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

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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 ± 6.1% and 40.9 ± 14.8%, respectively; $P < 0.05$). In slow-freezing, despite that DMSO had the highest percentage of morphologically normal follicles (77.7 ± 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.

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

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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 × 3 × 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 x 7 groups x 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 s 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 s 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 × objective magnification and an image capture system (LEICA Imaging Software, Wetzlar, Germany). The following end points