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Biochemical and morphological markers of oocyte quality in female dogs: the role of *cumulus* cells and reactive oxygen species

[Marcadores bioquímicos e morfológicos da qualidade oocitária em cadelas: papel das células do *cumulus* e espécies reativas de oxigênio]

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Abstract: The *in vitro* maturation (IVM) rate of canine oocytes is generally considered low, with less than 20 % achieving meiotic resumption, and oocyte quality being a determining factor. This study was performed to evaluate the oocyte quality of healthy bitches using *cumulus* cell (CC) viability, reactive oxygen species (ROS) analysis, and oocyte morphometry. Twelve bitches, with a mean age of 2.3 ± 1.3 years and mean weight of 9.4 ± 5.2 kg, underwent ovariosalpingohysterectomy for oocyte collection. Morphological classification and viability testing of the *cumulus–oophorus* complex (COCs) with Brilliant Cresyl Blue were subsequently performed. Viable COCs were divided into groups according to their morphological classification (GI, GII or GIII). An H2DCFDA probe was used to evaluate ROS levels in the oocytes, while Hoechst 33342 and propidium iodide probes were used to assess viable and apoptotic CCs, respectively. For morphometry, the total oocyte diameter, cytoplasmic diameter, zona pellucida thickness, radius, and perimeter were measured. Data were analysed using analysis of variance. The GI group had the highest percentage of viable oocytes, the greatest mean values for morphometric parameters, and the lowest levels of intracellular ROS. No difference was observed between the GI and GII groups in terms of the percentage of viable CCs. In conclusion, further investigation is required to establish acceptable ROS levels for selecting oocytes intended for IVM in bitches.

Keywords: Oocyte selection; meiotic competence; oocyte evaluation; *cumulus* cells; in vitro maturation.

Resumo: A taxa de maturação *in vitro* (MIV) de oócitos caninos é considerada baixa, com menos de 20 % de retomada meiótica, sendo a qualidade oocitária um fator determinante. O presente trabalho teve como objetivo avaliar a qualidade oocitária de cadelas saudáveis, utilizando a viabilidade das células do *cumulus* (CCs), a verificação das espécies reativas de oxigênio (EROs) e a morfometria dos oócitos. Doze cadelas, com idade média de 2,3 (± 1,3) anos e com peso médio de 9,4 (± 5,2) kg, foram submetidas à ovariosalpingohisterectomia (OSH) para coleta dos oócitos. Posteriormente, foram realizados a classificação morfológica e o teste de viabilidade com Azul Cresil Brilhante (ACB) do complexo *cumulus-oophorus* (CCOs). Os CCOs viáveis foram separados em grupos de acordo com sua classificação morfológica (GI, GII ou GIII). Foi utilizada a sonda H2DCFDA para a avaliação do nível de EROs intracelular e as sondas Hoechst 33342 e Iodeto de Propídeo para a avaliação das CCs viáveis e apoptóticas. Na morfometria, foram avaliados o diâmetro total do oócito, o diâmetro do citoplasma, a espessura da zona pelúcida, o raio e o perímetro. Foi utilizada a análise de variância para a análise dos dados. O grupo GI apresentou o maior percentual de oócitos viáveis, as maiores médias dos parâmetros morfométricos e o menor nível de EROS intracelular. Não houve diferença entre os grupos GI e GII em relação ao percentual de CCs viáveis. Em conclusão, torna-se necessário realizar maiores investigações para determinar os níveis de EROs aceitáveis para a seleção de oócitos que serão utilizados para MIV em cadelas.

Palavras-chave: seleção de oócitos; competência meiótica; avaliação oocitária; células do *cumulus*; maturação in vitro.

1. Introduction

Worldwide, 38 species in the canid family are at risk of extinction, with two already considered extinct. Of these, six species are native to or found in Brazil: the hoary fox (*Lycalopex vetulus*) [1], the short-eared dog (*Atelocynus microtis*) [2], the maned wolf (*Chrysocyon brachyurus*) [3], the pampas fox (*Lycalopex gymnocercus*) [4], the bush dog (*Speothos venaticus*) [5], and the crab-eating fox (*Cerdocyon thous*) [6]. The primary threats to these species are linked to habitat destruction and other human activities—such as hunting, the introduction of domestic species, disease transmission, and road collisions—which have both direct and indirect negative impacts.

The use of assisted reproduction techniques (ARTs) is essential for species preservation programs. The domestic dog (*Canis lupus familiaris*) is the primary experimental model for the canid family. However, the species' unique reproductive physiology still poses a challenge to *in vitro* maturation (IVM) and subsequent *in vitro* fertilization (IVF) procedures, which are critical steps in ARTs. Many authors have reported unsatisfactory results, with less than 20% meiotic competence in oocytes; as of 2007, no live offspring from IVM and IVF in canines had yet been reported. [7] Nevertheless, the technique has advanced over the years. In a study by Qin et al. [8] testing different progesterone (P4) concentrations during the IVM of canine oocytes, a maturation rate of 29.7% was achieved, leading to the birth of the first live puppies produced via IVM and IVF.

Canine oocyte meiotic competence during IVM is associated with various morphological aspects of the *cumulus-oocyte* complex (COCs). The recommended practice is to use good-quality oocytes exhibiting dark, homogeneous cytoplasm, a diameter greater than 100 μm (excluding the *zona pellucida*), and surrounded by two or more layers of *cumulus* cells (CCs) [9]. The Brilliant Cresyl Blue (BCB) stain test is also a widely used tool for selecting oocytes that have completed their growth phase and are therefore more viable for the IVM process [10]. However, these criteria have not been sufficient to select fully competent oocytes for IVM, necessitating further research into oocyte selection criteria.

Since the influence of CC apoptosis rate on oocyte developmental competence [11] and the deleterious effects of high intracellular levels of reactive oxygen species (ROS) on oocyte quality are already known, it is necessary to investigate these specific characteristics in bitches. Accordingly, this study aimed to perform a comparative analysis of oocytes of different quality grades from bitches, considering their morphological classification, morphometry, the BCB test, CC viability rate, and intracellular ROS levels.

2. Material and methods

2.1 2.1 Ethical aspects and animals used in the study

Data were obtained from elective ovariohysterectomy (OHE) procedures performed at the Prof. Sylvio Barbosa Cardoso Veterinary Hospital (HVSBC) in Fortaleza, Ceará, Brazil, from May to July 2024. The project was submitted to the Animal Use Ethics Committee (CEUA – UECE) under protocol number NUP 31032.003854/2024-80. All procedures were performed in accordance with animal care guidelines. Ovaries were collected from twelve healthy female dogs of various breeds, between one and five years of age, whose health was confirmed by clinical and laboratory examinations.

2.2 COC Collection

Following the surgical procedure, the ovaries were transported at 5 ºC in 50 mL Falcon tubes containing phosphate-buffered saline (PBS). The COCs were collected using the slicing technique. Briefly, the ovaries were isolated from the ovarian bursa and gently sliced into 1- to 2-mm-thick layers on a Petri dish containing PBS medium. This was performed with 14 cm rat-tooth tissue forceps and a No. 22 scalpel blade to release the COCs from the ovarian cortex. The COCs were then visualized under a stereomicroscope (Zeiss Stemi 305, Jena, Germany), collected, and transferred to 200 µL drops of PBS for counting and classification.

2.3 COC Classification

First, the COCs were morphologically classified according to the methodology proposed by Hewitt & England [12]. This assessment was performed by a single person to avoid inter-operator variability. Briefly, COCs with a homogeneous, strongly pigmented cytoplasm completely surrounded by two or more layers of granulosa cells were classified as grade I (GI); those with a lightly pigmented cytoplasm and incomplete layers of granulosa cells were classified as grade II (GII); and those with a weakly pigmented cytoplasm, no adherent *cumulus* cells, and an undefined shape were classified as grade III (GIII) and considered degenerated. After classification, the COCs were separated into groups by grade (GI, GII, and GIII) in 200 µL drops of PBS. Subsequently, the COCs were stained with 90 µL of Brilliant Cresyl Blue (BCB) for 60 minutes at 38.5 °C, 5% CO_2, and 100% relative humidity. Finally, the oocytes were washed twice in drops of PBS to facilitate the visualization and selection of BCB+ oocytes, i.e., those in which the cytoplasm was strongly stained blue, as shown in Figure 1.

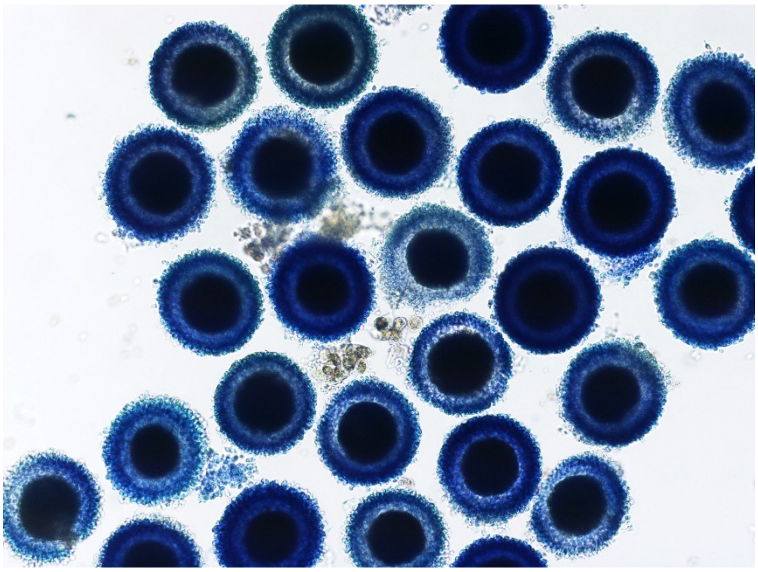

Figure 1. Grade I immature canine oocytes stained with Brilliant Cresyl Blue (BCB) and viewed under a stereomicroscope at 50x magnification. Most COCs feature a regularly shaped oocyte, an intact *zona pellucida*, and three or more layers of CCs. Oocytes with homogeneous, dark blue-stained cytoplasm are considered viable according to the BCB test.

2.4 Oocyte Morphometry

After the oocytes were separated into drops according to their grade, images of the oocyte groups were captured using a light microscope (Nikon E400, Tokyo, Japan) at 40x magnification. Measurements were performed using ImageJ software. The following parameters were measured in µm (Figure 2): total diameter (TD) (including the zona pellucida), oocyte diameter (OD) (excluding the zona pellucida), and zona pellucida (ZP) thickness. The oocyte radius (R) was calculated by dividing the TD by two, and this value was then used to determine the oocyte perimeter (OP) with the equation $P = 2\pi r$.

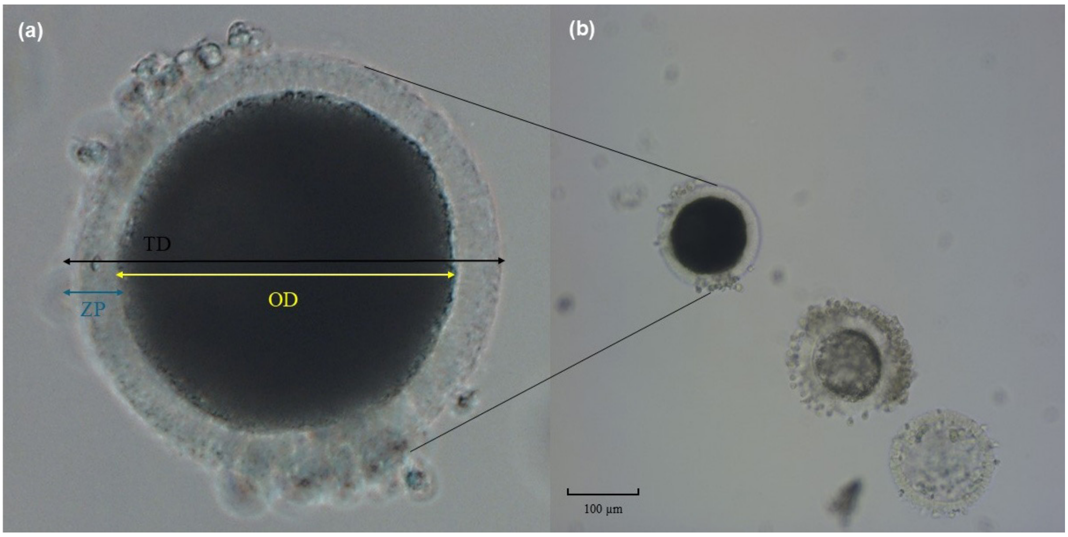

Figure 2. A grade I immature canine oocyte viewed by light microscopy at 10x magnification (b). The arrows indicate how the diameters for the morphometric parameters were defined and measured (a): total diameter (TD), oocyte diameter (OD), and *zona pellucida* (ZP) thickness.

2.5 Oxidative Stress and CC Viability

To assess oxidative stress, the COCs were labeled with the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe, as previously described by Shi et al. [13] (Figure 3).

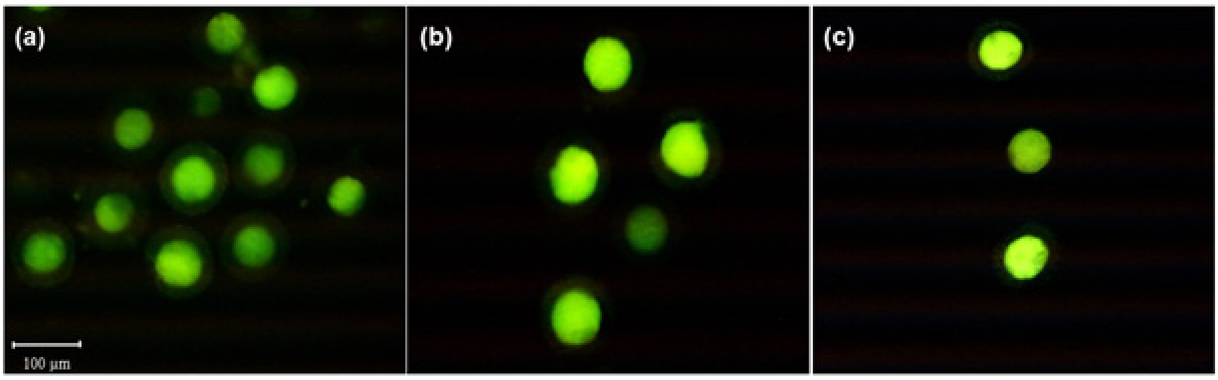

Figure 3. Grade I (a), grade II (b), and grade III (c) immature canine oocytes viewed using fluorescence microscopy at 10x magnification. The oocytes were stained with the fluorescent probe H_2DCFDA, an intracellular ROS marker. Fluorescence intensity was measured using ImageJ software. Higher intensity corresponds to a higher level of ROS.

First, the COCs were denuded by transferring them to PBS drops containing hyaluronidase (1 mg/mL in TCM199) (Sigma-Aldrich, St. Louis, USA) for 1 minute; subsequently, the CCs were removed by gentle pipetting with an automatic micropipette. For staining, the oocytes were incubated with 10 µM H_2DCFDA probe at 38.5 °C in 5% CO_2 for 20 minutes. The oocytes were then washed three times in 100 µL drops of PBS, transferred to a fluorescence microscope (Nikon E400, Tokyo, Japan) for visualization, and imaged for subsequent evaluation of fluorescence intensity using a filter with 497 nm excitation and 518 nm emission. The CCs obtained after denudation were centrifuged for 3 minutes at 600 g. To facilitate visualization, 100 µL of TCM-Hepes was added, and the mixture was homogenized. Next, a 10 µL sample was added to 20 µL of Hoechst 33342 (10 µg/mL; Sigma-Aldrich, City, USA), followed by 50 µL of Propidium Iodide (1 µg/mL; Sigma-Aldrich, City, USA). The solution was incubated for 10 minutes at 37 °C, protected from light. An aliquot was then placed on a slide with a coverslip and observed under a fluorescence microscope (Nikon E400, Tokyo, Japan) using emission filters of 617 nm for propidium iodide and 430 nm for Hoechst. To determine the viability rate, 200 cells across five distinct fields were evaluated and classified as either dead (stained exclusively red) or viable (stained blue) (Figure 4), as Propidium Iodide cannot cross the membrane of a viable cell. All images obtained by confocal microscopy were analyzed using ImageJ software.

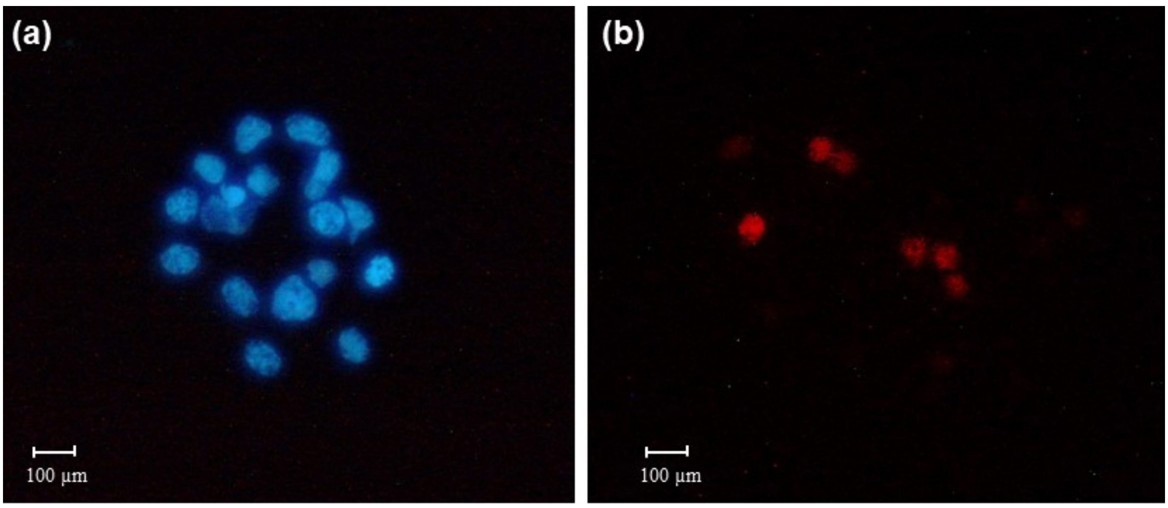

Figure 4. Fluorescence microscopy images (4x magnification) of *cumulus* cells from grade I canine oocytes. The cells were incubated with the fluorescent probes Hoechst 33342 (a) and Propidium Iodide (b). Red-stained cells, marked by Propidium Iodide, are considered apoptotic.

2.6 Statistical analysis

Data analysis was performed using JMP statistical software [14]. First, the Shapiro-Wilk test was used to confirm the normality of the data. Once this condition was met, an Analysis of Variance (ANOVA) was conducted, followed by Tukey's HSD test. Percentage data were analyzed using the chi-squared (χ^2) test.

3. Results

3.1 Morphological Classification of COCs and Oocyte Viability

A total of 642 COCs were recovered, of which 439 were classified as Group I, 140 as Group II, and 61 as Group III. The distribution between BCB+ and BCB- oocytes is shown in Table 1. This classification allowed for the selection of COCs containing developmentally competent oocytes. Group GI had the highest percentage of BCB+ oocytes (84.5%), followed by Group GIII (72.12%) and Group GII (63.57%). There was no significant difference in the percentage of BCB- oocytes among the groups.

Table 1. Distribution of canine oocytes by quality grade (GI to GIII) and according to BCB staining.

	Total Oocytes	Oocyte quality grades					
		GI		GII		GIII	
		No.	%	No.	%	No.	%
BCB+	504	371	84.5^a	89	63.6^c	44	72.1^b
BCB-	138	68	15.5^a	51	36.4^a	17	27.9^a

Different lowercase letters in the same row indicate a statistical difference (P < 0.05).

3.2 Oocyte Morphometry, *Cumulus* Cell (CC) Viability, and Intracellular Reactive Oxygen Species (ROS) Levels

Table 2 shows that group GI had the highest mean values for morphometric parameters, particularly total diameter (TD), radius (R), and perimeter (OP), which were statistically different from those of groups GII and GIII.

Table 2. Mean (± SD) of morphometric parameters for canine oocytes, according to their morphological classification. The parameters were: total diameter (TD) (including the zona pellucida), oocyte diameter (OD) (excluding the zona pellucida), zona pellucida thickness (ZP), oocyte radius (R), and oocyte perimeter (OP).

Oocyte classification	Morphometric parameters				
	TD	OD	R	ZP	OP
GI	120,78± 0,84^a	89,61± 4,12^a	60,35 ± 0,42^a	16,50± 2,31^a	378,98± 2,63^a
GII	108,9 ± 3,27^b	84,55± 4,52^a	54,45± 1,64^b	14,91± 1,51^a	341,95± 10,28^b
GIII	89,25 ± 6,77^c	63,54± 4,47^b	44,63± 3,38^c	12,12± 1,65^b	280,25± 21,25^c

Different letters in the same column indicate a statistical difference (P < 0.001).

Regarding the viability of CCs, no significant difference was observed between groups GI and GII (Table 3).

Table 3. Mean (± SD) of the number of *cumulus* cells labeled with the fluorescent probes Hoechst 33342 and Propidium Iodide in groups GI and GII, based on the morphological classification of oocytes.

Oocyte classification	**No. of cells**	
	HO	**IP**
GI	157,00 ± 9,17[a]	43,00 ± 9,17[a]
GII	145,67± 8,08 [a]	54,33 ± 8,08[a]

Different letters in the same column indicate a statistical difference (P < 0.05).

In contrast, the groups differed in their intracellular ROS levels. GI oocytes exhibited significantly lower fluorescence intensity, followed by groups GII and GIII, as shown in Table 4 and Figure 5.

Table 4. Mean (± SD) of intracellular reactive oxygen species (ROS) levels, determined by fluorescence intensity in H_2DCFDA-labeled oocytes, for groups GI, GII, and GIII, based on their morphological classification.

Fluorescence intensity	**Oocyte classification**		
	GI	**GII**	**GIII**
	62,09± 4,54[a]	88,54± 4,51[b]	110,95± 4,62[c]

Different lowercase letters in the same row indicate a statistical difference (P < 0.001).

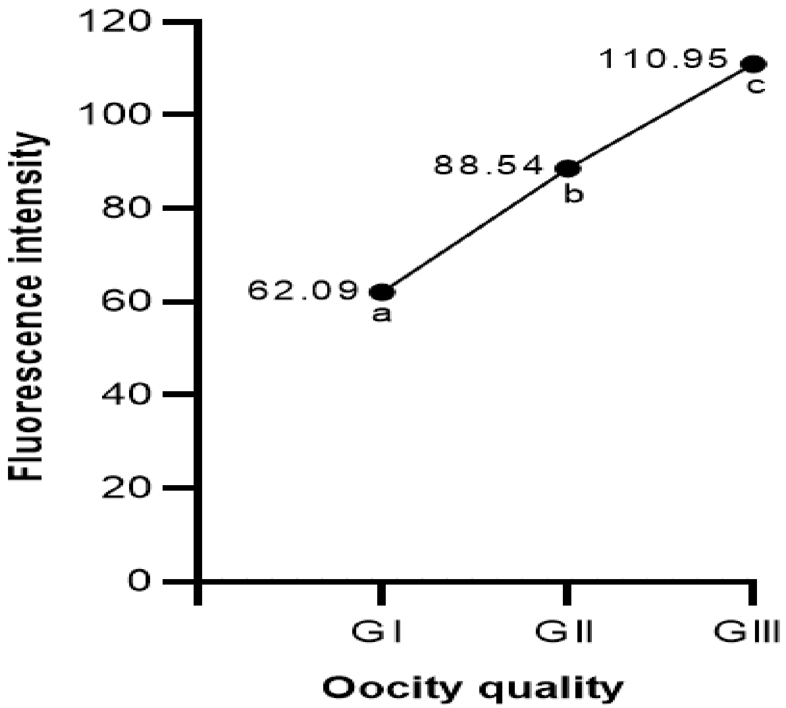

Figure 5. Graph comparing the mean fluorescence intensity among three groups of canine oocytes (GI, GII, and GIII) stained with the H_2DCFDA probe, an intracellular ROS marker. Different lowercase letters indicate a significant statistical difference between groups. GIII showed the highest level of fluorescence intensity.

4. Discussion

The present study aimed to evaluate canine oocyte quality in greater depth by analyzing the viability of CCs, among other functional and cellular morphological characteristics. In parallel with these analyses, we performed morphological classification of COCs, oocyte viability assessment with BCB, and oocyte morphometry—methods already widely used in routine *in vitro* reproduction for this species. This was done to enable a comparative study between these different approaches for assessing canine oocyte quality.

CCs play an important role in the resumption of meiosis and, consequently, in oocyte maturation. The oocyte and its surrounding CCs share a close relationship via gap junctions (GJ), which allow for the exchange of nutrients, ions, and small regulatory molecules, enabling synchronized nuclear and cytoplasmic maturation [15-16]. In this study, no significant difference was observed between the GI and GII groups in the percentage of viable CCs stained with Hoechst, a vital dye that marks the DNA of viable cells. Lee et al. [17], in a comparative study between COC+ and COC- in pigs, clearly demonstrated that the BCB+ group showed significantly lower levels of CC apoptosis and higher rates of nuclear maturation and subsequent embryo development, as higher-quality oocytes are known to reduce apoptosis in CCs. A study in the arctic fox (*Vulpes lagopus*) demonstrated that under culture conditions that preserved the GJs between the *corona* cells and the oocyte, even a very small population of CCs is capable of inhibiting the resumption of meiosis. Conversely, it was clearly observed that only oocytes with two or more complete layers of CCs continued their development in culture and extruded the polar body, whereas those with only a single layer of CCs degenerated [18]. In the present study, the viable CC rate was assessed only in BCB+ COCs, which may explain the similar values between the groups. However, the conformation of the CCs is a critical factor for maturation, underscoring the importance of selecting oocytes based on their morphological characteristics.

The production of ROS is a natural process of cellular metabolism. However, oxidative stress is characterized by an imbalance in the cell's redox system, involving a significant increase in intracellular ROS levels that has deleterious effects on the cell [19]. In female mammals, fertility declines with advancing age, accompanied by a reduction in the number of follicles and a decrease in oocyte quality. These oocytes exhibit numerous alterations in their cell biology, including biochemical and molecular changes such as an increased amount of ROS [20]. Excessive ROS levels are also linked to major reproductive diseases in women, such as endometriosis and polycystic ovary syndrome [21-22]. On the other hand, ROS are fundamental to mediating folliculogenesis, meiosis, ovulation, and embryonic development, where they act as secondary messengers for cell signaling. This makes the delicate balance of their intracellular and extracellular levels critically important for oocyte quality [23]. In a study by Silva et al. [24], the effect of different oxygen tension levels in the maturation culture medium on the viability of canine CCs was tested. The CC apoptosis rate varied significantly among the groups, with the highest rates found in the 20% oxygen tension group. This demonstrates that at this tension level, the oocytes' redox system was overwhelmed, causing deleterious effects on the COCs.

When oocytes are removed from the follicle and exposed to an artificial environment (e.g., a maturation medium), they are expected to undergo changes in response to the compounds to which they are subjected. Furthermore, apoptosis occurs spontaneously in COCs subjected to suboptimal culture conditions [25-27]. Previous studies on ROS levels in canine oocytes have primarily evaluated the influence of different culture media on the intracellular concentration of these molecules and their effects on oocyte quality. In the present study, we evaluated ROS levels in oocytes under identical external conditions, taking into account the varying ROS levels that can be found in oocytes of different quality grades, as classified by morphological characteristics. GI oocytes showed lower fluorescence intensity values, with a mean of 62.09 (± 4.54). This result was expected, as GI oocytes are considered to be of higher quality. This evaluation aimed to determine whether this method could be a potential tool for selecting oocytes for *in vitro* maturation, as *in vitro* environments typically increase cellular ROS production. Therefore, selecting cells that naturally have lower intracellular ROS levels prior to *in vitro* maturation could improve outcomes. However, more extensive studies are needed. These studies should involve the *in vitro* maturation of these oocytes to more precisely determine their meiotic competence and to establish optimal intracellular ROS ranges for oocytes intended for this procedure.

The population of recovered canine oocytes is highly heterogeneous, containing both competent and incompetent oocytes originating from developing or atretic follicles. Therefore, selection criteria must be applied to the oocytes intended for IVM to ensure the highest possible success rate. The criteria typically used are morphological, including the oocyte's morphology and diameter, and the conformation of the CCs. A typical feature of fully developed oocytes is a dark, homogeneous cytoplasm, which results from a high physiological concentration of lipids [(12)]. Oocyte diameter (excluding the *zona pellucida*) is related to the follicular stage at the time of collection and the oocyte's acquisition of meiotic competence. According to Hewitt and England [(28)], when oocytes were separated into three groups based on diameter (>100 µm, 100 µm, and <100 µm), only those with diameters >100 µm reached the MII stage in higher proportions than smaller oocytes. Subsequent studies more specifically concluded that oocytes with diameters >120 µm had a greater capacity to reach MII than those with diameters <110 µm [(29-30)]. In the present study, we measured the total oocyte diameter (including the zona pellucida), the oocyte diameter (excluding the *zona pellucida*), and other parameters not conventionally measured, such as oocyte radius, *zona pellucida* thickness, and oocyte perimeter. The oocytes in group GI showed the highest mean values for all parameters. However, compared to previous studies, the mean diameter of these oocytes was low (89.61 ± 4.12 µm), and according to those same studies, their meiotic competence rate would be predicted to be between 4-10%. Nevertheless, other studies have obtained similar results, with Grade I oocytes having diameters of less than 100 µm. In the study by Pereira et al. [(31)], the diameter of Grade I oocytes collected from bitches at different stages of the estrous cycle—anestrus and the luteal phase—was measured. The mean diameters were 77.62 µm and 78.64 µm, respectively, with no significant difference between them.

To assess viability during oocyte selection, the detection of glucose-6-phosphate dehydrogenase (G6PDH) remains the only reliable, non-invasive method. G6PDH is an enzyme synthesized by developing oocytes; therefore, oocytes with high G6PDH activity are still growing, whereas those that have completed their development show reduced enzyme activity. This activity can be observed using the Brilliant Cresyl Blue (BCB) test, which is based on the ability of highly active G6PDH to decolorize the blue dye. Thus, oocytes that stain dark blue (BCB+) are considered to have completed their development [(32)]. Furthermore, the study by Rodrigues et al. [(33)] observed that most stained oocytes are at the germinal vesicle (GV) stage, demonstrating that in the canine species, previously selected grade 1 oocytes are expected to be at this stage. Another characteristic that can be observed with the BCB test is the staining synchrony between the ooplasm and the *cumulus* cells. A difference in staining within the COCs may suggest a metabolic disconnection between the oocyte and its *cumulus* cells, leading to impaired glutathione synthesis. However, one hypothesis suggests that BCB asynchrony is a normal physiological feature of canine COCs [34]. For this study, only BCB+ oocytes were used for subsequent analyses. The oocyte viability results showed that Grade I (GI) oocytes had a significantly higher proportion of BCB+ cells (84.5%) compared to the other groups. This finding is likely because these same cells met all morphological selection criteria, with the exception of oocyte diameter, which on average did not exceed 100 µm. Despite this, they were still the largest oocytes compared to the other groups (GII and GIII), indicating that the majority had completed their development.

5. Conclusion

Although CCs play an important role in oocyte development and maturation, no significant difference in the viable CC rate was observed between the groups. Furthermore, further investigation is needed to determine the acceptable levels of ROS for the selection of oocytes to be used for IVM in bitches.

Conflict of interest statement

The authors declare no conflict of interest.

Data availability statement

The data will be provided by the corresponding author upon request.

Author contributions

Conceptualization: Teixeira, D. Í. A.; Formal analysis: Freitas, V. J. F., Teixeira, D. Í. A.; Investigation: Rodrigues, A. L. S., Arcce, I. M. L.; Methodology: Rodrigues, A. L. S., Arcce, I. M. L., Negreiros, N. A. B.; Resources: Freitas, V. J. F.; Supervision: Teixeira, D. Í. A.; Writing: Rodrigues, A. L. S., Teixeira, D. Í. A.

Generative AI use statement

The authors did not use generative Artificial Intelligence tools or technologies in the creation or editing of any part of this manuscript.

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