doi: 10.1016/j.theriogenology.2024.08.032

Nicole A. Luizari Stábile, Frederico Rocha de Oliveira, Ricardo Andrade Furtado, Carolina Barretto M.L. Felippe, Mariana Riboli Tavares, Paulo E.B. Martinelli, Carlos Eduardo Fonseca-Alves, Fabiana Ferreira de Souza, Martina Colombo, Gaia Cecilia Luvoni, Maricy Apparício

Cryopreservation of canine ovarian tissue by slow freezing and vitrification: Evaluation of follicular morphology and apoptosis rate,
Theriogenology, Volume 230, 2024, Pages 8-14,

Cryopreservation of canine ovarian tissue by slow freezing and vitrification: evaluation of follicular morphology and apoptosis rate

Nicole A. Luizari Stábile[1], Frederico Rocha de Oliveira[2], Ricardo Andrade Furtado[1], Carolina Barretto M.L. Felippe[2], Mariana Riboli Tavares[2], Paulo E. B. Martinelli[2], Carlos Eduardo Fonseca-Alves[3], Fabiana Ferreira de Souza[3], Martina Colombo[4], Gaia Cecilia Luvoni[4], Maricy Apparício[3*]

Affiliation and address of authors:

[1]Universidade de Franca, Franca-SP, Brasil;

[2]Universidade Estadual Paulista (UNESP), FCAV, Campus de Jaboticabal, Jaboticabal-SP, Brasil.

[3]Universidade Estadual Paulista (UNESP), FMVZ, Campus de Botucatu, Botucatu-SP, Brasil.

[4] Dipartimento di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, Milan, Italy;

Corresponding author:

*E-mail address: maricy.apparicio@unesp.br (M. Apparício)

Postal address: Rod. Dr. Walter Mauricio Correa, s/n CEP: 18618-681 Botucatu-SP, Brazil.

Abstract

In this study, we aimed to evaluate the efficacy of cryopreserving canine ovarian tissue using vitrification and slow freezing methods while investigating potential differences in cryotolerance based on follicular type and cryopreservation technique. Twenty-eight ovaries were collected from 14 anoestrus bitches of various breeds, aged between 2 and 5 years, and undergoing elective ovariohysterectomy. The ovaries were sectioned into small fragments and randomly assigned to three groups: vitrification, slow freezing, and a control group (fresh tissue). Vitrification was performed using cryotubes containing DAP 213 solution (2M DMSO, 1M acetamide, 3M propylene glycol) in two stages, while slow freezing involved cryotubes with 1.5M DMSO solution inserted into a programmable machine. The effects of cryopreservation were evaluated by histology and immunohistochemistry (cleaved caspase-3), to determine the percentage of cells undergoing apoptosis. Histological examination revealed that the slow freezing group exhibited a significantly higher percentage of intact follicles (45.75%) compared to those subjected to vitrification (38.17%; P=0.01). Immunohistochemical evaluation further indicated that 84.21% of the follicles in the slow freezing group did not express caspase-3, suggesting the absence of apoptosis. Conversely, vitrified samples exhibited significantly more apoptotic cells compared to other groups (P <0.001). Furthermore, early antral follicles displayed a higher susceptibility to degeneration regardless of the cryopreservation method employed. Nevertheless, when comparing the cryopreserved groups, early antral follicles showed greater degeneration in slow freezing group, while preantral follicles were the most affected in the vitrification group. In conclusion, slow freezing demonstrated superior preservation of viable follicles compared to vitrification and emerged as the preferred technique for cryopreserving canine ovarian tissue. These findings contribute valuable insights into optimizing cryopreservation methods for canine ovarian tissue, potentially benefiting reproductive technologies and fertility preservation in canines.

Keywords: bitches, gonadal tissue, immunohistochemistry, caspase-3, histology.

1. Introduction

Assisted reproduction techniques, such as artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and the preservation of gametes and gonadal tissues, present promising solutions for overcoming obstacles in natural reproduction. While successful cryopreservation of gametes has been achieved across various species, the susceptibility of canine oocytes to low temperatures poses a challenge, impairing their viability post-thaw [1]. Ovarian tissue cryopreservation, a more intricate process than gamete freezing, involves a diverse range of cell types with varying susceptibilities to cold-induced damage. However, research has indicated the potential for follicle development within cryopreserved ovarian tissue, progressing from early to advanced stages [2-4]. Nonetheless, investigations into this topic within the *Canidae* family remain scarce, and an optimal cryopreservation protocol has yet to be established.

Investigations on ovarian tissue cryopreservation in canine species holds crucial importance while awaiting the development of an improved *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) system, which, when available, could revolutionize assisted reproduction in dogs. The ability to successfully preserve and store ovarian tissue until the ideal system becomes available is particularly valuable for preserving breeds with exceptional characteristics, such as special guide dogs, enabling their genetics to contribute to future generations despite natural reproductive limitations. This approach not only benefits canine genetic resources but also plays a vital role in safeguarding endangered canid species, contributing to conservation efforts and supporting future breeding and reintroduction programs.

In light of this, our study evaluates two widely used techniques: slow freezing and vitrification, in preserving intact follicles. Slow freezing involves controlled cooling to prevent ice crystal formation and can damage cellular structure, while vitrification employs rapid cooling to transition cells into a glass-like state, minimizing ice crystallization. Both methods are chosen due to their distinct mechanisms of protection and potential to preserve cell viability, offering a comprehensive evaluation of their effectiveness in maintaining follicular integrity. Through morphological and immunohistochemical assessments of ovarian fragments obtained from domestic dogs, we aim to identify variances in cryotolerance among different follicular types, informing the development of optimized protocols for canine ovarian tissue preservation.

2. Material and methods

All chemicals in this study were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA) unless otherwise stated.

The study was approved by the Ethical Committee of the Institution (3171/2020).

2.1. Collection and processing of ovaries

Ovaries were obtained from 14 domestic bitches in late anoestrus submitted to elective ovariohysterectomy. The dogs were from different breeds, with ages ranging from 2 to 5 years, and were considered healthy after clinical examination.

The ovaries were transported to the laboratory at room temperature in Dulbecco´s PBS-PVA (phosphate-buffered saline with polyvinyl acetate) solution supplemented with 100 IU/mL of penicillin G sodium and 100 µg/mL of streptomycin sulphate, within 1-3h. Then, they were sectioned into small fragments of approximately 3 x 2 x 2 mm in length, width, and thickness, respectively.

Twenty-eight ovaries were sectioned into 75 fragments. Each ovary was treated as an individual unit, and the fragments were randomly assigned to Vitrification, Slow freezing, and Control groups.

2.2. Vitrification

Vitrification was performed according to [5]. Briefly, the ovarian cortex fragments were immersed in drops of 200 µL of modified Dulbecco´s PBS containing 1M DMSO at room temperature for 60 sec. Following this, one fragment was transferred to each cryotube containing 5 µL of PBS-DMSO and kept at 0 °C for 5 min. Next, 95µL of DAP 213 solution (composed of 2 M DMSO, 1 M acetamide, 3 M propylene glycol) was added to each cryotube and maintained at 0 °C for an additional 5 min. Subsequently, the cryotubes were plunged one at a time into liquid nitrogen, ensuring that each cryotube was completely submeged for storage.

For warming, after being kept at room temperature for 60 sec until the media was completed melted, the cryotubes were diluted in 900µL of PBS containing 0.25 M sucrose preheated to 37 °C. During this process, each cryotube was individually held in tongs in the air, one by one. Subsequently, the samples were washed repeatedly (5x) in HTF (human tubal fluid) with BSA (bovine serum albumin) medium before undergoing histological processing for further evaluation.

2.3. Slow Freezing

Slow freezing was performed according to the protocol described by [6] with modifications. The ovarian tissue fragments were equilibrated in cryotubes containing 1mL of Ham´s F10 medium, 1.5 M DMSO (dimethyl sulfoxide), 10% HSA (human synthetic albumin), and 0.1 M sucrose for 30 min at 4 °C. After this period, the samples were frozen using a programmable freezer (Cryogen; Neovet, Uberlândia, Brazil), being cooled to -7 °C at a rate of -2 °C/min, when the seeding was done. Then, the cooling rate decreased to -0.3 °C/min until it reached -30 °C when the cryotubes were immersed directly into the liquid nitrogen for storage.

For thawing, the cryotubes were individually exposed to room temperature for 2 min and then placed in a water bath at 38 °C for additional 2 min. Each vial was singly held in tongs during this process. Samples were then washed repeatedly in Ham´s F10 medium and submitted to histological processing for further evaluation.

2.4. Histological evaluation

All ovarian tissue samples from each experimental group (vitrified, slow frozen, and fresh) were fixed in 4% formaldehyde. For the control groups, fixation was performed immediately after the ovaries were sectioned. For the vitrified and slow frozen groups, fixation was carried out immediately after the samples were thawed and equilibrated. The fixed samples were then embedded in paraffin, sectioned in a series of 5 µm thickness, stained with hematoxylin-eosin (HE), and evaluated under a light microscope.

The developmental stage of each follicle was identified and classified into three categories based on [7]: (a) primordial follicles: oocytes surrounded by a single layer of flattened granulosa cells; (b) preantral follicles: oocytes surrounded by a layer or two or more completed layers of cuboidal granulosa cells; c) Early antral follicles: the presence of a small antrum within the multilaminar follicle.

Morphological alterations were classified according to [8] as follows: intact follicles were scored as 0; follicles presenting detachment of cells from the basement membrane (discrete degeneration) were scored as 1; follicles showing detachment of cells from the [basement membrane and degeneration of up to 30 % of oocytes (moderate degeneration) were scored as 2; follicles presenting accentuated degeneration with more than 30 % degenerated oocytes were classified as score 3.

2.5. Immunohistochemistry

Fresh and cryopreserved samples were evaluated to apoptosis rate following the procedures described by [8]. Briefly, tissue samples were mounted on positive-charged slides, deparaffinized in xylene, and hydrated in ethanol. The antigen retrieval was performed using citrate buffer (Target retrieval solution, Agilent, Santa Clara, CA, USA) in a pressure cooker. Samples were washed in PBS with 0.5 % tween 20 and the endogenous peroxidase activity was blocked with 3 % hydrogen peroxide, followed by another wash in PBS. To prevent non-specific antigen binding, the samples were incubated for 30 minutes at room temperature with 10 % goat serum. The rabbit primary anti-cleaved caspase 3 antibody (Asp175, code: #9661, Cell Signaling, Danvers, MA, USA) was placed and the samples remained overnight at 4°C. Polyclonal biotinylated immunoglobulin was used as the secondary antibody. The previous literature has provided the antibody cross-reactivity with canine tissue [9] [10] [11].

A cleaved caspase-3 antibody was used to detect apoptosis in the follicles. The number of follicles positive for caspase-3 was determined by the number of cells with stained nucleus or cytoplasm. For the negative control, the primary antibody was removed. Five fields were counted to establish the percentage of marked cells under 400x magnification. The final score was achieved using the methodology previously described [12]. Briefly, samples showing no caspase-3 expression were scored as 0, samples showing 1 up to 25 % were scored as 1, samples with 26 up to 50 % was scored 2, 51 % up to 75 % as score 3 and more than 75 %, as score 4.

2.6. Statistical analysis

Statistical analysis of the histological evaluation of the proportion of primordial and primary follicles with normal morphology was calculated per sample. Immunohistochemical evaluation was calculated as the proportion of follicles stained by caspase-3 per sample. The analysis was performed by Kruskal-Wallis test followed by Dunn's multiple comparison test. Values were considered significant when $P < 0.05$.

3. Results

3.1. Morphological evaluation of follicles

A total of 2,004 ovarian follicles were evaluated in the three experimental groups; 461 in the slow freezing group (196 primordial, 215 preantral follicles, and 50 early antral follicles; corresponding to 42.5 %, 46.7 %, and 10.8 %, respectively), 762 in the vitrified group (366 primordial, 313 preantral follicles and 83 early antral follicles; corresponding to 48 %, 41 %, and 11 %, respectively) and 781 in the control group (269 primordial, 414 preantral follicles and 98 early antral follicles; corresponding to 34.4 %, 53 %, and 12,6 %, respectively).

The results indicate that both slow frozen and vitrification methods resulted in higher rates of degeneration compared to fresh samples (54.2 %, 61.8 %, and 40.7 %, respectively; $p < 0.001$). When analysing follicular types, it was observed that the slow freezing group exhibited fewer degenerated primordial and preantral follicles compared to vitrification. Conversely, vitrification demonstrated fewer degenerated early antral follicles (Table 1). The main morphological alterations evaluated are illustrated in figure 1.

Figure 2 illustrates the relationship between degenerated follicles and normal follicles, in which degenerated follicles were considered as 1 and normal follicles as 0. Comparing the different groups in relation to primordial follicles it was noticed that the slow freezing and vitrification groups differ significantly from control ($p<0.001$), but not from each other. Concerning the preantral follicles, the vitrification group had the highest average of degeneration ($p < 0.0003$), while for early antral follicles the higher degeneration rates were observed in the slow freezing group ($p < 0.001$).

3.2. Immunohistochemical evaluation.

A total of 75 ovarian cortex was used for the evaluation of the caspase-3 expression. All fragments of the control group had 100 % follicles with a score 0 (Table 2). In the slow freezing group, 84.21 % of the evaluated follicles did not express caspase-3 (score 0, figure 3A and 3B) and in the vitrified group, 53 % of the early antral follicles, 56 % of the preantral follicles, and 62.5 % of the primordial follicles did not express caspase-3, and this group presented greater percentage of total damage (Figure 3C and 3D).

4. Discussion

In our study, the morphological evaluation revealed that slow freezing using 1.5M DMSO was more effective than vitrification in maintaining follicular integrity, as evidenced by a lower degeneration rate (54.2% vs. 61.8%). This finding is consistent with other studies in humans [13], and sheep [14], where slow freezing showed superior results compared to vitrification. Specifically, in bitches, Lopes et al. [15], reported that 1.5M DMSO was superior to propanediol for slow freezing, although still less effective than untreated controls. In contrast, Commin et al. [16] found no significant differences in canine follicular morphology between pre- and post- slow freezing.

Compared to previous reports, the post-thawing/warming degeneration rates observed in the present study were higher across all techniques. Notably, even control group follicles showed a degeneration rate of 40.7%, significantly higher than the rates reported by Commin et al. [16] (11%), Lopes et al. [15] (20%), and Jivago et al. [17] (approximately 10%). Several factors may account for these higher degeneration rates: 1) methodological differences: variations in fragment sizes, fixation solution, and fixation time may have contributed. If the fixation process did not optimally preserve the follicles, it could result in higher rates of degeneration; 2) Sample size: our study evaluated 28 ovaries, whereas other studies assessed fewer than 10. A larger sample size may capture a broader variability, potentially including more degenerated follicles; 3) Age of the animals: we used bitches aged 2 to 5 years, whereas other studies used animals under 2 years of age. Younger animals generally have a more robust follicular reserve, especially of early-stage follicles, which are smaller with low metabolic activity-traits that enhance cryopreservation tolerance [4]; 4) Estrus cycle stage: we exclusively used ovaries from bitches in late anestrus. Although this provides a uniform baseline for follicular size and activity, potentially facilitating cryopreservation standardization, it limits insights into preserving more advanced antral follicle stages; 5) Transport temperature: unlike others that transported samples at 4°C, allowing better preservation through slowed metabolic processes, our study maintained room temperature during transport, which could adversely affect tissue viability. Additionally, ovarian tissue might be sensitive to handling and manipulation, in line with findings on testicular fragments in cats [18].

In analyzing results by follicular type, vitrification and slow freezing both exhibited higher degeneration percentages compared to controls; however, they varied in their effectiveness depending on the follicular type preserved. Vitrification was particularly effective at preserving early antral follicles, whereas slow freezing minimized damage to preantral follicles. This may be due to the differing susceptibilities of follicular types to ice crystal formation. Early antral follicles, which are larger and more susceptible to ice-crystal induced harm, benefit from vitrification´s rapid cooling (that reduces crystal formation). Conversely, smaller preantral follicles, potentially more resilient to ice formation, may adapt better to slow freezing´s gradual temperature changes.

Currently, only two studies have directly compared vitrification and slow-freezing techniques in canine ovarian tissue. Jivago [17] found vitrification superior in preserving primordial and primary follicle ultrastructure, whereas Fujihara [19] observed that both techniques maintained follicular morphology, but only vitrified follicles retained morphology up to 5 weeks after xenotransplantation. Variability in protocol details, such as cryoprotectants associations and device usage, was significant across these studies. Our study utilized cryotubes for both methods, unlike Jivago´s use of a solid surface and Fujihara´s needle immersion. Cryotubes, made from low thermal conductivity materials like polypropylene, may slow cooling rates, affecting vitrification efficiency [20]. In contrast, needle immersion, an open system made from stainless steel, enhances rapid heat exchange, facilitating both cooling and warming rates. However, cryotubes provide a closed system advantage, preventing contamination and simplifying long-term liquid nitrogen storage. Additionally, the choice of cryoprotectants, including DMSO, remains critical. Most canine ovarian tissue cryopreservation studies, including ours, vary in cryoprotectant concentration and combination, limiting result comparability across research. Nonetheless, literature asserts that DMSO is generally safe as a cryoprotectant alone or combined, particularly in lower concentrations [21]. It is suitable for ovarian tissue preservation due to its lower molecular weight, which allows for faster penetration into the tissues compared to other cryoprotectants [22]. This rapid permeation is critical, as the extent of follicular survival is at least partly determined by how quick the cryoprotectant can diffuse into the cells, thus minimizing ice crystal formation and cellular damage.

According to Faustino et al. [23], although classical histological assessment is popular due to affordability and the ability to evaluate large follicle numbers, it only detects advanced signs of atresia, such as nuclear pyknosis, cytoplasmic damage, granulosa cell detachment, and basement membrane damage. Apoptosis, the primary underlying mechanism of follicular degeneration, plays a crucial role in tissue homeostasis [24]. Thus, immunohistochemistry for apoptosis rate evaluation can identify earlier tissue impairment stages [8], serving as a marker for cryopreservation protocol efficacy. Our immunohistochemistry data revealed that the slow-freezing group had a higher proportion of cells without apoptosis (84.21%) compared to the vitrified group (53.12% to 62.5%, depending on the follicular type). Conversely, the vitrified group showed a higher percentage of damage, reflected in the score 4, where total damage exceeded 36%. Similarly, Rahimi et al. [25] reported more apoptotic cells in vitrified human ovarian tissues compared to those preserved using slow freezing. In contrast, Hariya & Suzuki [26] found no apoptotic cells in primordial vitrified follicles in evaluations immediately after thawing using material from prepubertal bitches.

Thus, taking the caspase and histological evaluation results together, slow freezing emerges as the method that causes less damage and impairment to cryopreserved tissue. Moreover, our results corroborate what has already been reported by our research group concerning feline ovarian tissue [8], i.e. that morphologically normal follicles were positive for caspase-3. This finding may constitute confirmation of what has been reported by other researchers: follicles that are considered intact through morphological evaluation after cryopreservation/thawing may lose their developmental capacity, regardless of the technique used [17]. These findings underscore the imperative of additional assessment methods beyond histological analysis to accurately gauge cryopreservation-induced damage.

Culturing of ovarian tissue and xenografting in immunosuppressed mice are procedures of choice for verifying the viability of post-cryopreserved tissue. In humans, these methods have culminated in reports of live births following slow-frozen ovarian tissue autotransplantation [27], with over 100 documented cases worldwide. However, the requisite expertise for such procedures is possessed by only a few laboratories globally, limiting broader implementation and progress. Literature suggests slow-freezing techniques typically yield the best results. Conversely, vitrification, although requiring further refinement, presents significant potential for expansion due to its simplicity, speed, cost-effectiveness, and minimal equipment requirements, allowing wider adoption even in basic laboratory settings.

Conclusion

In conclusion, our study underscores the superiority of slow freezing as the optimal technique for preserving canine ovarian tissue. The impressive rates of follicular integrity and low levels of apoptosis observed with this method demonstrate its efficacy in maintaining the viability of ovarian tissue. Additionally, our findings reveal the differential cryotolerance of various follicular types, with early antral follicles favoring vitrification for improved preservation, while slow freezing proves more beneficial for safeguarding preantral follicles.

The successful cryopreservation of canine ovarian tissue holds immense promise for advancing assisted reproduction techniques in dogs. With the ongoing advancements in reproductive technologies, having cryopreserved ovarian tissue readily available will be crucial in unleashing the full potential of future IVM and IVF systems. This invaluable resource will not only ensure the preservation of genetic material from exceptional dog breeds but also contribute to the conservation of endangered canid species.

Acknowledgments

The authors would like to thank Roberta Vantini and José Luiz Souza for their technical and laboratory support.

This work was supported by São Paulo Research Foundation (FAPESP) [grant number 2017/23124-8] and Coordination for the Improvement of Higher Education Personnel (CAPES).

Conflict of interest

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

Author contributions

MA, NALS, FRO and GCL have designed the study and participated in the acquisition, interpretation of data, and drafting of the manuscript. RAR, CBMLF, MRT, PM, FFS, CEFA, and MC have participated in the acquisition and interpretation of data. All authors have read and approved the final version of the manuscript.

References

[1] Turathum B, Saikhun K, Sangsuwan P, Kitiyanant Y. Effects of vitrification on nuclear maturation, ultrastructural changes and gene expression of canine oocytes. Reprod Biol Endocrinol. 2010; 8: 70.

[2] Bosch P, Hernandez-Fonseca HJ, Miller DM, Wininger JD, Massey JB, Bracket BG. Cryopreservation and development of antral follicles in cat ovarian tissue after transplantation into NOD-SCID mice. Theriogenology. 2002; 57:62.

[3] Oktay K, Newton H, Mullan J, Gosden RG. Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle-stimulating hormone. Hum Reprod. 1998; 13:1133–1138.

[4] Gosden RG, Boulton MI, Grant K, Webb R. Follicular development from ovarian xenografts in SCID mice. J Reprod Fertil. 1994; 101:619–623.

[5] Ishijima T, Kobayashi Y, Lee DS, Ueta YY, Matsui M, Lee JY, Suwa Y, Miyahara K, Suzuki H. Cryopreservation of canine ovaries by vitrification. Journal of Reproduction Development. 2006; 52:293-299.

[6] Lima AKF, Silva AR, Santos RR, Sales DM, Evangelista AF, Figueiredo JR, Silva LDM. Cryopreservation of preantral ovarian follicles in situ from domestic cats (*Felis catus*) using different cryoprotective agents. Theriogenology. 2006; 66: 1664–1666.

[7] De los Reyes M, Palomino J, Parraguez VH, Ramirez F. Analysis of LH receptor in canine ovarian follicles throughout the estrous cycle. Theriogenology. 2017; 93:71–77.

[8] Gorricho CA, Tavares MR, Apparicio M, Fonseca-Alves CE, Macente BI, Mansano CFM, Toniollo GH. Vitrification of cat ovaries tissue;does fragment size matters? Reproduction in Domestic Animals. 2018; 53:125-132.

[9] Penzo-Méndez AI, Chen YJ, Li J, Witze ES, Stanger BZ. Spontaneous Cell Competition in Immortalized Mammalian Cell Lines. PLoS One. 2015; 10(7): e0132437.

[10] Pfankuche VM, Sayed-Ahmed M, Contioso VB, Spitzbarth I, Rohn K, Ulrich R, Deschl U, Kalkuhl A, Baumgärtner W, Puff C. Persistent Morbillivirus Infection Leads to Altered Cortactin Distribution in Histiocytic Sarcoma Cells with Decreased Cellular Migration Capacity. PLoS One. 2016; 11(12):e0167517.

[11] Ganier O, Schnerch D, Nigg EA. Structural centrosome aberrations sensitize polarized epithelia to basal cell extrusion. Open Biol. 2018; 8(6):180044.

[12] Fonseca-Alves CE, Rodrigues MM, de Moura VM, Rogatto SR, Laufer-Amorim R. Alterations of C-MYC, NKX3.1, and E-cadherin expression in canine prostate carcinogenesis. Microsc Res Tech. 2013; 76(12):1250-1256.

[13] Dalman A, Farahani NSDG, Pirjani R, Ebrahim B, Valojerdi MR. Slow freezing versus vitrification technique for human ovarian tissue cryopreservation: An evaluation of histological changes, WNT signaling pathway, and apoptotic genes expression. Cryobiology. 2017; 79:29-36.

[14] Vatanparast M, Khalili MA, Yarin N, Omidi M, Mohsenzadeh ME. Evaluation of sheep ovarian tissue cryopreservation with slow freezing or vitrification after chick embryo chorioallantoic membrane transplantation. Cryobiology. 2018; 81:178-184.

[15] Lopes CAP, Alves AMCV, Jewber K, Báo SN, Figueiredo JR. Cryopreservation of Canine Ovarian cortex using DMSO or 1,3 propanediol. Theriogenology. 2016; 86(5):1165-1174.

[16] Commin A, Buff S, Rosset E, Galet C, Allard A, Bruyere P, Joly T, Guerin P, Neto V. Follicle development in cryopreserved bitch ovarian tissue grafted to an immunodeficient mouse. Reprod, Fert. Develop. 2012; 24:461–471.

[17] Jivago JLPR, Paulini F, Silva RC, Araujo MS, Marinho APS, Marinho CM, Lucci CM. Cryopreservation and characterization of canine preantral follicles. Cryobiology. 2018; 81:34-42.

[18] Macente BI, Apparicio M, Mansano CFM, Tavares MR, Fonseca-Alves CE, Sousa BP, Bertolo PHL, Vasconcelos RO, Teixeira ES, Toniollo GH. Effect of cryopreservation on sperm DNA fragmentation and apoptosis rates in the testicular tissue of domestic cats. Animal Reproduction Science. 2019; 211: 106224.

[19] Fujihara M, Kaneko T, Inoue-Murayama M. Vitrification of canine ovarian tissues with polyvinylpyrrolidone preserves the survival and developmental capacity of primordial follicles. Scientific Reports. 2019; 9: 3970.

[20] Sansinena M, Santos MV, Zaritzky N, Chirife J. Comparison of heat transfer in liquid and slush nitrogen by numerical simulation of cooling rates for French straws used for sperm cryopreservation. Theriogenology. 2012; 77, 1717–1721.

[21] Cury-Silva TE, Nunes MEG, Casalechi M, Comim FV, Rodrigues JK, Reis FM. Cryoprotectant agents for ovarian tissue vitrification: Systematic review. Cryobiology. 2021; 103: 7–14.

[22] Borges EN, Silva RC, Futino DO, Rocha-Júnior CMC, Amorim CA, Báo SN, Lucci CM. Cryopreservation of swine ovarian tissue: Effect of different cryoprotectants on the structural preservation of preantral follicle oocytes. Cryobiology. 2009; 59 (2): 195-200.

[23] Faustino LR, Silva CMG, Rosseto R, Rodrigues GQ, Figueiredo JR, Rodrigues APR. Estado atual e desafios da criopreservação de tecido ovariano em mamíferos. Bras. Reprod. Anim. 2011; 35(1): 315.

[24] Husueh AJW, Billig H, Tsafriri A. Ovarian Follicle Atresia: A Hormonally Controlled Apoptotic Process. Endocr. Rev. 1994; 15 (6):707-724.

[25] Rahimi G, Isachenko V, Todorov P, Taawadros S, Mallmann P, Nawroth F, Isachenko E. Apoptosis in human ovarian tissue after conventional freezing or vitrification and xenotransplantation. CryoLetters. 2009; 30 (4):300-309.

[26] Hariya M, Suzuki H. Incidence of Apoptotic Cells After Vitrification in Canine Ovarian Tissues. J. Mamm. Ova Res. 2016; 33(1): 69-75.

[27] Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squiffl J. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet. 2004; 364:1405 – 1410.

Figures

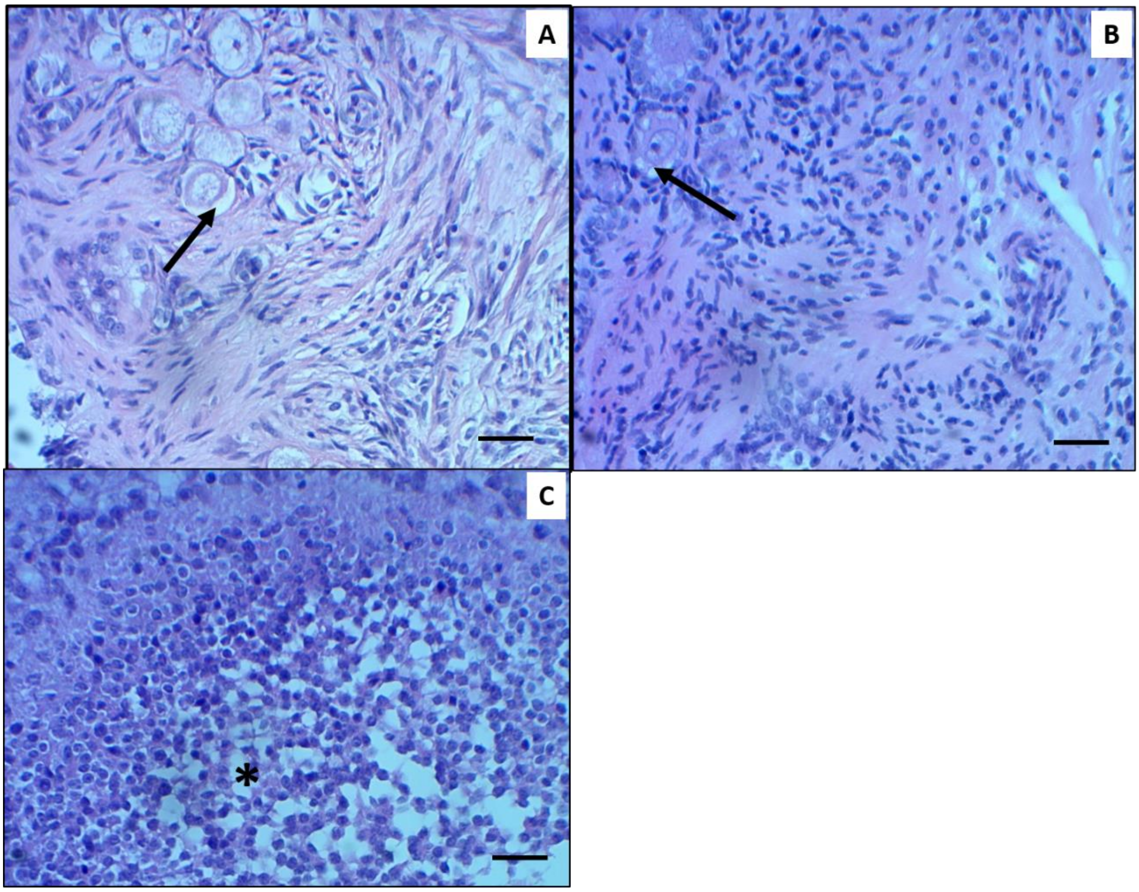

Fig 1. Photomicrograph of canine ovarian tissue submitted to cryopreservation, illustrating the main morphological changes. **A.** Detachment of basal membrane in the primordial follicle (arrow). **B.** Presence of vacuolizations in the primordial follicle (arrow). **C.** Presence of vacuolizations in the early antral follicle (*). Bars represent 50 μm. H.E. staining.

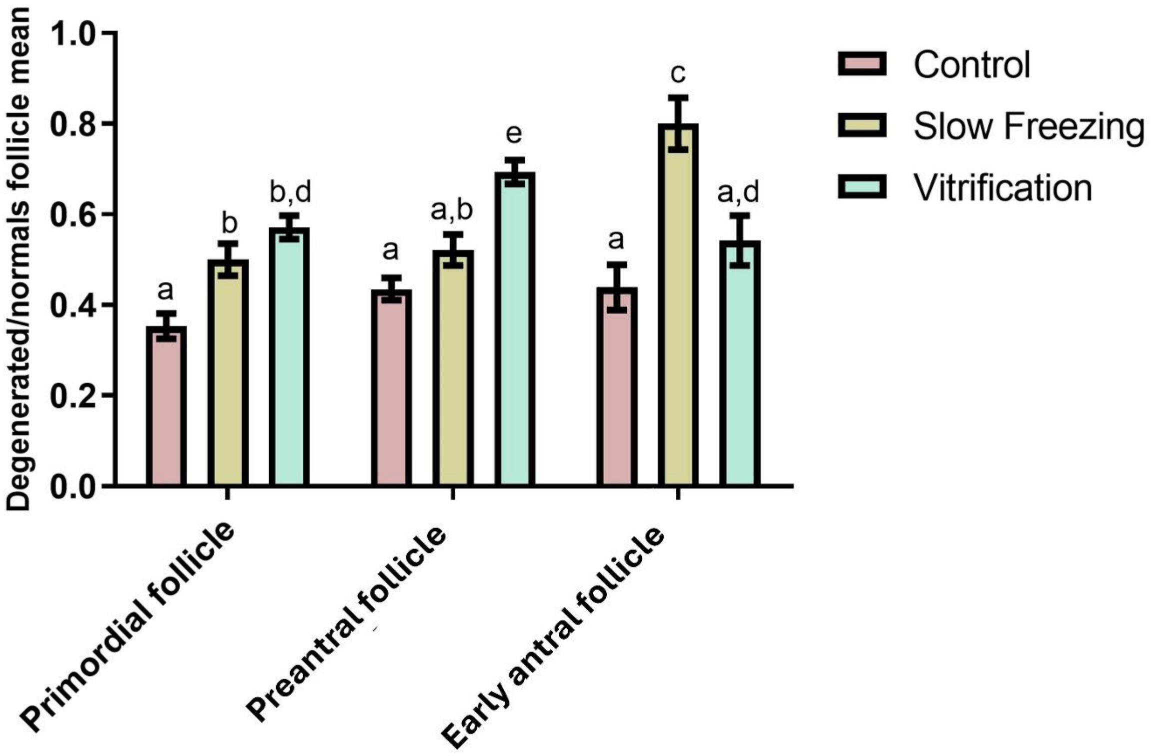

Fig 2. Mean ± standard error of the relationship between degenerated follicles and normal follicles obtained from canine ovarian tissue before (control) and after cryopreservation (slow freezing and vitrification). Analysis by the Kruskal-Wallis test followed by the Dunn's multiple comparison test. Different letters were considered significant for $p<0.05$.

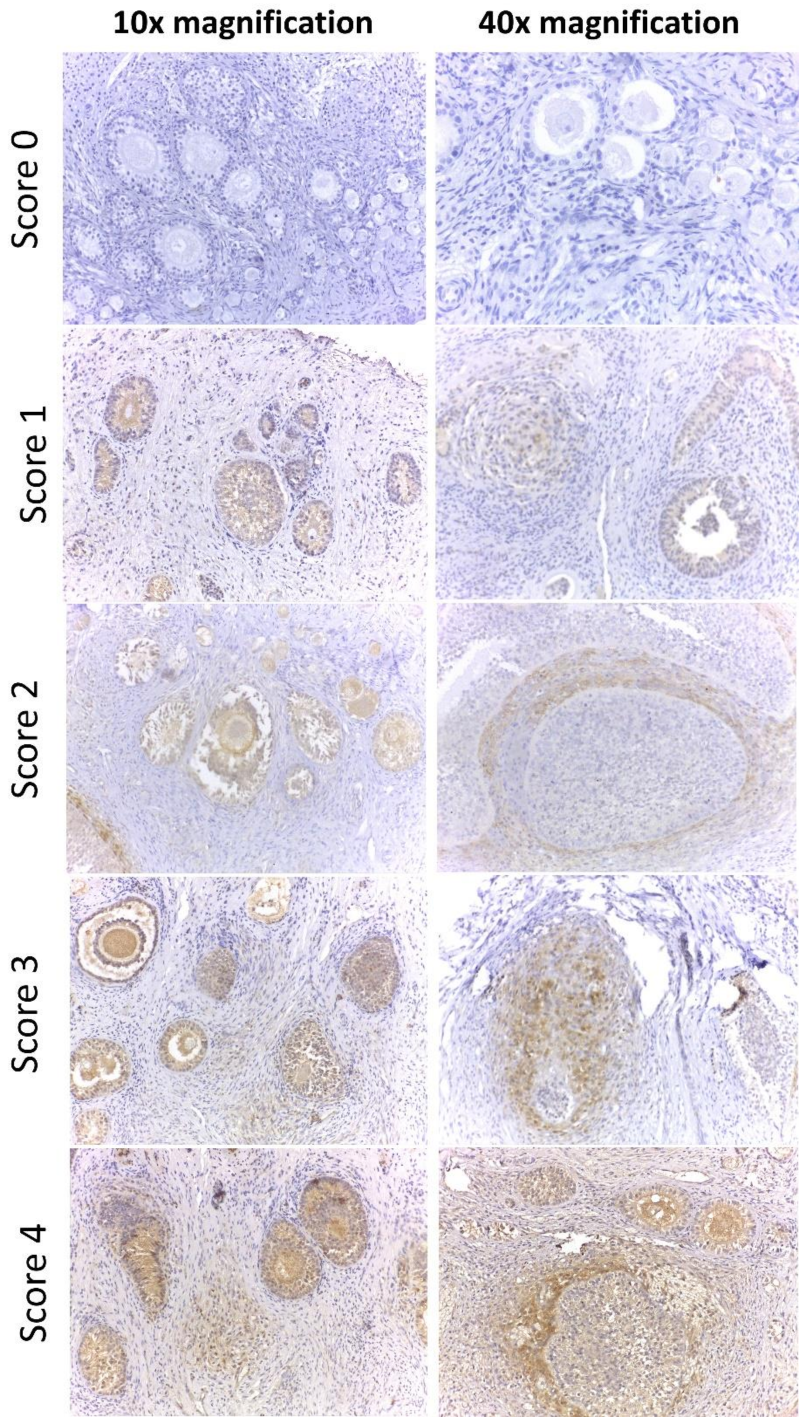

Fig 3. Photomicrograph of canine ovarian cortex submitted to cryopreservation and evaluated by immunohistochemistry for expression of cleaved caspase-3. Score 0 = absence of nuclear and cytoplasmic labeling in follicles; score 1= up to 25 % of marked cells; score 2= 26 % up to 50 % of marked cells ; score 3=51 % up to 75 %; Score 4 = more than 75 % of follicles labeled for caspase-3.

Tables

Table 1. Absolute and relative number (%) of primordial, preantral, and early antral follicles included in ovarian tissues before (control) and after cryopreservation (slow freezing and vitrification) morphologically evaluated by H&E stain.

Groups	**Primordial follicles**		**Preantral Follicles**		**Early antral Follicles**	
	Normal	**Degenerated**	**Normal**	**Degenerated**	**Normal**	**Degenerated**
Control (n=781)	174 (22.27)^{a}	95 (12.16)^{a}	234 (29.96)^{a}	180 (23.04)^{a}	55 (7.04)^{a}	43 (5.50)^{a}
Slow freezing (n=461)	98 (21.25)^{b}	98 (21.25)^{b}	103 (22.34)^{b}	112 (24.29)^{b}	10 (2.16)^{b}	40 (8.67)^{b}
Vitrification (n=762)	157 (20.60)^{c}	209 (27.42)^{c}	96 (12.59)^{c}	217 (28.47)^{c}	38 (4.98)^{a}	45 (5.90)^{a}

n = Number of follicles per group. a,b,c Different superscript letters in the same column differ from each other by the Kruskal Wallis test, $p < 0.05$.

Table 2. Immunohistochemical evaluation of canine ovarian follicles by caspase-3 expression.

GROUPS	**Follicles**	**EVALUATION OF APOPTOSIS RATES**					
		Score 0 (0-5%)	**Score 1 (6-25%)**	**Score 2 (26-50%)**	**Score 3 (51-75%)**	**Score 4 (>75%)**	**% Total damage**
Fresh (n=24)	**Primordial**	24 (100%)	0	0	0	0	0^{a}
	Preantral	24 (100%)	0	0	0	0	0^{a}
	Early antral	24 (100%)	0	0	0	0	0^{a}
Slow freezing (n=19)	**Primordial**	16 (84.21)	0	0	2 (10.53)	1 (5.26)	13.16^{b}
	Preantral	16 (84.21)	0	1 (5.26)	0	2 (10.53)	13.16^{b}
	Early antral	16 (84.21)	0	1 (5.26)	0	2 (10.53)	13.16^{b}
Vitrification (n=32)	**Primordial**	20 (62.5)	0	0	1 (3.12)	11 (34.37)	36.72^{c}
	Preantral	18 (56.25)	0	0	1 (3.12)	13 (40.62)	42.97^{c}
	Early antral	17 (53.12)	0	0	2 (6.25)	13 (40.62)	45.31^{c}

Kruskal-Wallis analysis followed by Dunn´s multiple comparisons, considering $p < 0.05$.