

Ovarian fragment sizes affect viability and morphology of preantral follicles during storage at 4°C

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Abstract

The method of transportation and the conditions imposed on the ovarian tissue are pivotal aspects for the success of ovarian tissue cryopreservation (OTC). The aim of this study was to evaluate the effect of the size of the ovarian tissue (e.g. whole ovary, biopsy size and transplant size) during different times of storage (0, 6, 12 and 24 h) on the structural integrity of equine ovarian tissue transported at 4°C. Eighteen pairs of ovaries from young mares (<10 years old) were harvested in a slaughterhouse and processed to simulate the fragment sizes (biopsy and transplant size groups) or kept intact (whole ovary group) and stored at 4°C for up to 24 h in α -MEM-enriched solution. The effect of the size of the ovarian tissue was observed on the morphology of preantral follicles, stromal cell density, DNA fragmentation and mitochondrial membrane potential. The results showed that (i) biopsy size fragments had more morphologically normal preantral follicles after 24 h of storage at 4°C; (ii) mitochondrial membrane potential was the lowest during each storage time when the whole ovary was used; (iii) DNA fragmentation rate in the ovarian cells of all sizes of fragments increased as storage was prolonged and (iv) transplant size fragments had increased stromal cell density during storage at cool temperature. In conclusion, the biopsy size fragment was the best to preserve follicle morphology for long storage (24 h); however, transportation/storage should be prior determined according to the distance (time of transportation) between patient and reproduction centers/clinics.

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Introduction

Transportation of ovaries or ovarian fragments has garnered increasing interest after the development of new techniques that are able to cryopreserve ovarian tissue and restore fertility followed by a transplantation procedure (Gosden *et al.* 1994, De Vos *et al.* 2014, Dolmans *et al.* 2014, Silber *et al.* 2015, Devi & Goel 2016). The method of transportation and the conditions imposed on the ovarian tissue are pivotal aspects for the success of ovarian tissue cryopreservation (OTC) observed by several reproductive centers around the world (Gosden *et al.* 1994, Hovatta *et al.* 1996, Newton *et al.* 1996, Gook & Edgar 1999) to preserve the fertility potential (Dittrich *et al.* 2012, 2015, Muller *et al.* 2012, Bastings *et al.* 2014, Jensen *et al.* 2015). Cryopreservation and ovarian tissue transplantation have shown successful results in humans and animals (Baird *et al.* 2004, Bordes *et al.* 2005, Donnez & Dolmans 2013, Isachenko *et al.* 2013,

Ting *et al.* 2013, Fabbri *et al.* 2016). The use of OTC prior to the 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 (Donnez & Dolmans 2015). Recently, OTC has been classified as an innovative treatment according to the criteria of the American Society for Reproductive Medicine (ASRM), European Society of Human Reproduction and Embryology (ESHRE) and the special interests groups 'Ethics and Law' and 'Safety and Quality in Assisted Reproductive Technology' and no longer been considered as an experimental technique (Provoost *et al.* 2014, Donnez & Dolmans 2015, Van der Ven *et al.* 2016). However, the majority of hospitals and/or clinics do not have the required trained staff, specialized equipment, designated space and time to prepare and freeze the ovarian cortex to perform OTC (Backhus *et al.* 2007, Practice Committee of American Society for Reproductive Medicine 2014). Furthermore, in the case of animals, OTC has been

applied to preserve germ cells of animals considered to have high genetic value and to endangered wild species (Comizzoli & Wildt 2013), and several times, the ovary or ovarian sample is collected in the wild, zoos or farms, usually at a great distance from reproductive centers. In those cases, a proper transport of the ovarian tissue is needed to preserve the fertility potential. Nevertheless, the method of transportation of ovarian specimens is still a barrier to overcome. Few studies have been conducted regarding transportation or storage of fresh specimens to use the maximum capability of the ovarian reserve (Isachenko *et al.* 2015). In fact, the number of viable primordial follicles in the ovarian tissue is decisive for a potential clinical benefit. Therefore, preservation of ovarian samples using special medium and adequate temperature during transportation have been necessary (Chaves *et al.* 2008, Tellado *et al.* 2014). Furthermore, because the OTC process *per se* leads to damages in the tissue and significant loss of preantral follicles, further ovarian tissue damage should be minimized during transportation of specimens (Schmidt *et al.* 2003).

Induced hypothermia to approximately 4°C has been the most common approach used for transport of organs to be preserved (Isachenko *et al.* 2009b, Cantu & Zaas 2011). Currently, protocols to transport ovaries have been proposed for different species, such as bovine (Celestino *et al.* 2008), caprine (Chaves *et al.* 2008), ovine (Matos *et al.* 2004), swine (Wongsrikeao *et al.* 2005) and equine (Gomes *et al.* 2012). However, studies have shown a large variation in fragment size from whole ovary (Kamoshita *et al.* 2016) to very small fragments (<1 mm thickness; Lan *et al.* 2010) submitted to transport, cryopreservation and/or transplantation. Consequently, a large variation in follicle survival has been noticed among studies using different animal models, such as goats (Silva *et al.* 2000), sheep (Barberino *et al.* 2016), cattle (Lucci *et al.* 2004), horses (Gomes *et al.* 2012), dogs (Lopes *et al.* 2009, Lima *et al.* 2010), mice (Kamoshita *et al.* 2016), non-human primates (Ting *et al.* 2011, Hornick *et al.* 2012) and women (Isachenko *et al.* 2009a, Lan *et al.* 2010, Sanfilippo *et al.* 2013, Wang *et al.* 2016). Nevertheless, the effect of ovarian fragment size and the time of storage during transportation have been considered a limiting factor briefly explored (Barberino *et al.* 2016). In human clinical situations, most of the clinicians harvest biopsy slices from the ovarian cortex or one whole ovary, preserving the contralateral ovary in the original site (Rice *et al.* 2008, Silber 2016). In veterinary medicine, the technique to harvest ovarian biopsy fragments is well established in cattle (Aerts *et al.* 2005) and horses (Haag *et al.* 2013a), facilitating the studies of ovarian tissue preservation. Therefore, the development of protocols to transport ovarian tissue for cryopreservation, culture and/or transplantation must be optimized to ensure the success of reproductive biotechnologies in animals and humans.

The use of human ovaries for OTC studies is scarce when compared to animal models due to ethical barriers and the limited availability of material for research. Considering the similarities between women and mares related to follicular waves and hormonal changes (Ginther *et al.* 2004, 2005, Mihm & Evans 2008, Baerwald 2009), preovulatory follicle characteristics before ovulation (Martinuk *et al.* 1992, Pierson & Chizen 1994, Gastal 2009), ovarian aging process (Carnevale 2008, Ginther *et al.* 2008, 2009, Alves *et al.* 2016b), acyclic conditions and anovulatory dysfunctions (Gastal *et al.* 2006, Ginther *et al.* 2007, Cuervo-Arango *et al.* 2011, Bashir *et al.* 2016), ovarian monovulatory function with a long follicular phase (Carnevale 2008, Gastal 2009, 2011), heterogeneity of preantral follicle density (Haag *et al.* 2013a,c, Alves *et al.* 2016b), preantral follicle survivability and growth rate after *in vitro* culture of fresh ovarian tissue (Haag *et al.* 2013b, Aguiar *et al.* 2016a,b), relationship of preantral follicle density and ovarian stromal cell density (Alves *et al.* 2016a) and similar permeability/toxicity of ovarian tissue to different cryoprotective agents (CPAs; Gastal *et al.* 2016), the mare can be considered an important animal model to advance knowledge regarding ovarian tissue transportation to be used in OTC and transplantation. Thus, studies with mare ovarian tissue may provide relevant information that could also be applied in the future on clinical human reproduction.

The aim of this study was to evaluate the effect of the size of the ovarian tissue (e.g., whole ovary, biopsy size and transplant size) during different times of storage (0, 6, 12 and 24 h) on the structural integrity of equine ovarian tissue transported at 4°C. The end points evaluated were ovarian tissue viability, mitochondrial membrane potential, ROS production, DNA fragmentation, preantral follicle morphology and class distribution and stromal cell density.

Materials and methods

Chemicals

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

Ovarian tissue collection and processing

Ovaries ($n=36$) of 18 young post-pubertal mares (3–10 years old) were harvested in a slaughterhouse. Briefly, after the removal of the mesovarium structures, ovaries were rinsed in alcohol 70%, followed by three washes in saline solution (0.9% NaCl) supplemented with antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin) at room temperature (22°C). Subsequently, ovaries were placed in the α -MEM solution 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 (Haag *et al.* 2013b) at room temperature for further processing and transportation.

The 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. The previously mentioned procedures were performed within 15 min.

Experimental design

Three sizes of ovarian tissue (treatment groups: whole ovary, biopsy size ($2 \times 2 \times 12$ mm) and transplant size ($0.5 \times 1 \times 1$ mm)) were compared after 0 (control group), 6, 12 or 24 h of storage at 4°C . The ovarian fragment simulation for the transplant size group was based in the literature (Aerts *et al.* 2008, Abir *et al.* 2009, Li *et al.* 2016), and for the biopsy size group, it was based on the size of the specimen notch of the needle used for *in vivo* biopsy collection performed by our research team (Haag *et al.* 2013a,b,c, Alves *et al.* 2016a,b, Gastal *et al.* 2016). A Thomas Stadie-Riggs Tissue Slicer (Thomas Scientific, Swedesboro, NJ, USA) was used to produce thin slices (0.5 mm) of ovarian fragments for the transplant size and control groups. Fragments for the biopsy and transplant size groups, and control group, were prepared using scalpels and tweezers. Fragments for the control group were sliced according to the transplant size group and immediately fixed in 4% paraformaldehyde. One ovary of each mare ($n=3$ /replicate) was processed immediately to produce the necessary fragments to be randomly distributed among the control (0 h), biopsy and transplant size groups and respective times (6, 12 or 24 h). The contralateral ovary of each mare ($n=3$ /replicate) was used for transportation at 4°C , being assigned as the whole ovary size group to one of the three times of transportation (6, 12 or 24 h). Ovaries (whole group) and ovarian fragments from the biopsy and transplant size groups were transported from the slaughterhouse to the laboratory (4-h trip) in a styrofoam box containing ice packs; temperature (4°C) was controlled with a digital thermometer. In the laboratory, specimens were maintained in the refrigerator (4°C) throughout the necessary times. After the time of storage for whole ovary and biopsy size treatments, fragments were cut and standardized in similar sizes ($0.5 \times 1 \times 1$ mm) before being fixed or submitted to tissue viability assays. The ovarian fragments were not checked for the presence of preantral follicles prior to assigning them to each treatment group. From our experience (data not shown), we know that $>90\%$ of the preantral follicles are located in the middle portion of the equine ovary; thus, we used this approach for preparing fragments. Therefore, the chance of a fragment with a given number of follicles was randomly distributed among treatment groups. For each replicate, six ovaries from three mares were used. Six replicates were performed.

Tissue viability assays

Ovarian fragments from every group and time were assessed by fluorescent probes for cell viability (5[6]-carboxyfluorescein diacetate succinimidyl ester, CFDA-SE, Molecular Probes, Invitrogen; Oktay *et al.* 1997, Newton *et al.* 1999, Chambers *et al.* 2010), mitochondrial membrane potential (JC-1, Molecular Probes, Invitrogen; Smiley *et al.* 1991) and reactive oxygen species (ROS, H2DCFDA; Fabbri *et al.* 2014).

For cell viability, fragments were incubated in $5.57 \mu\text{g}/\text{mL}$ CFDA-SE for 30 min, and then incubated in $100 \mu\text{g}/\text{mL}$ propidium iodide for 5 min at 37°C . For mitochondrial membrane potential, fragments were incubated in $2 \mu\text{L}/\text{mL}$ JC-1 for 30 min at 37°C . For ROS assay, fragments were incubated in $1 \mu\text{L}/\text{mL}$ H2DCFDA for 30 min, and then incubated in $100 \mu\text{g}/\text{mL}$ propidium iodide for 5 min at 37°C . Five fragments/group/replicate were used for each of the three assays described previously. After the incubation period of each fluorescent probe, fragments were fixed in 4% paraformaldehyde at room temperature for 15 min, and then transferred to PBS solution for up to 1 h at room temperature protected from light for fluorescent image analysis. Fragments were mounted on a slide for confocal laser scanning microscopy analyses (Zeiss LSM 710). For each sample, five randomly selected regions of interest with 1024×1024 pixels were drawn to measure the fluorescence intensity. Parameters related to fluorescence intensity, such as laser energy, signal detection (gain) and pinhole size, were maintained at constant values for all measurements. Fragments were observed at $630\times$ objective magnification under oil immersion. A helium/neon laser ray at 543 nm (551 nm excitation and 576 nm emission) was used to identify the propidium iodide and JC-1. An argon ion laser ray at 488 nm (495 nm excitation and 519 nm emission) was used to identify the CFDA-SE and H2DCFDA. Scanning was conducted with Z stack of 25 optical series from the top to the bottom of the fragments with a step size of 0.08 mm to allow three-dimensional distribution analysis.

Histological processing

Additional ovarian fragments ($n=5$ fragments) from every group and time were fixed in 4% paraformaldehyde for 1 h and then kept in 70% alcohol at 4°C until standard histological processing. The fragments were embedded in paraffin wax and totally cut into serial sections ($7 \mu\text{m}$; Alves *et al.* 2015). Every section was mounted and stained with periodic acid-Schiff (PAS) and counterstained in hematoxylin. Histological sections were analyzed using a light microscope (Nikon E200) 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), preantral follicle class distribution and ovarian stromal cell density.

Morphological classification of preantral follicles

For morphological classification, within each replicate, 5 ovarian fragments were used. The 5 fragments were blocked together, producing 8 slides, each with 8–10 sections evaluated (total = 75 sections per treatment per replicate; a total of 1350 sections were evaluated for preantral follicle morphology). 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; Hulshof *et al.* 1994). Preantral follicles were classified according to their developmental stages into primordial, transitional, primary and secondary (Haag *et al.* 2013c).

Ovarian stromal cell density

Ovarian stromal cell density was evaluated as described (Alves *et al.* 2016a). Briefly, a total of 10% of histological sections of all fragments of each group were analyzed. Four random fields (each with $50 \times 50 \mu\text{m} = 2500 \mu\text{m}^2$) per selected section were recorded to calculate the mean stromal cell density per ovarian fragment.

TUNEL assay

For TUNEL assay, within each replicate, 5 ovarian fragments were used. The 5 fragments were blocked together, producing 3 slides, each with 2 sections evaluated (total = 6 sections per treatment per replicate). TUNEL staining was carried out using a commercially available kit (DeadEnd Colorimetric TUNEL System; Promega) following the manufacturer's instructions. Tissue sections were examined under light microscope (Olympus BX 51, America Inc.), and six images of each group within each replicate ($n = 36$ images/group) were obtained in $20\times$ objective magnification to calculate the pixel intensity of TUNEL-positive cells using the ImageJ software (version 1.50f). Therefore, a total of 108 images were obtained from randomly selected regions of interest with 1280×960 pixels to measure the fluorescence intensity of TUNEL-positive cells. TUNEL-positive and -negative controls were included in all evaluations, according to the manufacturer's recommendations.

Statistical analysis

All statistical analysis were performed using R statistical software, version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Data for CFDA-SE, JC-1 and H2DCFDA were not normally distributed; therefore, data were transformed and presented in base 10 logarithm (Log10). Variables without normal distribution were analyzed by Kruskal–Wallis test and Wilcoxon–Mann–Whitney test. Mean percentage of normal preantral follicles, stromal cell density and TUNEL were analyzed by ANOVA and Tukey's test. 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.

Results

Ovarian tissue viability, mitochondrial membrane potential and ROS production

The ovarian tissue viability (CFDA-SE) in the whole ovary, biopsy size and transplant size groups did not differ ($P > 0.05$) from the fresh control group when stored at 4°C for up to 24 h (Fig. 1). However, after 6 h of storage, the transplant size group had a greater ($P < 0.05$) viability compared to that in the biopsy size group.

The mitochondrial membrane potential (JC-1) of the whole ovary group was lower ($P < 0.05$) at 6 and

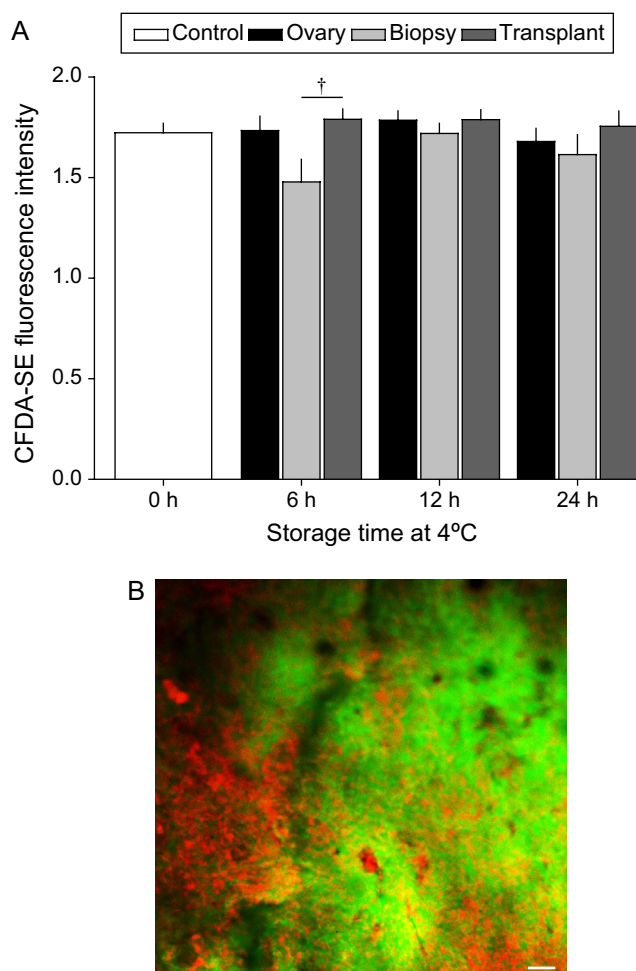


Figure 1 (A) Mean (\pm S.E.M.) fluorescence intensity of CFDA-SE (Log10) to evaluate tissue viability in different fragment sizes (whole ovary, biopsy or transplant) of equine ovarian tissue stored at 4°C for up to 24 h. †Indicates a difference ($P < 0.05$) between treatments at one time point. No difference ($P > 0.05$) between the control and treated groups was detected. (B) Illustrative image of merged CFDA and PI in equine fresh ovarian tissue; scale bar = 100 pixels.

24 h of storage compared to that in the fresh control group (Fig. 2). In the biopsy size group, the membrane potential was lower ($P < 0.05$) after 24 h compared to that in the control group. The transplant size group had a greater ($P < 0.05$) mitochondrial membrane potential at 6 and 12 h of storage and was similar ($P > 0.05$) at 24 h, compared with the control group. Within each time of storage, the whole ovary group had the lowest ($P < 0.05$) mitochondrial membrane potential and the transplant size group the greatest ($P < 0.05$). The ROS production (H2DCFDA) in the ovarian tissue did not differ ($P > 0.05$) among groups (data not shown).

DNA fragmentation

Regardless of treatment, the DNA fragmentation increased ($P < 0.05$) in ovarian cells after 24 h of storage at 4°C compared to that in the control group (Fig. 3).

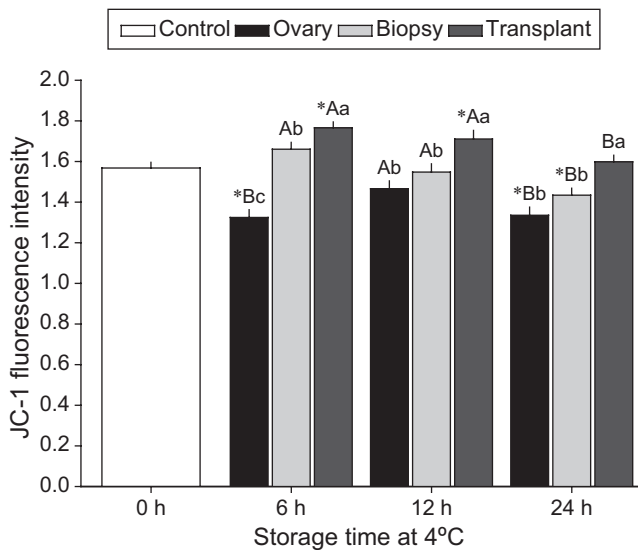


Figure 2 Mean (\pm S.E.M.) fluorescence intensity of JC-1 (Log10) to evaluate mitochondrial membrane potential in different fragment sizes (whole ovary, biopsy or transplant) of equine ovarian tissue stored at 4°C for up to 24 h. *Treatment groups differ from fresh control group ($P < 0.05$). ^{A,B}Within group, columns without a common superscript differed ($P < 0.05$). ^{a,b}Within time, groups without a common superscript differed ($P < 0.05$).

In addition, the whole ovary and biopsy size groups had an increase ($P < 0.05$) in DNA fragmentation between 6 and 24 h. However, the transplant size group had a similar ($P > 0.05$) DNA fragmentation throughout the storage time.

Preantral follicle class distribution and morphology

Overall, 3061 preantral follicles were recorded, and the majority was classified as primordial (69.3%), followed by transition (28.4%), primary (2.0%) and secondary (0.3%) follicles among all groups. The rate of morphologically normal follicles was 69.9% among all groups, with 86.3% being normal in the fresh control group. A mean of 306.1 ± 91.4 (range: 41–1012) preantral follicles were evaluated per group and time of storage. The mean percentage of normal follicles decreased ($P < 0.05$) in all groups in all storage times compared with the fresh control group, except for the whole ovary and transplant size groups at 6 h of storage at 4°C (Fig. 4). Moreover, after 6 h of storage, the whole ovary group had a greater ($P < 0.05$) percentage of morphologically normal follicles than the biopsy size and transplant size groups. However, after 12 and 24 h of storage, the percentage of normal follicles was greater ($P < 0.05$) in the biopsy size group compared with whole ovary and transplant size groups. When analyzing the groups across times of storage, the whole ovary and transplant size groups had lower ($P < 0.05$) percentages of morphologically normal follicles after 12 h and 24 h

of storage, whereas the biopsy size group was reduced ($P < 0.05$) only after 24 h of storage.

Stromal cell density

The ovarian stromal cell density differed ($P < 0.05$) among groups (Fig. 5). The ovarian stromal cell density in the fresh control group ranged from 15 to 46 cells/2500 μm^2 (CV% = 23.3%). The biopsy size and the whole ovary groups had lower ($P < 0.05$) stromal cell density after 12 and 24 h of storage respectively, when compared with the control group. The transplant size group had the greatest ($P < 0.05$) stromal cell density compared with that in the control group throughout all storage times.

Discussion

To our knowledge, this is the first study to report the effect of size of equine ovarian tissue stored at 4°C for up to 24 h on ovarian tissue viability, mitochondrial membrane potential, ROS production, DNA fragmentation, morphology and classification of preantral follicles and stromal cell density. The main findings of the present study were (i) biopsy size fragments had more morphologically normal preantral follicles after 24 h of storage at 4°C; (ii) the viability of ovarian cells and ROS production were not disturbed during storage at 4°C for up to 24 h regardless of the size of the ovarian fragments; (iii) mitochondrial membrane potential was the lowest during each time of storage when the whole ovary was used; (iv) DNA fragmentation rate in the ovarian cells of all sizes of fragments increased as storage was prolonged and (v) transplant size fragments had increased stromal cell density during storage at a cool temperature.

The present study has shown that ovarian biopsy size fragments had more than 60% morphologically normal preantral follicles after being stored at 4°C for 24 h in α -MEM solution enriched with BSA and pyruvic acid; the whole ovary and transplant size groups had less than 50% and 20% normal follicles after the same period of storage respectively. The only study in horses (Gomes *et al.* 2012) that has evaluated the quality of preantral follicles enclosed in ovarian tissue during transportation, but not the size of fragments, reported 27, 11 and 3% of morphologically normal follicles in fragments (1 \times 3 \times 3 mm) stored in MEM solution at 4°C after 4, 12 and 24 h respectively. However, the lower rates of morphologically normal follicles preserved in the study performed by Gomes and coworkers (Gomes *et al.* 2012) may not be suitable for transportation of ovarian specimens from large distances between animal (patient) and specialized centers. To our knowledge, the only study (Barberino *et al.* 2016) that has evaluated the effect of ovarian fragment size (whole ovary, 1/2, 1/4 or 1/8 of the ovary) stored at 4°C for up to 24 h was performed in sheep. The sheep study reported that the smallest

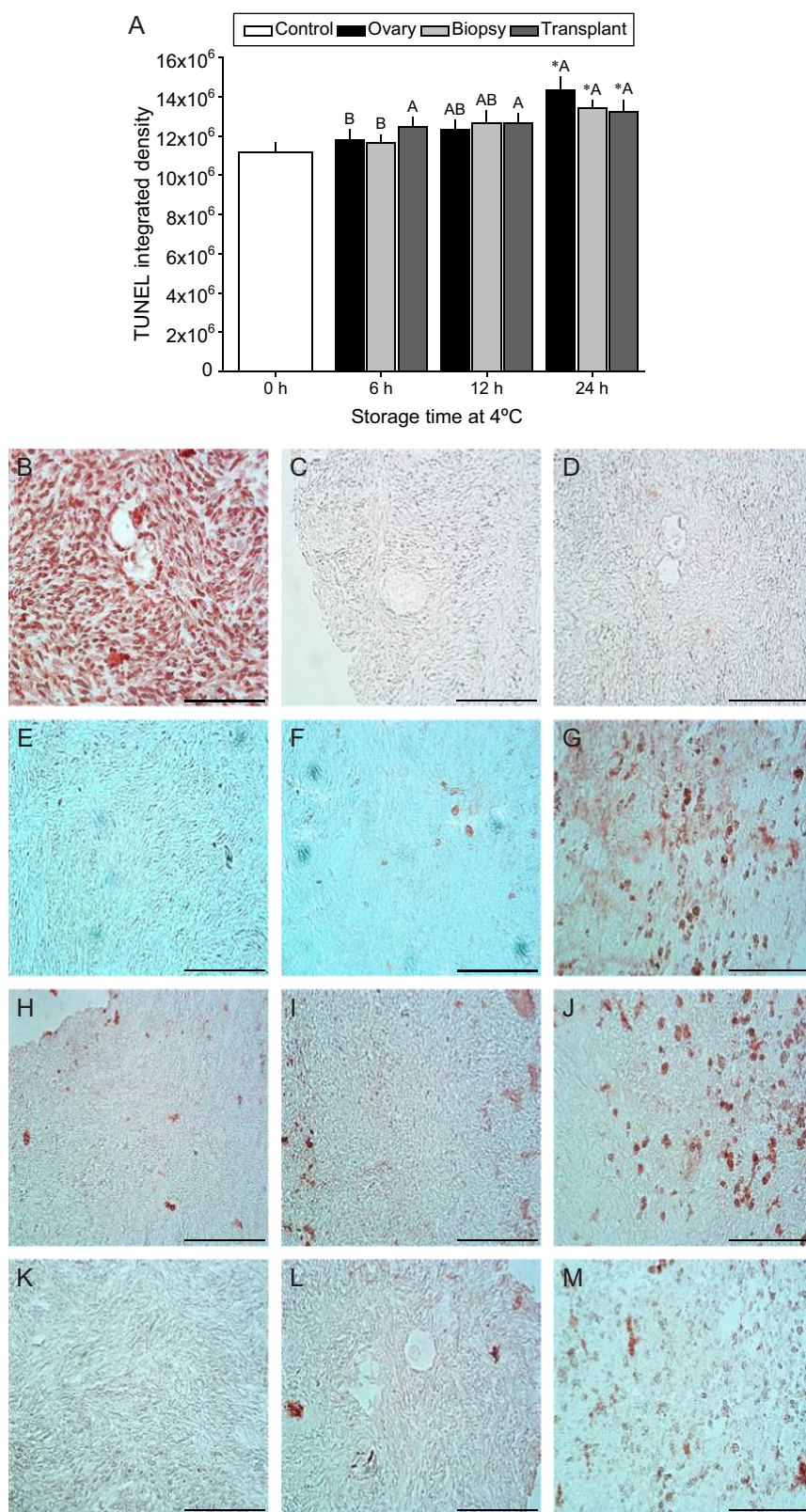


Figure 3 (A) Mean (\pm S.E.M.) intensity of TUNEL staining per unit area to evaluate DNA fragmentation in different fragment sizes (whole ovary, biopsy or transplant) of equine ovarian tissue stored at 4°C for up to 24 h. *Treatment groups differ from fresh control group ($P < 0.05$). ^{A,B}Within group, columns without a common superscript differed ($P < 0.05$). No difference ($P > 0.05$) among groups within each time point was detected. (B) Positive and (C) negative assay controls. For positive controls, the sections were incubated in DNase I (5 unit/mL) for 10 min; and negative controls, the sections were incubated in buffer without rTdT enzyme, as described in the manufacturer's instructions. (D) Control 0 h; whole ovary (E) 6 h, (F) 12 h and (G) 24 h; biopsy size (H) 6 h, (I) 12 h and (J) 24 h; and transplant size (K) 6 h, (L) 12 h and (M) 24 h; scale bar = 50 μ m.

fragments had more normal preantral follicles than the middle sizes and the whole ovary after 24 h of storage. However, the definition of fragment sizes used in the sheep study has not considered some important factors,

such as natural/individual variation of the ovarian size, and the presence of large ovarian structures (e.g., preovulatory follicle and corpus luteum) that may affect the ovarian dimension and, consequently, the exact

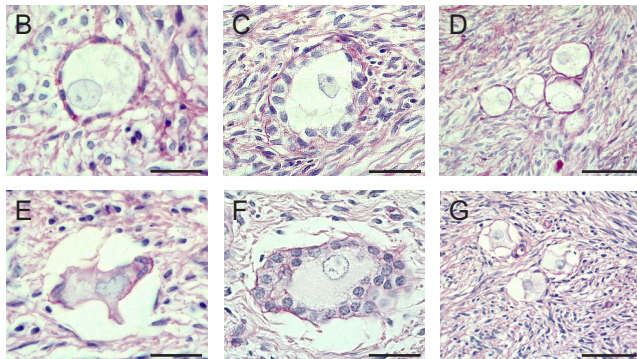
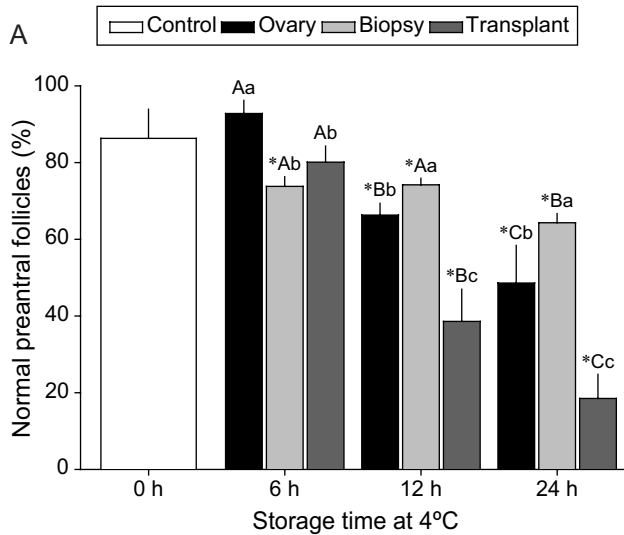


Figure 4 (A) Mean (\pm S.E.M.) percentage of morphologically normal preantral follicles enclosed in different fragment sizes (whole ovary, biopsy or transplant) of equine ovarian tissue stored at 4°C for up to 24 h. *Treatment groups differ from fresh control group ($P < 0.05$). ^{A,B}Within group, columns without a common superscript differed ($P < 0.05$). ^{a,b}Within time, groups without a common superscript differed ($P < 0.05$). The number of preantral follicles evaluated per group and time of storage ranged from 41 to 1012. (B, C, D, E, F and G) Illustrative images of equine preantral follicles enclosed in biopsy ovarian fragments stored for 24 h at 4°C; (B) normal and (E) abnormal primordial follicles; (C) normal and (F) abnormal primary follicles; (D, G) cluster of primordial follicles; (B, C, E, F) scale bar = 20 µm; (D, G) scale bar = 50 µm.

final size of the fragment. Furthermore, because the exact size of the fragments with stromal tissue was not defined, it is difficult to reproduce similar results to determine precisely the main effect of the size of the fragment on storage/transportation. In the present study, the biopsy and transplant fragment sizes have been standardized, allowing a precise interpretation of size effect. In addition, studies have evaluated different types of medium for transport and storage of ovarian fragments at cool temperatures and have shown relevant results regarding the preservation of preantral follicle morphology (Dittrich *et al.* 2012, Isachenko *et al.* 2012, Kamoshita *et al.* 2016). However, the aforementioned studies have shown a large variation in the results

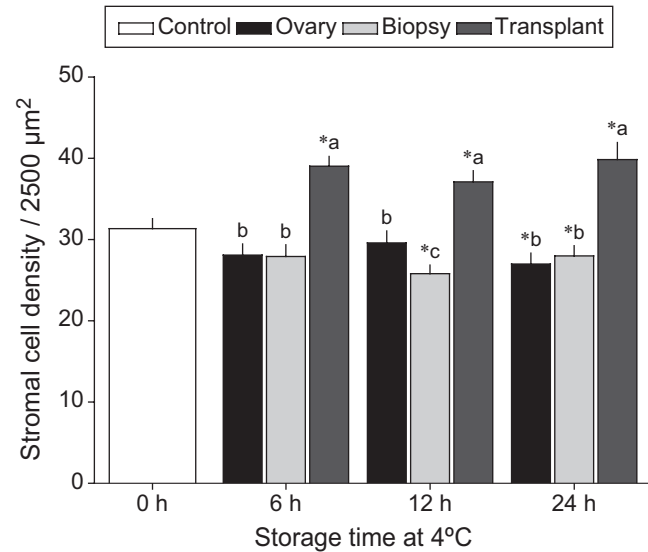


Figure 5 Mean (\pm S.E.M.) stromal cell density per area (2500 µm²) in different fragment sizes (whole ovary, biopsy or transplant) of equine ovarian tissue stored at 4°C for up to 24 h. *Treatment groups differ from fresh control group ($P < 0.05$). ^{a,b}Within time, groups without a common superscript differed ($P < 0.05$). No difference ($P > 0.05$) was observed across different times of storage for the same group.

(range, 40–90% of morphologically normal follicles), which might be due to different fragment sizes among studies. Moreover, the enriched solution used in our study might have contributed to preserving the ovarian tissue for a longer period. Better pH and osmolality control, and enrichment of nutrients, can increase the resistance of organs and tissues due to the lack of oxygenation during hypothermia, reduce REDOX activity and therefore, prolong the cell survivability (Guibert *et al.* 2011).

The most common organ preservation technique for transport is inducing hypothermia to approximately 4°C (Cantu & Zaas 2011). Cooling is necessary to reduce cellular metabolism and the requirements for oxygen to prevent tissue injury; however, at 4°C, there are some metabolic rates remaining (Guibert *et al.* 2011). In the present study, the cell viability and ROS production in the equine ovarian tissue stored at 4°C for up to 24 h have not been disturbed. However, mitochondrial membrane potential decreased after 24 h of storage in whole ovary and biopsy size fragments. Although ROS production in the equine ovarian tissue did not increase as storage was prolonged, this study has shown an increase of DNA damage in the ovarian cells only after 24 h. The lower metabolic rate due to cool temperatures reduces mitochondrial enzyme activity, which in turn reduces the accumulation of lactic acid and slows down the decrease in intracellular pH, proteolysis, lipolysis and lipid peroxidation associated with ischemia (Guibert *et al.* 2011). BSA has been commonly used in medium supplementation due to its important functions, such as solution stabilization during storage at cooling or freezing temperatures, binding and transport of important ligands

and as antioxidant (Francis 2010). Pyruvic acid has been reported to eliminate hydrogen peroxide and its toxic effects from the culture medium (Giandomenico *et al.* 1997) and maintain cell metabolism (Geshe *et al.* 2000). Therefore, we suggest that the enriched α -MEM medium with BSA and pyruvic acid used in this study was able to control and avoid the apoptosis caused by ROS production, prolonging the equine ovarian tissue preservation at 4°C. Nevertheless, when a proper transportation solution, control of temperature and standard of the fragment size are used within a feasible time of transport in cooling temperatures, the DNA fragmentation that occurs in the ovarian tissue during storage seems to be ROS independent.

In horses, stromal cell density has a strong relationship with the number and density of preantral follicles (Alves *et al.* 2016a, Gastal *et al.* 2016). The stromal cell density has a major role in protecting and supporting the recruitment and development of preantral follicles (Knight & Glister 2006). The present study is the first to evaluate the effect of ovarian fragment size during storage at cooling on stromal cell density. In this study, small fragments (0.5 × 1 × 1 mm) stored at 4°C had an increase in the ovarian stromal cell density compared with fresh control tissue. Conversely, larger-sized fragments (biopsy size and whole ovary groups) had a decrease in stromal cell density during storage compared to those in fresh tissue. These effects might have been caused by surface-to-volume ratio during the cooling stage (Herraiz *et al.* 2016). Therefore, small fragments may shrink before penetration and equilibration of the solution within the cell, whereas in larger tissues, the cooling curve occurs slowly, and thus, the solution can better penetrate the cells leading to swelling and reduction of stromal cell density per area. Although variation in stromal cell density in the different fragment sizes after storage was observed, the stromal cells were still physiologically viable based on the parameters studied herein.

In conclusion, the findings described in the present study provide new perspectives for transporting equine preantral follicles enclosed in ovarian tissue for long distances. Therefore, the biopsy fragment size was the best to preserve follicle morphology for long storage (24 h). Translation of this work to other animal species and humans may lead to significant advancements regarding methodologies for fertility preservation. The size of ovarian fragment played a major role in preserving preantral follicles during ovarian tissue transportation. The enriched transport medium herein used was potentially beneficial to preserve the ovarian cell viability during storage at 4°C for up to 24 h, thus allowing greater chances of success for future cryopreservation and/or grafting processes. Therefore, the size of the ovarian fragment for transportation/storage should be prior determined according to the distance (time of transportation) between patient and reproduction center/clinics.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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