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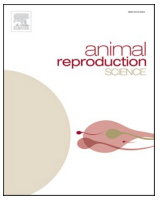

In vitro culture of canine preantral follicles after slow freezing

Bence Somoskői [a,1,*], Dóra Török [a,1], Giovanni M. Lacalandra [b], Lilla Bordás [a], Sándor Cseh [a]

[a] Department of Obstetrics and Food Animal Medicine Clinic, University of Veterinary Medicine, Budapest, Hungary
[b] Department of Veterinary Medicine, University of Bari "Aldo Moro", Bari, Italy

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ABSTRACT

The objective of this study was to analyse the effect of slow freezing on the morphology and viability of canine isolated preantral follicles. Ovaries were collected from ovariohysterectomy bitches being in different ages. Preantral follicles were isolated from ovarian cortex using collagenase-based digestive solution and randomly divided into two groups: fresh control and slow frozen. Fresh and frozen/thawed preantral follicles were cultured individually in 100 µl drops of culture medium for 10 days at 38.5 °C with 6.5 % CO_2, and half of the medium was changed and samples from culture media were collected on Day 2, Day 5 and Day 10 for hormonal analysis. Post-thaw live cell rate, normal morphology rate, area change, and estradiol and progesterone production were examined. Frozen/thawed follicles have lower number of live cells than that of fresh ones (94.69 % (± 6.97) vs 98.58 % (± 1.71), respectively [$p < 0.05$]). Normal morphology rate was different only on Day2 (91.2 % in fresh and 50 % in frozen/thawed, $p < 0.05$) and showed decreasing tendency in both groups. Differences in follicular growth were found on Day5 and 10, when fresh follicles showed lower area than that of frozen/thawed ones (5.84 vs12.62 mm^2 [$p < 0.005$] and 6.47 vs 10.75 mm^2 [$p < 0.05$], respectively). Regarding the hormonal production, estradiol and progesterone concentrations were lower in frozen/thawed samples than that of fresh follicles throughout the culture period. In conclusion, our data suggest that slow freezing can provide canine follicular survival, however, the quality and viability of the follicles are reduced.

1. Introduction

In the field of assisted reproduction and conservation biology, there is an increased need to develop effective methods to store and maintain the reproductive potential of species that are either endangered or possess significant genetic value (Pukazhenthi et al., 2005; Herrick, 2019). The cryopreservation of preantral follicles has emerged as a promising fertility preservation approach. These immature follicles contain oocytes at an early developmental stage. Therefore, they are more resistant to freezing-induced damage compared to fully grown mature oocytes (Abedelahi et al., 2013; Kagawa et al., 2009). Since mammalian ovaries contain large number of preantral follicles, culturing frozen/thawed follicles could be a viable method to obtain a considerable quantity of oocytes. These oocytes can undergo maturation and fertilization in vitro, with the resulting embryos being suitable for either immediate transfer or

* Corresponding author.
E-mail addresses: somoskoi.bence@univet.hu (B. Somoskői), torok.dora@univet.hu (D. Török), giovannimichele.lacalandra@uniba.it (G.M. Lacalandra), bordas.lilla@univet.hu (L. Bordás), sandor.cseh@univet.hu (S. Cseh).
[1] These authors contributed equally.

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cryopreservation for future use (Temerario et al., 2023). While cryopreservation techniques for oocytes and embryos are well-established in species like humans and cattle, the cryopreservation of dog ovarian tissue, follicles, and oocytes has presented several unique challenges (Reynaud et al., 2020).

The cryopreservation of canine oocytes and follicles has predominantly focused on vitrification, with relatively few studies exploring the application of slow freezing in this context (Abe et al., 2008, 2010; Turathum et al., 2010). While slow freezing has been used successfully in other mammalian species, its application to canine reproductive material remains limited, and the outcomes have been inconsistent (Songsasen and Wildt, 2007). Slow freezing offers certain advantages over vitrification, particularly in reducing the risk of cryo-induced damage caused by osmotic stress, toxicity of high concentration of cryoprotectants and rapid cooling. Unlike vitrification, which relies on high concentrations of cryoprotectants to prevent ice crystal formation, slow freezing allows for a more controlled and gradual dehydration process, potentially preserving cellular integrity more effectively (Vajta, 2000). This approach may be particularly advantageous for complex structures like ovarian follicles, where the balance between cryoprotectant penetration and intracellular ice formation is critical (Luvoni and Colombo, 2020). However, the unique characteristics of canine oocytes, including their high lipid content and sensitivity to cryopreservation, present challenges that necessitate further optimization of slow-freezing protocols for this species (Nagashima et al., 2019; Nagashima and Songsasen, 2021).

Post-thawing survival and growth of preantral follicles in dogs have demonstrated promising outcomes, though the ability to achieve complete follicle maturation and successful fertilization remains limited (Luvoni and Colombo, 2020; Luvoni et al., 2005; Paris et al., 2004). Unlike other mammals, dogs have a prolonged proestrus and anestrus phase, which affects follicular dynamics and oocyte maturation timing (Reynaud et al., 2020). Moreover, canine oocytes are ovulated at the germinal vesicle stage and require significant in vivo maturation, making in vitro approaches more complex (Heru et al., 2004). To the best of our knowledge, there have been no documented live births resulting from cryopreserved dog oocytes or follicles. Dogs thus serve as pivotal models for understanding ovarian cryopreservation and advancing reproductive biology (Jewgenow and Songsasen, 2014).

In our previous experiment (Somoskői et al., 2023), the authors investigated the effect of different vitrification techniques (cryotube and open pulled straw) on the survival of canine preantral follicles after thawing. To complement previous findings, slow freezing was conducted as an additional cryopreservation approach. The objective of the study was to assess the post-thaw viability and function of preantral follicles with a hypothesis that slow freezing can serve as a suitable tool for preservation of canine PAFS, however, with different success rates than that of vitrification.

2. Methods

2.1. Collection of ovaries

Ovaries were collected from ovariohysterectomized bitches (N = 10; undefined crossbreds) being in different ages (2.57 ± 1,26 years). Ovariohysterectomies were carried out at the Small Animal Clinic of the Department of Obstetrics and Food Animal Medicine Clinic, University of Veterinary Medicine, Budapest. After collection, ovaries were placed in sterile 50 ml centrifuge tubes, containing filtered Dulbecco phosphate buffered saline (DPBS; Merck KGa, Darmstadt, Germany) + 10 % FBS (fetal bovine serum; Merck KGaA Darmstadt, Germany). Samples were stored at room temperature and delivered to the laboratory within 2 hours (Somoskői et al., 2023).

2.2. Isolation of preantral follicles

The ovarian cortex of each ovary was sliced to approximately 1 mm^2 pieces with a surgical blade, then placed in digestive solution (HEPES-modified Medium 199 + 3 mg/ml collagenase [both from Merck KGa, Darmstadt, Germany]) and incubated for 90 min at 37 °C. Following the enzymatic digestion, preantral follicles were isolated manually with 28G needles attached to 1 ml syringes. After isolation, morphologically normal secondary follicles (multiple layers of evenly distributed granulosa cells, centralized, circular, dark oocyte, intact basement membrane and lack of antrum-like structure; lack of highly dense granulosa cell) were selected and randomly divided into two groups: fresh control (n = 127) and slow frozen (SF; n = 97). The whole procedure was carried out under stereo-microscope (Olympus SZX7, Olympus Corporation, Tokyo, Japan).

2.3. Slow freezing and thawing

Slow freezing was carried out based on a protocol which is used for embryo cryopreservation (Somoskoi et al., 2015). Each experiment was carried out in 4 replicates.

Preantral follicles were equilibrated in medium containing 5 % glycerol (DPBS + 10 % FCS + 10 % glycerol, both from Merck KGa, Darmstadt, Germany) for 10 min on room temperature, followed by an equilibration in medium with 10 % glycerol. Then, the follicles (4/straw) were sucked up into conventional mini straws (Minitüb GmBH, Tiefenbach, Germany) which then were transferred into a Planer freezing machine (PLANER R 205, Planer, Sunbury-on-Thames, Middlesex UK) pre-cooled to minus 7°C. During a 10 min waiting period, the samples were allowed to cool down to minus 7 °C (3 °C/min). Once reached this temperature, artificial induction of the ice formation with a pre-cooled forceps was performed (seeding). After 10 min waiting, cooling down the follicles to minus 35 °C was performed with cooling rate 0.3 °C/minute. Finally, the straws containing the follicles were transferred into liquid nitrogen (LN2) and stored for one week. Thawing was performed by transfer the straws in warm water (25 °C) for 30 s. Cryoprotectant (CPA) was removed from the follicles in DPBS-based medium in 4 steps: 5 min in medium containing 6 % glycerol + 0.3 M sucrose; 5 min in

medium containing 3 % glycerol + 0.3 M sucrose; 5 min in medium containing 0.3 M sucrose and, finally, 5 min in DPBS + 20 % FCS (500 μl each).

2.4. Post-thaw viability of follicles and oocytes

Viability assessment was carried out based on our previous studies (Somoskői et al., 2023). After cryopreservation, several follicles from each group were randomly selected for viability assessment, which was performed with LIVE/DEAD Viability/cytotoxicity Kit (Thermo Fisher Scientific Inc, Waltham, USA), according to the manufacturer's protocol. Briefly, follicles were incubated in 100 μl drops containing DPBS (Merck KGa, Darmstadt, Germany) + 2 μM ethidium homodimer 1 + 4 μM calcein AM for 15 minutes. Following incubation, samples were washed in DPBS (Merck KGa, Darmstadt, Germany), then fixed with 4 % paraformaldehyde, and analysed with a fluorescent microscope (400X magnification; dual filter for green and red fluorescence; excitation 475–490 nm/emission 500–540 and 540–565/575–660, respectively) Olympus IX73, Olympus Corporation, Tokyo, Japan). Staining of fresh samples was carried out immediately after collection. Follicle viability was determined by calculating the ratio of live/dead cells. To separate the green (live cell) areas from the red ones (dead cells), threshold adjustment was made. Then, green area-to-whole follicle rate was calculated (based on pixel/area counts) in each sample and referred to as live cell rate. Image analysis was performed with Image J software (NIH, Bethesda MD, USA).

2.5. In vitro culture

Culture conditions were based on our previous studies (Somoskői et al., 2023). Fresh and frozen/thawed preantral follicles were cultured individually in 100 μl drops of culture medium, using 18-well culture dish (ibiTreat: #1.5 polymer coverslip, Ibidi GmBH, Grafelfing, Germany). Culture medium consists of Opti MEM (Thermo Fisher Scientific Inc, Waltham, USA), supplemented with 5 % FBS, 1 % ITS (ITS-G,100X, Thermo Fisher Scientific Inc, Waltham, USA), 0.5 % antibiotic-antimycotic solution (Thermo Fisher Scientific Inc, Waltham, USA) and 100 mIU/ml initial concentration of rFSH (R&D Systems Inc, Minneapolis, USA), which was elevated by 2-fold on every medium change (dynamic culture). Follicles were cultured for 10 days at 38.5 °C with 6.5 % CO_2, and half of the medium was changed on Day 2, Day 5 and Day 10.Samples from culture media (50 μl) were collected on the same days for hormonal analysis.

Rate of normal and abnormal follicles was analysed by calculating the rate of follicles showing atresia (irregular shape and dark, dense cell material) by Day 10.

In vitro growth (area change) of follicles was examined during the IVC period. Only those follicles were involved in the measurement that were considered to be live (not showing atresia). Simultaneously with the medium change, images were taken of each sample. To evaluate growth, follicular area was measured (mm^2) and analysed with Image J (NIH, Bethesda MD, USA), using the "Area Measurement" function.

Estradiol (17β-estradiol) production of each follicle was also evaluated. Aspirated culture medium was collected in Eppendorf tubes and stored at −20 °C until measurement. Concentration of estradiol in the samples was measured with ELISA (DE2693, Demeditec Diagnostics, Kiel, Germany; analytical sensitivity 10.6 pg/ml; intra-assay CV<5 %; inter-assay CVs were 14.9 % and 6.9 % for low and high controls, respectively). Beside estradiol production, progesterone concentration in each treatment group was measured with ELISA (DE1561, Demeditec Diagnostics, Kiel, Germany; analytical sensitivity 0.045 ng/ml; intra-assay CV<5 %; inter-assay CVs were 10 % and 5.6 % for low and high controls, respectively).

2.6. Statistical analysis

Data analysis was performed with version 4.1.1 of R (R Core Team, 2019).

Normality of data was analysed prior to the statistical analyses. Since none of the data showed normal distribution, we applied non-parametric methods. Therefore, in the case of growth and hormonal production, we calculated with the median instead of the mean values.

Differences in the in vitro growth and hormonal (estradiol and progesterone) production between treatments on Day 2, 5 and 10 were analysed with Mann-Whitney test.

Differences in the growth and hormonal (estradiol and progesterone) production within treatments were analysed with Friedman test, using post-hoc Wilcoxon rank sum test.

Differences in viability (live cell rate) between treatments were analysed with Wilcoxon rank sum test with continuity correction.

Chi-squared test was used to analyse the differences of in vitro survival rate among treatments.

Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Post-thaw viability

Post-thaw viability analysis showed that slow freezing resulted in lower number of live cells than that of follicles in control group (94.69 % [± 6.97] VS 98.58 % [± 1.71], respectively. $p < 0.05$, Wilcoxon rank sum test).

3.2. Morphology

Rate of follicles showing normal morphology can be found in Table 1. Differences between the two groups can only be found in the case of Day2, where the fresh follicles showed a higher rate of normal morphology. However, this difference disappeared by the end of the culture period. While the normal morphology rate decreased constantly in the fresh group, slow frozen/thawed follicles did not show this pattern.

Beside the daily change of normal morphology rate, we analysed the area of normal and abnormal follicles within each group (Fig. 1). Abnormal follicles showed lower size both in the fresh and frozen/thawed groups.

3.3. Follicular growth

During the 10-day long IVC, differences in follicular growth were found between the treatment groups, however, only from Day5 (Fig. 2). On Day 2 the median area of fresh and frozen/thawed follicles were similar (4.4 [range: 0.465–13.49] vs. 3.67 mm^2 [range: 0.4–6.99], respectively; $p = 0.52$). On Day 5, follicles of fresh control group showed lower area (5.84 mm^2 [range: 0.2–14.4]) than that of frozen/thawed follicles (12.62 mm^2 [range: 6.37–14.34], $p < 0.005$). Frozen/thawed follicles showed higher median on Day 10 than control ones (10.75 mm^2 [range: 2.66–14.14] vs. 6.47 mm^2 [range: 0.61–13.9], respectively, $p < 0.05$).

Regarding the area change within groups, both the fresh and SF follicles showed overall significant increasing from Day 0 to Day 10. However, PAFs reached their peak size by Day 5 and remained unchanged until Day 10 (Fig. 2).

3.4. Estradiol production

17-β-estradiol production of preantral follicles was overall remarkably higher throughout the 10-day long IVC period. However, the variances, analysed by Levene-test, were significantly lower in SF group ($p < 0.01$). (Fig. 3)

The changes in the estradiol production within the different treatment groups were analysed throughout the 10-day long IVC. Follicles of fresh control showed slightly lower level of estradiol production on Day 10 than on Day 2, while remained at the initial concentration in the SF group.

3.5. Progesterone production

Progesterone production of preantral follicles did not differ on Day2, but significantly lower in SF group on Day 5 and Day10. (Fig. 4)

Changes of progesterone concentrations within the different treatment groups were analysed throughout the 10-day long IVC. Control follicles showed a peak concentration on Day 2, although it decreased on Day 10. Slow-frozen follicles did not show this tendency, and hormonal production remained unchanged.

4. Discussion

In this study, we analysed the effect of slow freezing on canine preantral follicles from different aspects. We found lower live cell rate in slow frozen PAFs after thawing than that of control (94.7 % vs 98.6 %, respectively). These data are in contrast with our previous findings with vitrified PAFs, where OPS method provided similar live cell rate of thawed samples to control ones (80.3 % (± 23.5) vs 83.6 % (± 17.6), respectively) (Somoskői et al., 2023). When samples were cryopreserved in different holder (cryotube), however, cell rate decreased to 58.7 %. In a recent study by Hartzler et al. (2023), ovarian tissue slices of African painted dog were cryopreserved with slow freezing, conducted in cryotubes with media routinely used in vitrification protocols (DMSO + EG and DMSO +EG + sucrose supplementation). They found that slow freezing in case of both freezing solutions induced elevated apoptotic index and increased the non-viable follicle rate. These data show that slow freezing in conventional straws is a superior technique to cryotube freezing to preserve the highest number of viable granulosa cell, which is a key factor to support the developing oocyte.

Despite many attempts to find the most appropriate cryopreservation method which prevents cryodamage in canine preantral follicles, most of the studies examined the survival of follicles within ovarian slices. In a recent study by Luizari Stábile et al. (2024), morphology of follicles was analysed following thawing of cryopreserved canine ovarian tissue. Authors found 55.7 % normal morphology rate in control/fresh ovarian slices and 47.9 % in slow-frozen samples, which resulted in significant decreasing. The same

Table 1
Rate of follicles showing normal morphology in control and slow freezing groups throughout the 10-day long culture period. (A,B) $p < 0.05$, differences between treatments within the same day; (a,b,c) $p < 0.05$, differences between days within control and slow frozen groups, respectively. Fisher's exact test. SF=slow freezing.

Treatment	D2	D5	D10
Control	91.2 %$^{A, a}$ (57/62)	77.4 %^{A,b} (48/62)	54.8 %^{A,c} (34/62)
SF	50 %^{B,a} (12/24)	58.4 %^{A,a} (14/24)	37.5 %^{A,a} (9/24)

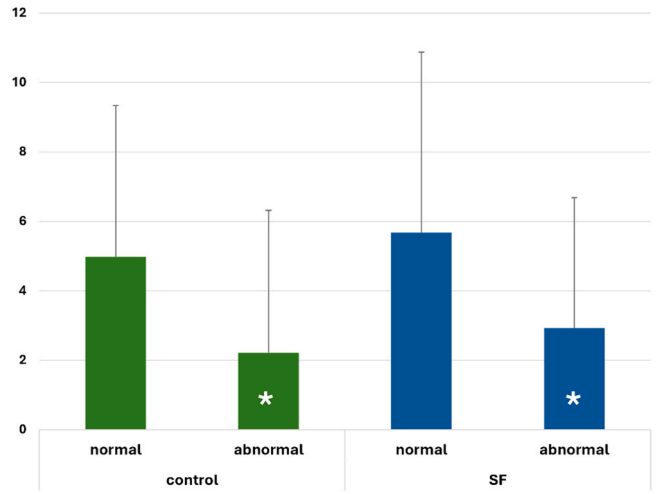

Fig. 1. Mean area of normal and abnormal follicles in control and slow frozen groups. (*) $p < 0.05$, difference within treatment, Mann-Whitney test.

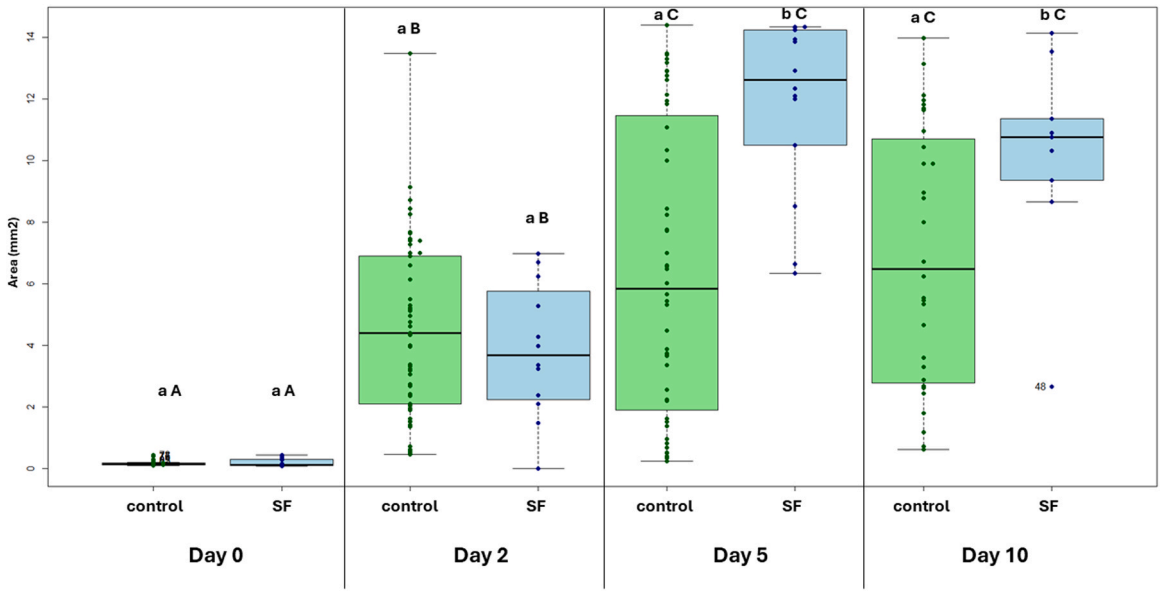

Fig. 2. Changes in median area of fresh and slow-frozen follicles, during the IVC period. (a,b) $p < 0.05$; differences among groups were analysed with Mann-Whitney test. (A,B) $p < 0.05$; differences within groups on each day. Friedman test with post hoc Wilcoxon ranked sign test for pairwise comparison. SF=slow freezing.

tendency was detectable when they analysed apoptotic rate which was also higher in SF group (0 % vs. 13.16 % in control and SF, respectively.). Another study by Lopes et al. (2016) analysed ovarian slices cryopreserved with slow freezing. They found that 65.7 % (± 1.7) of PAFs (isolated after thawing) were viable after thawing, which was lower than that of control group (77.7 ± 2.9 %). In both studies DMSO was added to the freezing medium. Our results suggest that glycerol is a suitable cryoprotectant when the goal is the preservation of PAFs. Jivago's research team assessed the effect of slow freezing and vitrification on normal morphology. Despite the normal morphology rate was significantly lower in vitrified samples (68.14 ± 12.75 %) than that of control and slow frozen groups (93.66 ± 6.81 % and 86.16 ± 11.05 %, respectively), the vitrification was more effective in preserving ultrastructure (mitochondria, ER, nuclei, etc.) of PAFs (Jivago et al., 2018). In our study, we found a relatively high rate of morphologically normal follicles in the control group on Day2 (91.2 %), which was decreased day by day, diminished to 54.8 %. In case of SF group, survival rate was lower (50 %) on Day 2, but did not differ at the end of culture period. The compromised morphology of control follicles after Day 5 indicates that there is a room for the refinement of culture conditions. Beside the general morphological markers (abnormal oocyte structure, highly dense granulosa cell area or damaged basement membrane), we assessed the size of follicles which were referred to as abnormal and found lower size in both treatment groups. Although these results are not directly comparable to cryosurvival of tissue samples yet provide deeper insights in the effects of cryodamage.

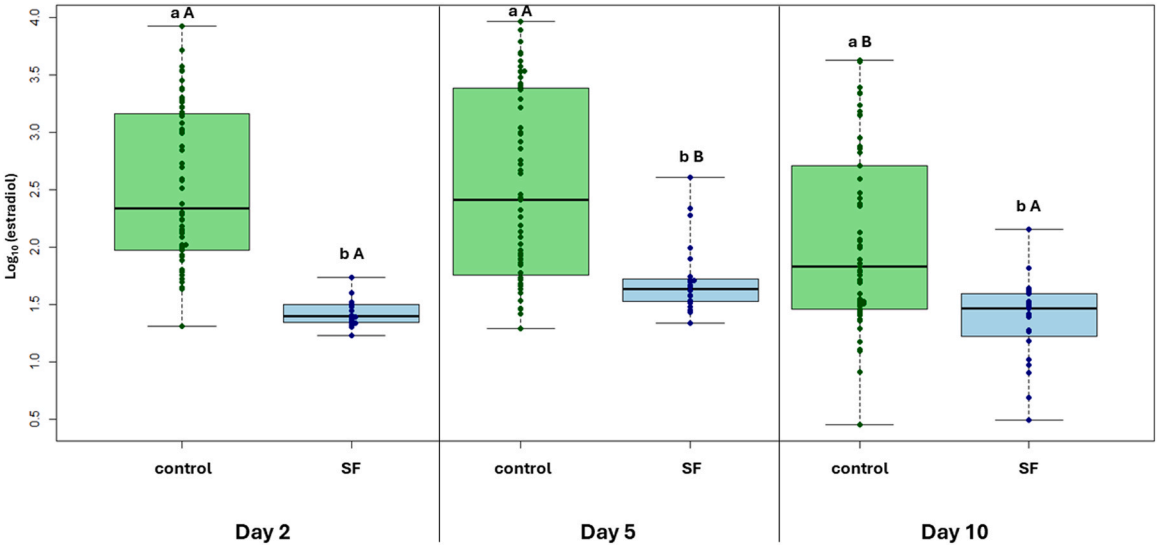

Fig. 3. Changes in estradiol production (median $\log_{10}$ (estradiol) concentrations) in each group, during the IVC period. (a,b) $p < 0.05$; differences between groups were with Mann-Whitney test. (A,B) $p < 0.05$; differences within groups on each day. Friedman test with post hoc Wilcoxon ranked sign test for pairwise comparison. SF = slow freezing.

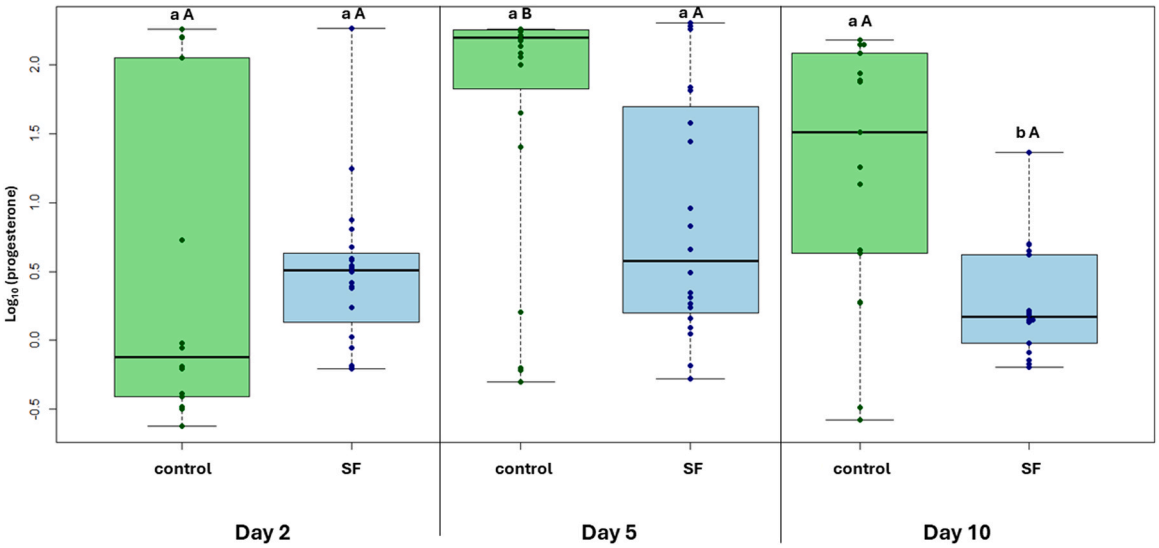

Fig. 4. Changes in progesterone production (median $\log_{10}$ (progesterone) concentrations) in each group, during the IVC period. (a,b) $p < 0.05$; differences between groups were with Mann-Whitney test. (A,B) $p < 0.05$; differences within groups on each day. Friedman test with post hoc Wilcoxon ranked sign test for pairwise comparison. SF=slow freezing.

Area was measured as a simple morphological tool to assess the growth capacity of follicles. On Day 0, median areas of follicles were not different, which was the result of random dividing of samples (0.142 mm^2 [range: 0.086–0.434] and 0.118 mm^2 [range: 0.067–0.437], in fresh and SF groups, respectively [$p = 0.12$]). Interestingly, we found higher area and lower range of SF/thawed follicles on Day 5 and Day 10 than that of control ones. Although the reason is not fully understood, we suggest that SF can act as a selection factor, corroborating the "only the strong will survive" statement, resulting in higher growing capacity after cryo-stress.

Measuring hormonal content of culture medium is a useful, non-invasive method to evaluate normal function of follicles (Demeestere et al., 2002; Dorphin et al., 2012), however, only a limited number of studies provides information on PAF hormonal production in vitro in dogs. We found that both fresh and slow frozen PAFs produce estradiol and progesterone, however, in lower concentrations in SF/thawed samples. In a study by Nikiforov, authors assessed the effect of slow freezing and 4 types of vitrification on isolated ovine follicles cultured for 10 days after thawing. They found that the follicles of the control group produced significantly higher concentrations of estradiol and progesterone each day (Day 4,6,8 and 10) than that of the slow frozen and vitrified ones.

Slow-frozen PAFS, however, produced higher level of the hormone than all the VF samples. In their culture system, estradiol production reached a peak level on Day 6 before start to decrease, while progesterone production increased continuously (Nikiforov et al., 2018). In contrast, we found the estradiol peak on Day 5 only in the SF group, while control PAFs produced constant estradiol concentration until Day 5 before decreasing. Regarding the progesterone production, we found peak concentration on Day 5 in control, and constant levels in SF group. According to Boland et al. (1993), estradiol secretion increases as follicular size increases. This phenomenon was quite controversial in dog PAF IVC conducted by two research groups. Songsasen et al. (2011) evaluated the growing capacity, estradiol and progesterone production of canine PAFs in alginate hydrogel 3D culture, supplemented with 1.5 IU/ml eCG. Although they found continuous follicular growth throughout the 8-day long culture period, estradiol and progesterone production stagnated between Day 6 and Day 10 (Songsasen et al., 2011). Serafim et al. (2015) found that Day 18 follicular diameter was significantly higher than that of Day 6 in FSH-supplemented culture medium (481.01 μm vs 404.85 μm, respectively), and the same tendency was found with estradiol secretion (66.21 ± 38.91 pg/ml vs. 4.66 ± 3.43 pg/ml, respectively). Our data shows that follicular area was constant from Day 5 to Day 10 in both groups, which resulted in decreasing estradiol production and decreasing in progesterone secretion in fresh control follicles.

In conclusion, our data suggest that slow freezing can provide canine follicular survival, and follicles can grow in vitro after thawing; however, the quality and viability of the follicles (normal morphology and hormonal production) are reduced compared to fresh samples. These facts indicate that slow freezing is a suitable tool for gene preservation in emergency cases, although further refinement of the culture system is needed in order to improve the condition for long-term storage (> 2 weeks). These findings, complemented with our previous studies in this topic can provide comprehensive information for professionals working on the field of gene preservation and biobanking.

CRediT authorship contribution statement

Lilla Bordás: Methodology, Investigation. **Bence Somoskoi:** Writing – original draft, Methodology, Investigation, Conceptualization. **Sándor Cseh:** Writing – review & editing, Conceptualization. **Giovanni M. Lacalandra:** Writing – review & editing. **Dóra Török:** Methodology, Investigation.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used [OpenAI. (2025). ChatGPT-4: AI Language Model [Large Language Model]] in order to make the „Introduction" chapter more comprehensive and to improve its readability and language. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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