini and co-workers [27]. However, since oocytes were collected from bitches at various stages of the estrous cycle, it was unclear if those few GV oocytes with pattern B were recovered from donors at a follicular phase, or merely represented oocytes in advanced stages of cytoplasmic maturity. In agreement with our observation in reference to pattern A, an ultrastructural study using electron microscopy, revealed that before the LH surge (i.e. GV stage), few mitochondria dispersed in small clusters were present in the cytoplasm of canine oocytes [8]. Since pattern A was detected mostly in GV oocytes, the highest rate of oocytes with pattern A was in the non-matured group, and this pattern was not present in ovulated oocytes, we inferred that the homogeneous fine distribution of mitochondria indicated the starting point of maturation in canine oocytes. In contrast, in other species, e.g. swine [23] and cattle [13], aggregations of mitochondria in the cortex were a common feature of GV oocytes. Apparent differences among species make it difficult to ascertain the real contribution of mitochondrial localization to normal oocyte maturation; nevertheless, the capacity of mitochondria to produce ATP by oxidative phosphorylation could vary among species, perhaps due to site-specific requirements within the ooplasm in the maturing oocyte [24].

As expected, the GV stage was mainly observed in non-matured oocytes; during IVM a decreasing proportion of oocytes at the GV stage was detected over time, as previously reported [31,35]. Furthermore, relocation of mitochondria occurred during incubation, with an increasing proportion of oocytes with resumption of meiosis at the nuclear level. In cattle, this movement of mitochondria appeared to be correlated with the degree of developmental competence acquired by the oocyte [10]. Progressive maturation proceeded and mitochondrial clustering around the nucleus and in peripheral

Table 1
Mitochondrial distribution patterns in non-matured, *in vitro* matured (IVM) and ovulated canine oocytes. Number (% in each maturing group).

Oocyte Groups	Cytoplasmic distribution Mitochondrial Pattern	Nuclear stages GV	GVBD	MI	MII	Total
		n = 338	n = 208	n = 319	n = 100	n = 972
	A	169 (72.2)^{ax}	—	—	—	169 (72.2)
Non matured	B	59 (25.2)^{bAx}	6 (2.6)^{Bx}	—	—	65 (27.8)
n= 234	C	—	—	—	—	—
	A	38 (27.5)^{aAy}	9 (6.5)^{aB}	—	—	47 (34)
IVM 48	B	23 (16.7)^{bAy}	44 (31.9)^{bBy}	22 (15.9)^{Cx}	2 (1.4)^{Dx}	91 (65.9)
n=138	C	—	—	—	—	—
	A	27 (9.8)^{az}	—	—	—	27 (9.8%)
IVM 72	B	12 (4.4)^{bAz}	61 (22.2)^{aBz}	83 (30.2)^{aBy}	20 (7.3)^{aCy}	176 (64)
n=275	C	—	11(4)^{bx}	52 (18.9)^{b}	9 (3.3)^{b}	72 (26.2)
	A	—	—	—	—	—
IVM 96	B	10 (3.4)^{Az}	55 (18.5)^{aBz}	92 (31)^{aCy}	32 (10.8)^{aDy}	189 (63.6)
n= 297	C	—	22 (7.4)^{bAy}	70 (23.6)^{bB}	16 (5.4)^{bA}	108 (36.4)
	A	—	—	—	—	—
Ovulated	B	—	—	—	28 (100)^{z}	28 (100)
n=28	C	—	—	—	—	—

Nuclear stages: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, first metaphase; MII, second metaphase. Mitochondria distribution patterns: A, homogeneous fine; B, homogeneous granular; C, heterogeneous peripheral and central.
$^{a–c}$Within a column and sampling time, numbers without a common superscript differed ($P < 0.05$).
$^{A–D}$Within a row, numbers without a common superscript differed ($P < 0.05$).
$^{x–z}$Within a column and mitochondrial pattern, numbers without a common superscript differed ($P < 0.05$).

position (pattern C) was observed only after 72 h of culture in oocytes resuming meiosis (GVBD) and in oocytes at MI and MII stages, suggesting therefore, a major relocation of mitochondria with increased duration of incubation. The perinuclear aggregation of mitochondria in swine was related to the establishment of the circular bivalent configuration that subsequently developed into the first meiotic metaphase spindle [36]. In human oocytes, mitochondrial clustering around the peripheral cytoplasm seemed important for controlling intracellular pH and protein function and cytoskeletal organization [25,37]. In addition, accumulation of mitochondria in a peripheral position and around the nucleus has been considered as a developmental necessity for cytoplasmic maturation [38]. In goats, 78% of oocytes that reached MII after 20 h of IVM had mitochondria around the perinuclear region [39]. In the present study, although approximately one third of the oocytes at MII had pattern C, this mitochondrial localization appeared to represent intermediate stages of development, since the major proportion of MII oocytes *in vitro* and the totality *in vivo* matured oocytes displayed pattern B, with large granulations spread throughout the cytoplasm. Similarly, 3 d after ovulation in the bitch, mitochondria were homogeneously distributed in the cytoplasm [8]. It is likely that aggregation of mitochondria throughout the cytoplasm in canine oocytes represented a more advanced stage in terms of cytoplasmic maturation. Similarly, in metaphase I and II human oocytes, mitochondria were also spread throughout the cytoplasm [40].

Although the functional significance of this distribution of mitochondria in the canine oocytes it is not clear, especially at the final stages of oocyte maturation, perhaps energy metabolism and modulating calcium concentrations are important factors. In that regard, the distribution of mitochondria and lipid droplets, used as an intracellular energy source, are involved in delivering ATP to specific regions in oocytes to support normal development [41,42], including modulating the calcium release pattern. Moreover, the homogeneous aggregation of mitochondria in bovine oocytes had a positive correlation with ATP content [43] and the ATP content in porcine oocytes increased significantly at the end of IVM [23]. Thereby, pattern B may reflect the high mitochondrial contribution to ATP concentrations and calcium requirements to support high energy demand, including metabolic adaptations needed to satisfy the requirements for fertilization and the initial stages of cell division and differentiation.

Although MII oocytes can be in the oviduct as early as 52 h after ovulation [4], in the present study, ovu-

lated oocytes were obtained at approximately 72 h post ovulation, since this interval was more consistently associated with oocyte maturation [5]. All ovulated oocytes had normal morphology [8], without indications of aging, displaying pattern B with large granulations of mitochondria spread throughout the cytoplasm and chromosomes at MII. In contrast, in IVM groups, pattern B did not exceeded 65% of the oocytes after 48 h of culture, with no subsequent increase. Similarly, *in vitro* conditions may cause incomplete movement of mitochondria in porcine oocytes, and thus, oocytes matured *in vitro* were less competent than those matured *in vivo* [23]. In addition, mitochondrial granulations observed in oocytes displaying pattern B were somewhat smaller in IVM versus ovulated oocytes, which could be due to fewer mitochondria developing *in vitro* than *in vivo*. Although molecular mechanisms of the aggregation of mitochondria have not been elucidated, the reduced aggregation following IVM may have contributed to the maturation delay. In the current study, only 4, 11, and 17% of the IVM oocytes that displayed pattern B after 48, 72, and 96 h respectively, reached MII, indicating that, although some IVM oocytes may have been able to display a pattern of mitochondrial distribution similar to that in oocytes matured *in vivo*, the oocytes cannot always proceed to MII. The minor mitochondrial aggregation might implicate abnormal ATP content in oocytes during culture, which may result in poor developmental competence. *In vitro* matured human [44] and bovine [12] oocytes had reduced protein content compared to *in vivo* matured oocytes. In that regard, spindle formation and chromosome behavior depend on the expression and activity of proteins, which use ATP as their energy source. Furthermore, the low mitochondria and ATP contents have been associated with the failure of meiotic spindle visualization in IVM human oocytes [42]. Indeed, changes in intracellular energy in the form of ATP, calcium and pH may abolish the association between mitochondria and micro-tubules required for concurrent nuclear and cytoplasmic maturation [16,45,46]. These alterations can reflect a deficiency in the intrinsic ability of the IVM oocyte per se, the maturation medium, or both, to support mitochondrial maturity, which may partially explain the reduced potential development of oocytes matured *in vitro* compared with those matured *in vivo*.

In conclusion, mitochondria in canine oocytes developed the ability to move and became progressively aggregated during IVM. Although this ability was related with nuclear maturation, these processes were less complete than *in vivo* matured oocytes, and the oocytes did not always proceed to MII. It is expected that future studies will elucidate the interplay between mitochondria organization and chromatin configuration in the development of canine oocytes.

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