Equine ovarian tissue viability after cryopreservation and *in vitro* culture

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Short title: Cryopreservation of equine ovarian tissue

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Abstract

Ovarian tissue cryopreservation allows the preservation of the female fertility potential for an undetermined period. The objectives of this study were to compare the efficiency of cryoprotective agents (CPAs; dimethyl sulfoxide, DMSO; ethylene glycol, EG; and propylene glycol, PROH) using slow-freezing and vitrification methods, and evaluate the viability of cryopreserved equine ovarian tissue after 7 days of culture. Fresh and cryopreserved ovarian fragments were evaluated for preantral follicle morphology, stromal cell density, EGFR, Ki-67, Bax, and Bcl-2 protein expression, and DNA fragmentation. Vitrification with EG had the highest rate of morphologically normal preantral follicles, while DMSO had the lowest ($76.1 \pm 6.1\%$ and $40.9 \pm 14.8\%$, respectively; $P < 0.05$). In slow-freezing, despite that DMSO had the highest percentage of morphologically normal follicles ($77.7 \pm 5.8\%$), no difference among the CPAs was observed. Fluorescence intensity of EGFR and Ki-67 was greater when vitrification with EG was used. Regardless of the cryopreservation treatment, DMSO had the highest ($P < 0.05$) Bax/Bcl-2 ratio; however, DNA fragmentation was similar ($P > 0.05$) among treatments after thawing. After *in vitro* culture, the percentage of normal follicles was similar ($P > 0.05$) between slow-freezing and vitrification methods; however, vitrification had greater ($P < 0.05$) stromal cell density than slow-freezing. In summary, equine ovarian tissue was successfully cryopreserved, increasing the viability of the cells in the ovarian tissue after thawing when using DMSO and EG for slow-freezing and vitrification methods, respectively. Therefore, these results are relevant for fertility preservation programs.

Keywords: ovary; slow-freezing; vitrification; preantral follicles; stromal cells

1. Introduction

The use of ovarian tissue cryopreservation (OTC) in studies to develop and improve methods of female fertility preservation has been able to generate several offspring in animal models (mice, [1]; sheep, [2]; and monkeys, [3]) and consequently corroborated to advances made in the human reproductive field. In 2004, the first baby was born [4] after OTC and ovarian fragment transplantation (graft). Since then, OTC has been indicated for fertility preservation of prepubertal females and before cancer treatment that cannot be delayed [5]. The use of OTC prior to initiation of cancer treatments and graft of cryopreserved ovarian fragments after treatment has become more common and allowed the birth of more than 60 babies worldwide [6]. Currently, studies have been focusing on improving the recovery rate of healthy oocytes from frozen-thawed ovarian tissues [7]. Therefore, the use of animal models in OTC studies is essential to develop better protocols for potential translational studies and applications in humans.

The mare has been strongly endorsed as an important comparative animal model for studying the mechanisms of antral and preantral follicle dynamics in women. The remarkable similarities between mares and women in follicular waves and hormonal changes [8-11], preovulatory follicle characteristics before ovulation [12-14], ovarian aging process [15-18], acyclic conditions and anovulatory dysfunctions [19-22], ovarian monovulatory function with a long follicular phase [14, 15, 23], heterogeneity of preantral follicle density [18, 24, 25], preantral follicle survivability and growth rate after *in vitro* culture of fresh ovarian tissue [26-28], relationship of preantral follicle density and ovarian stromal cell density [29], and similar permeability/toxicity of ovarian tissue to different cryoprotective agents (CPAs; [30]), advocate

the importance of the mare as an experimental model for the studies of antral and preantral folliculogenesis.

The cryopreservation process of individual cells or tissues seeks to protect the cellular structure and organelles during the cooling and warming steps in order to preserve the cell functionality when submitted to *in vitro* culture or transplant [31]. However, the decrease and subsequent increase in temperature often result in cryoinjuries [32, 33] and cell death, especially in the temperature zone between -15°C and -60°C [34]. Thus, to survive at low temperatures, the cells need to become sufficiently dehydrated, which normally requires the action of CPAs [35]. Therefore, to protect from intracellular ice formation, a CPA with a high concentration must penetrate the cells, implying that the molecule should be small, highly water soluble, and with no or very low toxicity [35]. The most tested CPAs for OTC have been dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propylene glycol (PROH) [5]. In addition, different types of CPAs have been combined to reduce toxicity and improve ovarian tissue quality post-thawing [5, 36, 37]. However, cryoprotectant solutions should be chosen according to the cryopreservation method (slow-freezing or vitrification) to be used for OTC [5]. Despite all technical advances made with OTC, this is still an experimental procedure and needs considerable improvement for several species. To the best of our knowledge, no study has evaluated the effect of different cryoprotective agents using slow-freezing and vitrification methods to preserve equine preantral follicles enclosed within ovarian tissue.

The aims of this study were: (1) to compare the efficiency of CPAs (DMSO, EG, and PROH) using different cryopreservation methods (slow-freezing vs. vitrification) to preserve equine ovarian tissue after thawing (Experiment 1); and (2) to evaluate the efficiency of the two cryopreservation methods associated with the best CPAs to preserve the viability of

cryopreserved tissue after *in vitro* culture (Experiment 2). The following end points were assessed: (i) preantral follicle morphology and class distribution, (ii) ovarian stromal cell density, (iii) expression of cell proliferation, and (iv) early and late apoptotic markers (Experiment 1); and (v) preantral follicle morphology after *in vitro* culture of cryopreserved fragments, and (vi) stromal cell density of cultured cryopreserved equine ovarian tissue (Experiment 2).

2. Materials and methods

2.1. Experiment 1. Effect of different cryoprotective agents using slow-freezing and vitrification methods

2.1.1. Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.1.2. Ovarian tissue collection and processing

The use of animals and procedures were approved by Mississippi State University Institutional Animal Care and Use Committee. Ovaries of five Quarter horse type mares (7 – 19 years old) slaughtered at Mississippi State University were harvested. Briefly, ovaries were rinsed in alcohol 70%, followed by three washes in saline solution (0.9% NaCl) supplemented with antibiotics (100 IU/ml penicillin and 100 µg streptomycin/ml). Subsequently, ovaries were placed in a petri dish with α -MEM containing 1.25 mg/ml bovine serum albumin (BSA), 100 µg/ml penicillin, 100 µg/ml streptomycin, 0.047 mM sodium pyruvate, and 2.5 mM Hepes [26].

Ovaries were divided into three longitudinal portions (two laterals and one middle); only the middle portion of the ovary was used to collect fragments for this study. Large fragments were sliced in small fragments ($3 \times 3 \times 0.5$ mm, L×W× H respectively) using scalpels, tweezers, and the Thomas Stadie-Riggs Tissue Slicer (Thomas scientific®, Swedesboro, NJ, USA) to obtain a standard thickness (0.5 mm). Mares were slaughtered on different days. One ovary of each mare was sufficient to harvest fragments for all groups and considered as a replicate. Therefore, five replicates were performed.

2.1.3. Experimental design

This study was designed to determine the effect of three cryoprotective agents (Dimethyl sulfoxide, DMSO; Ethylene glycol, EG; Propylene glycol, PROH) under two cryopreservation methods (slow-freezing, SF vs. vitrification, VIT) on equine ovarian tissue. Therefore, seven treatment groups were compared: Control (fresh tissue), SF-DMSO, SF-EG, SF-PROH, VIT-DMSO, VIT-EG, and VIT-PROH. Fifty-six small fragments from each ovary were randomly distributed among groups ($n = 8$ fragments $\times$ 7 groups $\times$ 5 replicates; total = 280 fragments).

2.1.4. Slow-freezing method

The ovarian fragments were placed in 1.5 ml cryovials containing the following cryoprotectant solution: α -MEM supplemented with 2.5 mM Hepes, 10% fetal equine serum (FES), 0.25 M sucrose, and 1.5 M of CPA (DMSO, EG, or PROH) [38-41]. The cryopreservation protocol [42] was adapted for this study. Briefly, the following steps were performed: equilibration time (20 min) in the CPA at room temperature (RT; 20°C), and cryovials were placed in a programmable freezing machine (Bio-Cool IV40 - Controlled Rate

Freezer, SP Scientific Company, Warminster, PA, USA); the cooling curve was programmed for a rate of 2°C/min to -7°C and the seeding was done manually by touching the vials with a forceps dipped into liquid nitrogen (LN2); then, the freezing curve was set for a rate of 0.3°C/min to -40 °C, and vials were plunged in LN2 and stored for 1 week. For the thawing process, the cryovials were exposed to RT for 30 sec and then immersed in water at 37°C for 1 min. Fragments frozen-thawed were washed in three step solutions (5 min each) in the following order: α -MEM + 2.5 mM Hepes + 10% FES + 0.5 M sucrose; α -MEM + 2.5 mM Hepes + 10% FES + 0.25 M sucrose; and α -MEM + 2.5 mM Hepes + 10% FES.

2.1.5. Vitrification method

Fragments submitted to vitrification were placed in petri dishes containing the following solutions and periods of equilibration: 1st step: 0.3 M CPA (DMSO, EG, or PROH) and 0.5 M trehalose in the base medium (α -MEM + 2.5 mM Hepes + 6% FES) for 3 min at RT; 2nd step: 1.5 M CPA (DMSO, EG, or PROH) in base medium for 1 min at RT; and 3rd step: 3 M CPA (DMSO, EG, or PROH) in base medium for 1 min at RT [38, 43]. Fragments were lightly dried, placed in cryovials (1 ml), plunged in LN2, and stored for 1 week. For the thawing process, the cryovials were exposed to RT for 30 sec and then immersed in water at 37°C for 1 min. Immediately, fragments were submitted to decreasing concentrations of sucrose (0.5, 0.25, and 0 M in the base medium) for 5 min within each solution to remove the cryoprotectant solution.

2.1.6. Histological processing

Fresh and cryopreserved fragments were fixed in 4% paraformaldehyde for 4 h and then kept in 70% alcohol at 4°C until standard histological processing. The fragments were embedded in

paraffin wax and cut into serial sections (7 μm ; [44]). Every section was mounted and stained with Periodic Acid-Schiff (PAS) and counterstained with hematoxylin. Histological sections were analyzed using a light microscope (Nikon E200, Tokyo, Japan) at 40 $\times$ objective magnification and an image capture system (LEICA Imaging Software, Wetzlar, Germany). The following end points were recorded: preantral follicle morphology (normal and abnormal) and class distribution, and ovarian stromal cell density.

2.1.7. Morphological classification of preantral follicles

Preantral follicles with visualized oocyte nucleus were counted and morphologically classified as either normal (follicles containing an intact oocyte and oocyte nucleus surrounded by granulosa cells well organized in one or more layers) or abnormal (follicles with a retracted cytoplasm or disorganized granulosa cell layers detached from the basement membrane and oocyte with pyknotic nucleus; [45]). Preantral follicles were classified according to their developmental stage into primordial, transitional, primary, and secondary [25].

2.1.8. Ovarian stromal cell density

Ovarian stromal cell density was evaluated as described [29]. Briefly, a total of 10% of all histological sections of each group were analyzed. Five random fields (each with $50 \times 50 \mu\text{m} = 2,500 \mu\text{m}^2$) per selected section were recorded to calculate the mean stromal cell density per ovarian fragment.

2.1.9. Western immunoblotting

Remaining fresh equine ovarian tissue from ovaries collected for the experiment were snap-frozen in LN2 and kept in -80°C for western immunoblotting assay. Ovarian tissue was thawed at RT and processed as previously described [46, 47]. Briefly, total proteins were extracted using complete radioimmunoprecipitation assay buffer (RIPA buffer) containing enzyme inhibitor cocktail (Santa Cruz Biotechnology; Santa Cruz, CA), followed by total protein quantification (Pierce BCA kit, Thermo Fisher Scientific, Rockford, IL). Equivalent amounts of protein (20 µg) were loaded and resolved onto 4–12.5% SDS-PAGE NuPage gels and transferred to PVDF membranes (0.2 µm; Millipore Corp., Belford, USA). The MagicMark™ XP Western Protein Standard (10 µl/well; Invitrogen Co., Carlsbad, CA) was also loaded to determine the protein sizes. Subsequently, membranes were incubated with 500× diluted anti-human Bax antibodies (orb312174; Biorbyt, Berkeley, CA, USA), Bcl-2 (sc-492; Santa Cruz Biotechnology, Dallas, TX, USA), or EGFR (sc-03-G; Santa Cruz Biotechnology, Dallas, TX, USA), which immunogenicities with equine tissues have been described by the companies. Proteins of interest were detected using the WesternBreeze® Chromogenic Kit (Invitrogen Co., Carlsbad, CA). The specificity of Ki-67 protein has been described by the manufacturer and previously reported for horses [48, 49].

2.1.10. Immunohistochemistry

Ovarian tissue sections mounted on slides were deparafinized, submitted to antigen retrieval in sodium citrate buffer for 30 min in a steamer, washed with PBS and submitted to immediate standard *in situ* immunofluorescence detection of Bax, Bcl-2, EGFR, and Ki-67 proteins, as previously described [46, 47]. Briefly, slides were permeabilized in 1% Triton -X100 for 30 min, non-specific binding sites blocked in 1% BSA solution for 60 min, and incubated with 100×

diluted rabbit polyclonal anti-human Bax (orb312174; Biorbyt, Berkeley, CA, USA), Bcl-2 (sc-492; Santa Cruz Biotechnology, Dallas, TX, USA), EGFR (sc-03-G; Santa Cruz Biotechnology, Dallas, TX, USA), or Ki-67 (PA5-19462; Thermo Fisher Scientific Inc., Waltham, MA USA) antibodies for 60 min. Then, slide sections were incubated with FITC labeled goat anti-rabbit secondary antibody (200x dilution) for 60 min. Three washes with PBS solution were performed between all steps and all procedures were undertaken at RT. Slides were immediately covered with a DAPI-contained mounting medium to counterstain cell nuclei for a fluorescence evaluation using a fluorescent microscope (EVOS FL Cell Imaging System, Thermo Fisher Scientific Inc., Waltham, MA, USA). Images were obtained in 20x objective magnification and analyzed using ImageJ software (version 1.50f).

2.1.11. TUNEL assay

TUNEL staining was carried out using a commercially available kit (DeadEnd™ Fluorometric TUNEL System, Promega©, Madison, WI, USA) following the manufacturer's instructions. Tissue sections were examined, and five images from randomly selected fields of one section/treatment/replicate were obtained in 20x objective magnification to calculate the fluorescent intensity of TUNEL positive cells using the ImageJ software. TUNEL positive and negative controls were included in all evaluations, according to the manufacturer's recommendations.

2.2. Experiment 2. In vitro culture of ovarian biopsy fragments after cryopreservation using slow-freezing and vitrification methods

2.2.1. Ovarian tissue collection and processing

All experimental procedures were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training (<http://grants.nih.gov/grants/olaw/references/phspol.htm>). The research protocol was approved by the Institutional Animal Care and Use Committee of Southern Illinois University. Quarter horse mares (n = 9; 5-10 years old) weighing between 400 and 600 Kg were used. No hormonal treatments were administered during the experimental period. The ovaries and uterus of all mares were scanned using a transrectal ultrasound scanner (Aloka SSD-900, Aloka Co., LTD., Wallingford, CT, USA) equipped with a 5 to 10 MHz linear array transducer (Aloka UST-5821-7.5). Animals with a healthy reproductive tract, and no large ovarian structures (preovulatory follicle or corpus luteum) to allow space for the biopsy needle to collect good size fragments, were selected on different days of the estrous cycle based on previous history of successful biopsy procedures providing ovarian fragments with many preantral follicles. For each replicate, three mares were used, and biopsy fragments (n = 6, size $\sim 1.5 \times 1.5 \times 10$ mm) were collected from both ovaries (left and right) of each mare via the biopsy pick-up technique [24]. Immediately after the ovarian biopsy procedure, full biopsy fragments from every replicate were mixed and maintained in α -MEM supplemented with 0.4% BSA, 0.1 M sucrose, and 10 mM Hepes (base medium) [26] for up to 60 min at RT, cut in small fragment size ($\sim 1.5 \times 1.5 \times 2$ mm) using scalpel and tweezers, and randomly distributed to the following treatment groups: fresh noncultured, fresh cultured, SF noncultured, SF cultured, VIT noncultured, and VIT cultured. Fragments were then fixed for histological analysis. Six replicates were performed.

2.2.2. Cryopreservation methods

Protocols for SF and VIT methods were performed as previously described in Experiment 1, with some slight modifications. Briefly, the fragment ($\sim 1.5 \times 1.5 \times 2$ mm), although maintaining the same cubic dimension, had different sizes because of the notch of the biopsy needle [24]. Furthermore, DMSO was used as the CPA, and fragments were stored and cryopreserved in 0.5 ml straws for the slow-freezing method. For the vitrification method, EG was used as the CPA, and fragments were cryopreserved in droplets of 100 μ l solution by a solid-surface method and then stored in cryovials [50].

2.2.3. *In vitro* culture

Ovarian tissue fragments from all *in vitro* culture (IVC) treatments (e.g., fresh, SF, and VIT) were placed in culture plates (6 fragments per well) containing 1000 μ l of medium composed of α -MEM supplemented with insulin (10 ng/ml), transferrin (5 ng/ml), selenium (5 ng/ml), glutamine (2 mM), hypoxanthine (2 mM), BSA (1.25 mg/ml), ascorbic acid (50 μ g/ml), recombinant follicle stimulating hormone (50 μ g/ml), epidermal growth factor (50 ng/ml), penicillin (100 μ g/ml), and streptomycin (100 μ g/ml) [26-28, 51, 52]. Fragments were cultured for 7 days at 39°C in a humidified atmosphere with 5% CO₂ in air. Culture medium was completely replaced on days 2, 4, and 6 of culture.

2.2.4. *Histological processing and analysis*

Ovarian fragment processing and morphological analyses for preantral follicles and stromal cell density were performed as previously described in Experiment 1.

2.3. *Statistical analyses*

All statistical analyses were performed using R statistical software version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Data for end points that were not normally distributed were transformed to natural logarithms or square root. Variables with normal distribution were analyzed by ANOVA. Variables without normal distribution were compared by Kruskal-Wallis test. When appropriate, t-Test and Mann-Whitney U test were used to compare mean values between groups. A probability of $P < 0.05$ indicated that a difference was significant, and $P > 0.05$ and ≤ 0.1 indicated that a difference approached significance.

3. Results

3.1. Experiment 1

3.1.1. Preantral follicle morphology and class distribution

A total of 319 preantral follicles were recorded with a mean of 45.6 ± 11.6 follicles per treatment. Considering all treatments, 304 follicles were classified as primordial and 15 as developing (transition, primary, or secondary) follicles. Therefore, results regarding follicle morphology involve the total number of preantral follicles. Overall, the percentage of normal follicles differed ($P < 0.05$) among treatments (Fig. 1). The VIT-DMSO and VIT-PROH groups had lower ($P < 0.05$) percentages of normal follicles than the control fresh tissue. The percentages of normal follicles were not different ($P > 0.05$) among CPAs under the SF method. However, VIT-EG had a greater ($P < 0.05$) percentage of normal follicles compared to VIT-DMSO and VIT-PROH. Furthermore, only DMSO in the VIT method had a lower ($P < 0.05$) percentage of normal follicles when compared with the SF method.

3.1.2. Stromal cell density

The stromal cell density in the fresh ovarian fragments varied from 15 to 43 cells/2500 μm^2 (CV = 29.0%). Cryopreserved ovarian fragments in the SF-DMSO and VIT-DMSO had similar ($P > 0.05$) stromal cell density to fresh ovarian fragments; however, the stromal cell density in the SF-DMSO treatment was greater ($P < 0.05$) than in all VIT treatments. Cryopreserved fragments in the EG and PROH treatments in both cryopreservation methods had lower ($P < 0.05$) stromal cell density than the fresh control and SF-DMSO treatments (Fig. 2).

3.1.3. TUNEL, Western Immunoblotting, and Immunohistochemistry assays

DNA fragmentation in fresh and cryopreserved ovarian tissue did not differ ($P > 0.05$) among treatments (Fig. 3). Immunodetection of Ki-67, EGFR, Bax, and Bcl-2 proteins, and negative control are illustrated (Fig. 4A-E). The specificity of protein targets using EGFR, Bcl-2, and Bax antibodies was confirmed by western immunoblotting (WIB) in fresh ovarian tissues (Fig. 4F), followed by immunofluorescence. Detection levels of EGFR and Ki-67 proteins in cryopreserved ovarian fragments did not differ ($P > 0.05$) from fresh tissue (Fig. 5A and 5B, respectively). The expression levels of EGFR and Ki-67 in the VIT-EG treatment were stronger ($P < 0.05$) than in the SF-EG. The expression of Bcl-2 protein was greater ($P < 0.05$) in the SF-EG, VIT-EG, and VIT-PROH treatments, demonstrated by the lower mean ratios of Bax/Bcl-2 when compared with the fresh control treatment (Fig. 5C). SF-DMSO and VIT-DMSO had greater ($P < 0.05$) Bax protein expression, demonstrated by the greater mean Bax/Bcl-2 ratios when compared with the other CPA treatments. In the VIT-EG treatment, positive correlations were found between Bcl-2 and EGF ($r = 0.96$, $P < 0.01$), and Bcl-2 and Ki-67 values ($r = 0.79$, $P > 0.05$).

3.2. Experiment 2

3.2.1. Preantral follicle morphology and class distribution

A total of 411 preantral follicles were recorded with a mean of 68.5 ± 22.9 follicles per treatment. Considering all treatments, 325 follicles were classified as primordial and 86 as developing (transition, primary or secondary) follicles. Overall, the percentage of normal preantral follicles differed ($P < 0.05$) among groups (Fig. 6). The morphology of preantral follicles enclosed in fresh fragments cultured for 7 days was similar ($P > 0.05$) to noncultured fresh control fragments. In addition, the morphology of follicles in cryopreserved ovarian fragments in the SF method did not differ ($P > 0.05$) from the fresh noncultured and cultured controls. Noncultured frozen-thawed SF fragments had a greater ($P < 0.05$) percentage of normal follicles than VIT fragments. Regardless of the cryopreservation method, the morphology of preantral follicles was negatively affected ($P < 0.05$) after the culture period.

The percentage of developing preantral follicles in the noncultured fresh control (16.7%) increased ($P < 0.05$) after the cultured period (55.0%). However, the percentage of developing preantral follicles in the SF noncultured (28.7%) and VIT noncultured (30.8%) decreased ($P < 0.05$) after the culture period for both SF (2.9%) and VIT (4.2%) methods.

3.2.2. Stromal cell density

The stromal cell density in the fresh ovarian fragments varied from 27 to 44 cells/2500 μm^2 (CV = 19.4%). Fresh fragments cultured for 7 days had similar ($P > 0.05$) stromal cell density to noncultured fresh control treatment (Fig. 7). Regardless of cryopreservation method, noncultured cryopreserved-thawed fragments had lower ($P < 0.05$) stromal cell density than the noncultured and cultured fresh control treatments. However, cultured SF and VIT fragments had greater ($P <$

0.05) stromal cell density than noncultured controls. Cultured VIT fragments had greater ($P < 0.05$) stromal cell density compared with fresh noncultured and cultured fragments.

4. Discussion

To date, cryopreservation of equine preantral follicles enclosed in ovarian tissue has not been studied. To the best of our knowledge, this is the first study to report the feasibility of cryopreserving equine preantral follicles enclosed in ovarian tissue by slow-freezing and vitrification methods.

In our study, markers related to tissue growth and survival such as EGFR, Ki-67, Bax, and Bcl-2 were expressed after the cryopreservation and thawing processes, demonstrating tissue functionality. Regardless of the cryopreservation method, the types of CPAs herein tested have not affected the nuclear antigen for Ki-67 in equine ovarian cells. In addition, we observed a high positive correlation between EGFR and Ki-67 in fresh and frozen-thawed equine ovarian tissue. Studies have shown that the EGF/EGFR signaling upregulates mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, stimulating proliferation, growth, and survival of various cell types, including cumulus cells and oocytes [53]. Moreover, Ki-67 antibody recognizes a nuclear antigen which is expressed in all stages of the cell cycle except G0, so it represents an acceptable index of cell proliferation for cryopreserved tissue after thawing [54]. Contrarily to findings in the human ovary [54], our study has demonstrated positive labeling of Ki-67 on frozen-thawed ovarian stromal cells. Thus, the cryopreservation methods herein tested preserved EGFR and Ki-67 receptors in the equine ovarian tissue, receptors that are important for cell proliferation and essential for growth and viability of the primordial follicles.

Apoptosis of the equine ovarian tissue was assessed by Bax and Bcl-2 antibodies and TUNEL assay. Regardless of the CPA tested, cryopreservation by slow-freezing or vitrification has not significantly damaged the DNA of equine ovarian cells. However, Bax/Bcl-2 ratio was affected by the CPAs tested according to the method of cryopreservation. Cryopreservation using EG maintained greater expression of Bcl-2 either in the SF or VIT method. On the other hand, cryopreservation using DMSO increased Bax expression. Similar results have been reported for human ovaries [54, 55]. Moreover, DMSO has been shown [56-58] to have a considerable toxicity effect on morphology of ovarian germ cells and cell proliferation. Our previous study of cryoprotectant toxicity on equine ovarian tissue has shown a moderate toxicity of DMSO when compared to EG and PROH [30]. Although our results do not clarify the mechanism(s) by which the toxicity effects of CPAs trigger Bax activation, which may lead to a later apoptotic process in the ovarian cells when submitted to *in vitro* culture, the present study supports the hypothesis that Bcl-2 seems to regulate Bax protein once the tissue has adapted to the *in vitro* conditions. To evade apoptosis, the anti-apoptotic protein Bcl-2 must prevent Bax activation. Therefore, Bax/Bcl-2 ratio controls a critical step in commitment to apoptosis by regulating permeabilization of the mitochondrial outer membrane [54].

Morphology of preantral follicles enclosed in ovarian tissue was not affected by the SF method regardless of the CPAs tested. However, the morphology of the follicles was affected by DMSO or PROH when ovarian tissue was vitrified. In addition, although a great percentage (range, 70 to 90%) of equine preantral follicles cryopreserved by SF and VIT methods survived after the thawing process, a small percentage (range, 8 to 10%) did survive after 7 days of *in vitro* culture. Due to the higher concentrations of the CPAs and short time of incubation in the VIT method, the toxicity effect of the CPA may be playing a detrimental role as previously

reported [30]. Nevertheless, a wide range of results have been found in the literature regarding follicle morphology after cryopreservation either by the slow-freezing [59-61] or vitrification [59, 61, 62] method. The inconsistent results previously reported might have been influenced by differences in thickness of the tissue, CPA concentration, and time of incubation. Similar results were found in human follicles cryopreserved by either slow-freezing or vitrification and *in vitro* culture for up to 8 days [61]; the cryopreservation by slow-freezing and vitrification methods delayed follicle growth throughout culture, down-regulating mRNA levels of ZP3 and CYPI IA compared to fresh tissue, but no differences were seen between slow-freezing and vitrification. Therefore, further analysis of cell functionality of cryopreserved equine ovarian tissue should be carried out to understand the low percentage of normal follicles after *in vitro* culture.

It is noteworthy to highlight that this study also shows for the first time an adequate culture medium able to support follicle survival ($\geq 78\%$ morphologically normal follicles) of equine fresh ovarian tissue for up to 7 days of *in vitro* culture. Previous studies using equine ovarian tissue have reported between 27.0 to 43.5% normal preantral follicles after 6 to 7 days of culture [26-28, 63]. The addition of FSH in the medium has been shown to promote activation and survivability of equine primordial follicles [27]. Moreover, the use of EGF in culture medium has been related with cell growth and differentiation [64]. The association of EGF and FSH has been shown to promote follicular growth in other species [65, 66]. However, the association of FSH and EGF in the culture medium for equine ovarian tissue has not been demonstrated yet. Therefore, we assume that the association of FSH and EGF in the culture medium for equine preantral follicles is beneficial and has promoted the survival and growth of ovarian tissue.

In the present study, stromal cell density was reduced after cryopreservation by SF and VIT methods. However, when cryopreserved ovarian fragments were cultured for 7 days, a

significant increase in stromal cell density was seen. Similar findings were observed in goat and sheep ovarian tissue after cryopreservation [67]. The reduction of stromal cell density might be partially caused by physical process and stretching of the tissue after the thawing process. As earlier observed in this study, receptors of growth and proliferation were maintained intact after cryopreservation; therefore, we assume that the cryopreserved ovarian tissue may need to reorganize itself to start growing again during *in vitro* culture.

In conclusion, this study demonstrated the feasibility of cryopreserving equine ovarian tissue by slow-freezing and vitrification methods, preserving the tissue viability. The use of DMSO and EG increased the viability of the cells in the ovarian tissue after thawing when equine ovarian tissue was cryopreserved by slow-freezing and vitrification, respectively. Although a small percentage (~ 9%) of morphologically normal follicles of cryopreserved fragments after 7 days of culture was seen, the ovarian stromal cell density had increased. Therefore, further studies are needed to investigate the factors involving stimulatory and inhibitory effects on proliferation and apoptosis of ovarian cells during cryopreservation and *in vitro* culture to improve the survivability and the development of cryopreserved preantral follicles enclosed in ovarian tissue. The results herein presented are relevant to equine veterinary medicine and fertility preservation programs.

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Figure captions

Figure 1. Mean ($\pm$ S.E.M.) percentage of morphologically normal preantral follicles in fresh, and frozen-thawed (slow-freezing method) versus vitrified-thawed (vitrification method) equine ovarian tissues using different CPAs (DMSO, EG, or PROH). * CPAs differ from fresh control group ($P < 0.05$). ^{A,B} Within CPA, cryopreservation methods differed ($P < 0.05$). ^{a,b} Within cryopreservation method, CPAs differed ($P < 0.05$).

Figure 2. Mean ($\pm$ S.E.M.) stromal cell density per area ($2500 \mu\text{m}^2$) in fresh, and frozen-thawed (slow-freezing method) versus vitrified-thawed (vitrification method) equine ovarian tissues using different CPAs (DMSO, EG, or PROH). * CPAs differ from fresh control group ($P < 0.05$). ^{A,B} Within CPA, cryopreservation methods differed ($P < 0.05$). ^{a,b} Within cryopreservation method, CPAs differed ($P < 0.05$).

Figure 3. Mean ($\pm$ S.E.M.) DNA fragmentation (TUNEL assay) in fresh, and frozen-thawed (slow-freezing method) versus vitrified-thawed (vitrification method) equine ovarian tissues using different CPAs (DMSO, EG, or PROH). No difference ($P > 0.05$) among groups was detected.

Figure 4. Representative micrographs of immunofluorescence detection of (A) Ki-67, (B) EGFR, (C) Bax, and (D) Bcl-2 proteins in fresh control equine ovarian fragments; (E) negative control micrograph; and (F) specificity of antibodies using western immunoblotting.

Figure 5. Mean ($\pm$ S.E.M.) immunofluorescence quantification in fresh, and frozen-thawed (slow-freezing method) and vitrified-thawed (vitrification method) equine ovarian tissues using different CPAs (DMSO, EG, or PROH). (A) epidermal growth factor receptor (EGFR), (B) Ki-67, and (C) Bax/Bcl-2 ratio. * CPAs differ from fresh control group ($P < 0.05$). ^{A,B} Within CPA, cryopreservation methods differed ($P < 0.05$). ^{a,b} Within cryopreservation method, CPAs differed ($P < 0.05$).

Figure 6. Mean ($\pm$ S.E.M.) percentage of morphologically normal preantral follicles in fresh, frozen-thawed (DMSO, slow-freezing method), and vitrified-thawed (EG, vitrification method) equine ovarian tissues before and after *in vitro* culture for 7 days. * Differ from fresh noncultured group ($P < 0.05$). ^{A,B} Within group (noncultured or cultured), treatments differed ($P < 0.05$). ^{a,b} Within treatment, groups differed ($P < 0.05$). Representative micrographs of morphologically normal preantral follicles in (A) fresh, (B) slow-freezing, and (C) vitrification cultured groups. Scale bar = 20 μm .

Figure 7. Mean ($\pm$ S.E.M.) stromal cell density per area (2500 μm^2) in fresh, frozen-thawed (DMSO, slow-freezing method), and vitrified-thawed (EG, vitrification method) equine ovarian

tissues before and after *in vitro* culture for 7 days. * Differ from fresh noncultured group ($P < 0.05$). ^{A,B} Within group (noncultured or cultured), treatments differed ($P < 0.05$). ^{a,b} Within treatment, groups differed ($P < 0.05$). Representative micrographs of (**A**) low and (**B**) high stromal cell density in the fresh noncultured and vitrification cultured groups, respectively.

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