Incidence of Apoptotic Cells After Vitrification in Canine Ovarian Tissues

—Brief Note—

Incidence of apoptotic cells after vitrification in canine ovarian tissues

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Abstract: Although vitrification and follicular survival subsequent to xenotransplantation of canine ovarian tissues have been reported, the degree of cryoinjury after the vitrification has not yet been fully examined. Since cryoinjury after vitrification of ovaries has been reported in bovine and humans, this study evaluated canine ovarian tissues after vitrification by immunohistochemistry and TUNEL assay. Ovaries were collected from immature bitches. The ovarian tissue cubes were pretreated with 1 M DMSO. Subsequently, DAP 213 solution (2 M DMSO, 1 M acetamide, 3 M propylene glycol) was added. The cryotubes containing the ovarian tissues were placed on ice for 5 min, then plunged directly into liquid nitrogen. After warming with 0.25 M sucrose, cryopreserved ovarian tissues were fixed and subsequently stained with hematoxylin-eosin, proliferation cell nuclear antigen (PCNA) antibody, or active caspase-3 (AC-3) antibody, and evaluated by the TUNEL assay. There were no differences in the numbers of primordial, primary, secondary and antral follicles/mm^2 between the fresh and vitrified-warmed ovarian tissues. Percentages of PCNA and AC-3 antibody positive cells were similar regardless of the developmental stage of the follicles and experimental group. A few TUNEL positive cells were detected in both experimental groups. These results suggest that vitrification with DAP213 does not induce cryoinjury in ovarian tissues from immature bitches.
Key words: Cryopreservation, Dog, Follicle, Ovary

Introduction

The cryopreservation of ovarian tissues is a potentially significant technology for the preservation of the genetic resources of dogs as well as other animals. Because of the simplicity of vitrification, it has used for the cryopreservation of ovarian tissues as well as embryos and oocytes as an alternative to slow freezing. Successful ovarian cryopreservation by the vitrification method has been reported for mice [1–3] and sheep [4]. In humans, although the establishment of ovarian tissue banks for patients has been proposed [5], and live births after transplantation of cryopreserved ovarian tissues by slow-freezing have been reported [6–9], no live births derived from vitrified ovarian tissues have yet been recorded. It has been reported that slow freezing is more promising than vitrification for human ovarian tissues [10, 11]. However, many studies have supported vitrification as the cryopreservation method of choice for human ovarian tissues [12].

In dogs, although vitrification and follicular survival subsequent to xenotransplantation of ovarian tissues has been reported [13–15], the degree of cryoinjury after vitrification has not yet been fully examined. Since cryoinjury after vitrification of ovaries has been reported in bovine [16], and rats [17], as well as humans [18, 19], this study evaluated canine ovarian tissues after vitrification by immunohistochemistry and TUNEL assay.

Materials and Methods

Vitrification of canine ovaries

Canine ovaries were collected from an 8-month-old Chihuahua (BW: 2.4 kg), a 6-month-old mixed breed (BW: 11.5 kg), and a 6-month-old mixed breed (BW: 8.6 kg) undergoing routine ovariohysterectomy. The ovaries from these immature bitches were transported to the laboratory in a jar containing sterile saline at 37C. The vitrification and warming procedures were performed according to the method of Ishijima *et al.* [13]. The fresh canine ovaries were dissected in a 60-mm-diameter Petri

dish (#150326, Nunc, Thermo Fisher Scientific KK, Yokohama, Japan), half-filled with PBI medium [20] at 37C, to separate the cortex and medulla. The cortex was further sectioned into 1.0–1.5 mm cubes. The ovarian cubes were pretreated with PBI medium containing 1 M dimethyl sulfoxide (DMSO; #049–07213, Sigma-Aldrich Japan, Tokyo, Japan) at room temperature for 60 sec. The cubes were transferred into a 1-ml cryotube (#375418, Nunc) containing 5 μl of 1 M DMSO, which was then placed in ice water for 5 min to allow DMSO to thoroughly bathe the ovaries. Subsequently, 95 μl of DAP 213 solution (2 M DMSO, 1 M acetamide, 3 M propylene glycol) [21] precooled on ice were added to each cryotube. The cryotubes were placed in ice water for 5 min, then plunged directly into liquid nitrogen and stored until use. As a control, fresh ovarian tissues were fixed with 10% buffered formalin for 24 h. For warming, the tubes were taken from the liquid nitrogen, allowed to stand at room temperature for 60 sec, then diluted with 900 μl of PBI medium (37C) containing 0.25 M sucrose (#196–00015, Wako Chemical Industries, Ltd., Osaka, Japan). The recovered ovaries were transferred to PBI medium and washed 5 times with PBI, then fixed with 10% buffered formalin.

Histological evaluation

The fixed tissues were embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. To evaluate the effects of vitrified-warming, follicles that visibly contained an ovum (oocyte) with a nucleus were counted according to the classification of Kerr *et al.* [22]: primordial follicles, follicles containing an oocyte partially or completely encapsulated by squamous pregranulosa cells; primary follicles, follicles in which all of the granulosa cells exhibit enlargement and a single layer of granulosa cells; Secondary follicles, follicles containing an oocyte encapsulated by more than 2 layers of granulosa cells with no antrum formation; antral follicles, follicles containing an oocyte encapsulated by more than 2 layers of granulosa cells with antrum formation. For both fresh and cryopreserved ovarian tissues, 10 cubes were prepared for a tissue specimen (a block) in each experimental group. In total, five ovarian tissue sections (5 μm in thickness) were examined in each block. The distance between sections was 100 μm. The number of follicles in each section was counted, and the area of each section was calculated using an All-in-one Fluorescence Microscope (BZ-9000, Keyence, Osaka, Japan). The number of follicles in each stage in each section was expressed as the number per square millimetre. The data from 10 cubes were expressed as the mean ±standard deviation of the mean.

Next, sections originating from both fresh and vitrified-warmed ovarian tissues were randomly selected and evaluated for immunolocalization of proliferating cell nuclear antigen (PCNA) [13], and active caspase-3 antigen as an apoptosis marker. Furthermore, to evaluate DNA fragmentation derived from apoptosis, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed. One section from each block, in total 10 sections, were examined in each experimental group. For immunostaining of PCNA, an anti-PCNA mouse monoclonal antibody (#412801, prediluted, Nichirei Biosciences Inc., Tokyo, Japan) was used as the primary antibody. Deparaffinized and hydrated sections were heated in 10 mM citrate buffer (pH 6.0) for 15 min at 98C to enhance antigen retrieval. For immunostaining of active caspase-3 antigen, an anti-active caspase-3 rabbit polyclonal antibody (bs-0081R, diluted to 1:500, Bioss Inc., Boston, MA, USA) was used as the primary antibody. To enhance antigen retrieval for active caspase-3, deparaffinized and hydrated sections were treated with protease solution for 15 min at room temperature.

The sections were incubated with 3% H_2O_2–methanol solution for 15 min to remove endogenous peroxidase and subsequently treated with 2.5% normal horse serum (MP-7500, Funakoshi Co., Ltd., Tokyo, Japan) to block nonspecific binding of the antibody. They were then incubated with 100 μl of primary antibodies for 1 h at room temperature in a humidified chamber, washed with PBS (pH 7.5) and subsequently incubated with 100 μl of ImmPRESS anti-mouse/rabbit Ig antibody (MP-7500, prediluted, Funakoshi Co., Ltd.) as the secondary antibody for 30 min at room temperature. After washing with PBS, to detect binding of the primary antibody, sections were treated with 100 μl of diaminobenzidine (DAB) solution for 30 sec according to the manufacturer's instructions (#425011, Nichirei Biosciences Inc.). Afterwards, the sections were washed with distilled water, and the nuclei were counterstained with Mayer-hematoxylin (#30002, Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Labeled cells were distinguished from unlabeled ones based upon the brown DAB precipitate. The primary antibodies were replaced by 10% normal horse serum in negative control sections.

A DeadEnd Colorimetric Apoptosis Detection System Kit (G7130, Promega KK, Tokyo, Japan) was used for TUNEL staining. Deparaffinized and hydrated sections were fixed with 10% buffered formalin. After washing with PBS, the sections were treated with 100 μl of proteinase solution (20 μg/ml) for 20 min at room temperature. Then, after washing with PBS, the sections were fixed with 10%

Table 1. Number of healthy follicles in canine ovarian tissues after vitrificaion

Dog	Treatment	Mean number of healthy follicles ± SD /mm^2				
		Primordial	Primary	Secondary	Antral	Total
Chihuahua (8M, 2.4 kg)	Fresh	3.0 ± 2.35[a]	1.3 ± 0.60[a]	0.2 ± 0.18[a]	0[a]	4.4 ± 2.59[a]
	Vitrified	2.2 ± 2.17[a]	1.1 ± 0.80[a]	0.1 ± 0.11[a]	0[a]	3.5 ± 2.80[a]
Mixed breed (6M, 11.5 kg)	Fresh	3.2 ± 2.95[a]	0.7 ± 0.47[a]	0.1 ± 0.14[a]	0.002 ± 0.01[a]	4.0 ± 3.43[a]
	Vitrified	3.2 ± 2.74[a]	0.8 ± 0.39[a]	0.1 ± 0.14[a]	0[a]	4.2 ± 2.95[a]
Mixed breed (6M, 8.6 kg)	Fresh	10.7 ± 6.67[a]	1.3 ± 0.54[a]	0.5 ± 0.26[a]	0[a]	12.5 ± 6.92[a]
	Vitrified	10.5 ± 8.40[a]	1.0 ± 1.13[a]	0.3 ± 0.42[a]	0[a]	11.8 ± 12.22[a]

Values in the same column with different letters differ significantly (p<0.05) in each dog.

buffered formalin again. Subsequently, the sections were treated with 100 μl of equilibration buffer for 10 min after washing with PBS. Then, 100 μl of incubation buffer containing equilibration buffer, biotinylated nucleotide mix and rTdT enzyme was added to the sections, and incubated for 1 h at 37C. To terminate the reaction, the sections were treated with 2 x SSC (standard saline citrate) for 15 min. The sections were further incubated with 3% H_2O_2 in PBS for 5 min to remove endogenous peroxidase, washed with PBS, and treated with 100 μl of horse radish peroxidase labeled streptavidin (diluted to 1:500) for 30 min at room temperature. After washing with PBS, the sections were treated with 100 μl of DAB solution for 30 sec and washed with distilled water, then the nuclei were counterstained with Mayer-hematoxylin. As a positive control, sections were treated with DNase I to induce apoptosis after the refixation. The rTdT mixed with distilled water instead of rTdT enzyme was used as a negative control.

For immunohistochemistry, the percentages of antibody positive cells and the intensity of staining were analyzed in both follicular and stroma cells. Follicles with at least one stained granulosa cell were considered positive [23]. Stromal positivity for the primary antibodies was counted in a total of 5 fields of view in each section at 400 X magnification under an All-in-one Fluorescence Microscope. The mean intensity of staining in both follicular and stroma cells was measured and counted from the luminous intensity of the pixels using an All-in-one Fluorescence Microscope; higher pixel values indicate stronger immunoreactivity.

Statistical analysis was performed using the Wilcoxon signed rank test (software version 3.0.2 for Windows). *P* values less than 0.05 were considered to be significant. The animals used in this study were treated under the Guiding Principles for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Results

As shown in Table 1, there was no difference in the mean number of follicles/mm^2 of each follicular stage between fresh and vitrified-warmed ovarian tissues in the three dogs examined. Furthermore, proportions of atretic/abnormal follicles in ovarian tissue were not different between the fresh (17.9–24.9%) and vitrified-warmed (18.2–22.2%) ovarian tissues in each dog. These results indicate that the vitrification procedures utilized in this study did not affect the morphology of the follicles in the canine ovarian tissues.

The percentages of follicular cells positive for PCNA (Fig. 1) did not significantly differ between fresh and vitrified-warmed ovarian tissues in the three dogs examined. Approximately ninety percent of primordial (87.3–91.2%), primary (98.5–100%) and secondary (100%) follicles in ovarian tissues were positive for PCNA antibody in both experimental groups for each dog. Similarly, in stroma cells of ovarian tissues, there was no difference in the percentages of PCNA antibody positive cells between fresh and vitrified-warmed ovarian tissues of the examined dogs (ranging from 23.2% to 31.9%). The luminous intensity of pixels representing PCNA in both follicular (ranging from 124.5 to 136.4) and stromal cells (ranging from 79.4 to 88.6) was not significantly different between the experimental groups in all three dogs. A few positive follicular (0–2.0%) and stromal (0–0.01%) cells for active caspase-3 antibody were detected in both fresh and vitrified-warmed ovarian tissues in the three dogs (Fig. 1). The staining intensity of active caspase-3 ranged from 39.5 to 46.6 pixels in both experimental groups in all three dogs. These values appear to be the background level of the immunohistochemistry for active caspase-3 under our experimental conditions. Similar to active caspase-3 staining, a few follicular (0–0.3%) and stromal (0.01–0.03%) cells positive for the TUNEL assay were detected in both fresh and vitrified-warmed ovarian tis-
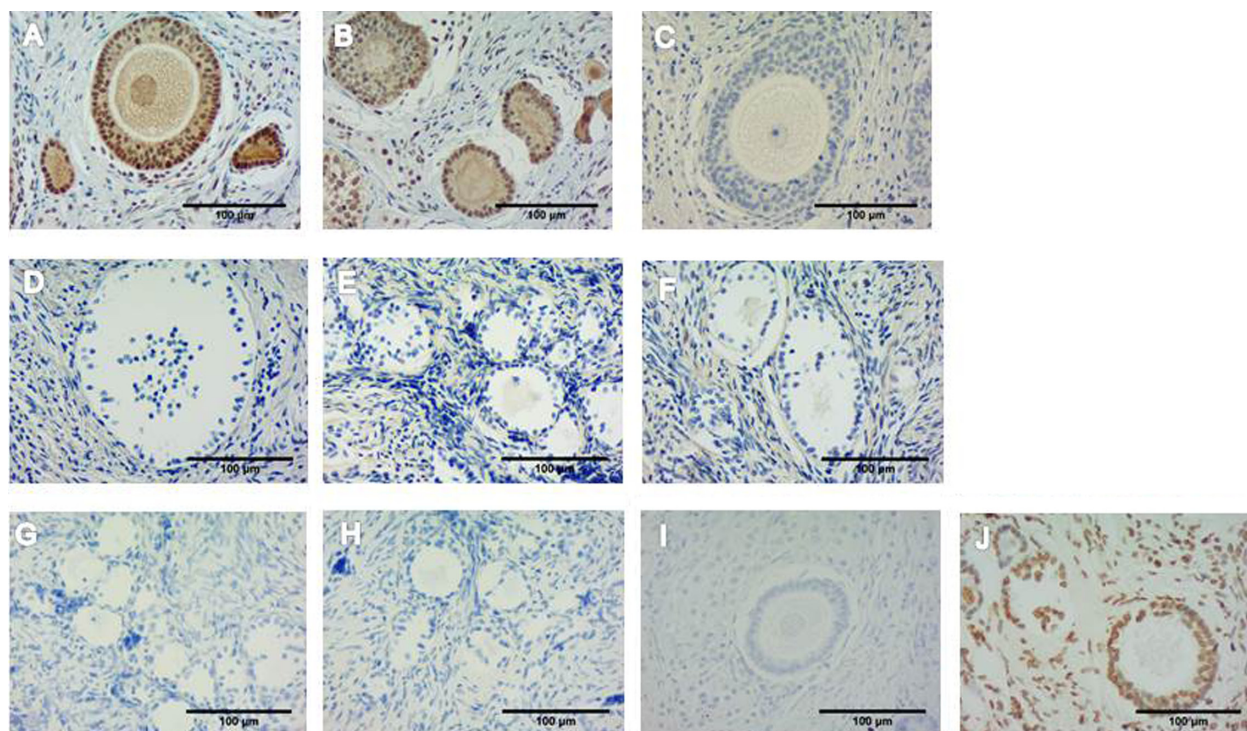
Fig. 1. Immunohistochemistry for PCNA (A, B and C), active caspase-3 (D, E and F), and the TUNEL assay (G, H, I, and J) of canine ovarian tissues before (A, D and G) and after vitrification (B, E and H). Panels C, F and I are negative controls for PCNA, active caspase-3 and TUNEL staining, respectively. Panel J is a positive control for the TUNEL assay.

sues in the three dogs (Fig. 1). These results suggest that the vitrification method used in this study did not induce cryoinjury in the ovarian tissues from the three immature bitches.

Discussion

Cryopreservation of sperms, oocytes and embryos, ovaries and testes is one of the most important assisted reproductive techniques. It allows efficient breeding and reproduction in animals, as well as infertility treatment and fertility preservation in humans. The advantage of cryopreservation of the ovary or ovarian tissue is the ability to conserve large numbers of immature oocytes within the protective environment of the original ovarian tissue and the avoidance of hormonal stimulation to increase the number of retrieved oocytes. In addition, because primordial follicles are small and have a simple structure, they are much more tolerant to cryoinjury in freeze-thaw procedures than large growing follicles [24, 25]. Currently, live births resulting from cryopreserved ovarian transplantation in humans are based on programmed slow cryopreservation [6–9]. Research into the vitrification of ovarian tissues in humans and many other

mammals is still at the developmental stage. However, vitrification seems to be an attractive method that has a simple and fast protocol which does not require special equipment, and can be performed in any laboratory, or in the field. Although the results of vitrification of ovarian tissues in many animal species including humans seem to be similar to those of slow freezing [12], several reports concerning porcine [10], bovine [10], and human [10, 11, 18, 19] ovarian tissues have indicated that vitrification is less efficient than slow freezing. Moreover, it has been reported that vitrification causes morphological damage to the follicular cells of ovarian tissues from rats [26], pigs [10], cows [10] and humans [10]. In addition, although there was no difference in the follicular morphologies of the vitrified and frozen human ovarian tissues, glyceraldehyde 3-phosphate dehydrogenase gene expression in vitrified tissues was decreased compared to its expression in tissues cryopreserved by conventional slow freezing after culture [11]. It has been shown that the numbers of apoptotic cells, assessed by immunohistochemical staining with the anti-caspase-3 antibody, in vitrified human ovarian tissues is significantly higher than in tissues cryopreserved by slow freezing [18]. In addition, TUNEL assays have indicated that the incidence of

apoptotic cells in human ovarian tissue after vitrification was significantly higher than that in fresh tissue [19]. In contrast, morphological analysis by electron microscopy has demonstrated that follicles are similarly preserved after both vitrification and slow freezing followed by *in vitro* culture for 24 h, and that ovarian stroma cells are significantly better preserved after vitrification than after slow freezing in human ovarian tissue [27]. Moreover, recent reports have concluded that vitrification does not affect the incidence of apoptosis in human ovarian tissue based on the results of techniques such as transmission microscopy, TUNEL assay, anticaspase-3 immunolabelling, and DNA laddering [28, 29]. In terms of the efficacy of cryopreservation, previous studies have reported that the differences between the vitrification approach, type and concentration of cryoprotectants, carrier system and/or warming rate might be factors behind the inconsistencies in results.

In dogs, this aspect of vitrification has recently been investigated [13–15, 30]. Our previous report indicated that canine ovarian tissue after vitrification was morphologically normal and positive for the PCNA antibody at 4 weeks after xenotransplantation [13]. However, the degree of cryoinjury of ovarian follicles after vitrification had not been examined. In the present study, we showed that the numbers of healthy primordial, primary, secondary and antral follicles were not different between fresh and vitrified-warmed canine ovarian tissues (Table 1) and the percentages of follicular cells positive for PCNA antibodies were the same regardless of the experimental group, or the developmental stage of the follicle and stroma cells. Furthermore, few active caspase-3 antibody and TUNEL-positive cells were detected in the experimental groups. Interestingly, apoptotic cells were not detected in primordial follicles of the fresh and vitrified ovarian tissues from all of the three examined dogs. However, it might be necessary to interpret these results with care, since our present study was conducted as soon as the tissues were warmed; therefore, the ovarian tissues might not have had time to exhibit any damage caused by vitrification.

The results presented in this study suggest that vitrification by DAP213 does not induce cryoinjury in ovarian tissues from immature bitches. Many reports have suggested that damage of cryopreserved ovarian grafts might be induced by initial ischemia and/or ischemic perfusion injury rather than from vitrification-associated processes [30–38]. Several attempts have been made to prevent the follicular loss of cryopreserved ovarian tissues after transplantation [14, 15, 38–43]. In the dog, the effect of desialylated erythropoietin on the follicular

survival of vitrified ovaries after xenotransplantation has been reported [14, 15]. However, further studies will be required before we are consistently able to enhance the survival of transplanted cryopreserved ovarian follicles in canine.

Acknowledgments

References

1) Takahashi, E., Miyoshi, I. and Nagasu, T. (2001): Rescue of a transgenic mouse line by transplantation of a frozen-thawed ovary obtained postmortem. Contemp. Top. Lab. Anim. Sci., 40, 28–31. [Medline]

2) Migishima, F., Suzuki-Migishima, R., Song, S.Y., Kuramochi, T., Azuma, S., Nishijima, M. and Yokoyama, M. (2003): Successful cryopreservation of mouse ovaries by vitrification. Biol. Reprod., 68, 881–887. [Medline] [CrossRef]

3) Chen, S.U., Chien, C.L., Wu, M.Y., Chen, T.H., Lai, S.M., Lin, C.W. and Yang, Y.S. (2006): Novel direct cover vitrification for cryopreservation of ovarian tissues increases follicle viability and pregnancy capability in mice. Hum. Reprod., 21, 2794–2800. [Medline] [CrossRef]

4) Bordes, A., Lornage, J., Demirci, B., Franck, M., Courbiere, B., Guerin, J.F. and Salle, B. (2005): Normal gestations and live births after orthotopic autograft of vitrified-warmed hemi-ovaries into ewes. Hum. Reprod., 20, 2745–2748. [Medline] [CrossRef]

5) Bahadur, G., and Steele, S.J. (1996): Ovarian tissue cryopreservation for patients. Hum. Reprod., 11, 2215–2216. [Medline] [CrossRef]

6) Donnez, J., Dolmans, M.M., Demylle, D., Jadoul, P., Pirard, C., Squifflet, J., Martinez-Madrid, B. and van Langendonckt, A. (2004): Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet, 364, 1405–1410. [Medline] [CrossRef]

7) Meirow, D., Levron, J., Eldar-Geva, T., Hardan, I., Fridman, E., Zalel, Y., Schiff, E. and Dor, J. (2005): Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. N. Engl. J. Med., 353, 318–321. [Medline] [CrossRef]

8) Sánchez-Serrano, M., Crespo, J., Mirabet, V., Cobo, A.C., Escribá, M.J., Simón, C. and Pellicer, A. (2010): Twins born after transplantation of ovarian cortical tissue and oocyte vitrification. Fertil. Steril., 93, 268.e11–268.e13. [Medline] [CrossRef]

9) Donnez, J., Squifflet, J., Jadoul, P., Demylle, D., Cheron, A.C., Van Langendonckt, A. and Dolmans, M.M. (2011): Pregnancy and live birth after autotransplantation of frozen-thawed ovarian tissue in a patient with metastatic disease undergoing chemotherapy and hematopoietic stem cell transplantation. Fertil. Steril., 95, 1787.e1–1787.e4. [Medline] [CrossRef]

10) Gandolfi, F., Paffoni, A., Papasso Brambilla, E., Bonetti, S., Brevini, T.A. and Ragni, G. (2006): Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. Fertil. Steril., 85,(Suppl 1): 1150–1156. [Medline] [CrossRef]

11) Isachenko, V., Lapidus, I., Isachenko, E., Krivokharchenko, A., Kreienberg, R., Woriedh, M., Bader, M. and Weiss, J.M. (2009): Human ovarian tissue vitrification versus conventional freezing: morphological, endocrinological, and molecular biological evaluation. Reproduction, 138, 319–327. [Medline] [CrossRef]

12) Amorim, C.A., Curaba, M., Van Langendonckt, A., Dolmans, M.M. and Donnez, J. (2011): Vitrification as an alternative means of cryopreserving ovarian tissue. Reprod. Biomed. Online, 23, 160–186. [Medline] [CrossRef]

13) Ishijima, T., Kobayashi, Y., Lee, D.S., Ueta, Y.Y., Matsui, M., Lee, J.Y., Suwa, Y., Miyahara, K. and Suzuki, H. (2006): Cryopreservation of canine ovaries by vitrification. J. Reprod. Dev., 52, 293–299. [Medline] [CrossRef]

14) Suzuki, H., Ishijima, T., Maruyama, S., Yanagimoto Ueta, Y., Abe, Y. and Saitoh, H. (2008): Beneficial effect of desialylated erythropoietin administration on the frozen-thawed canine ovarian xenotransplantation. J. Assist. Reprod. Genet., 25, 571–575. [Medline] [CrossRef]

15) Hirayama, Y., Inoue, K. and Suzuki, H. (2011): Effect of intraperitoneal administration of desialylated erythropoietin on the follicular survival in cryopreserved canine ovaries after xenotransplantation. J. Mamm. Ova Res., 28, 143–147. [CrossRef]

16) Faheem, M.S., Carvalhais, I., Baron, E. and Moreira da Silva, F. (2013): Effect of bovine ovarian tissue vitrification on the structural preservation of antral follicles. Reprod. Domest. Anim., 48, 774–780. [Medline] [CrossRef]

17) Milenkovic, M., Diaz-Garcia, C., Wallin, A. and Brännström, M. (2012): Viability and function of the cryopreserved whole rat ovary: comparison between slow-freezing and vitrification. Fertil. Steril., 97, 1176–1182. [Medline] [CrossRef]

18) Rahimi, G., Isachenko, V., Todorov, P., Tawadros, S., Mallmann, P., Nawaroth, F. and Isachenko, E. (2009): Apoptosis in human ovarian tissue after conventional freezing or vitrification and xenotransplantation. Cryo Lett., 30, 300–309. [Medline]

19) Zhou, X.H., Wu, Y.J., Shi, J., Xia, Y.X. and Zheng, S.S. (2010): Cryopreservation of human ovarian tissue: comparison of novel direct cover vitrification and conventional vitrification. Cryobiology, 60, 101–105. [Medline] [CrossRef]

20) Whittingham, D.G. (1974): Embryo banks in the future of developmental genetics. Genetics, 78, 395–402. [Medline]

21) Nakagata, N. (1989): High survival rate of unfertilized mouse oocytes after vitrification. J. Reprod. Fertil., 87, 479–483. [Medline] [CrossRef]

22) Kerr, J.B., Duckett, R., Myers, M., Britt, K.L., Mladenovska, T. and Findlay, J.K. (2006): Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply. Reproduction, 132, 95–109. [Medline] [CrossRef]

23) Fabbri, R., Venturoli, S., D'Errico, A., Iannascoli, C., Gabusi, E., Valeri, B., Seracchioli, R. and Grigioni, W.F. (2003): Ovarian tissue banking and fertility preservation in cancer patients: histological and immunohistochemical evaluation. Gynecol. Oncol., 89, 259–266. [Medline] [CrossRef]

24) Hsu, S.Y., and Hsueh, A.J. (2000): Tissue-specific Bcl-2 protein partners in apoptosis: An ovarian paradigm. Physiol. Rev., 80, 593–614. [Medline]

25) Oktay, K., Newton, H., Mullan, J. and Gosden, R.G. (1998): Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone. Hum. Reprod., 13, 1133–1138. [Medline] [CrossRef]

26) Fathi, R., Valojerdi, M.R. and Salehnia, M. (2013): Effects of different cryoprotectant combinations on primordial follicle survivability and apoptosis incidence after vitrification of whole rat ovary. Cryo Lett., 34, 228–238. [Medline]

27) Keros, V., Xella, S., Hultenby, K., Pettersson, K., Sheikhi, M., Volpe, A., Hreinsson, J. and Hovatta, O. (2009): Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. Hum. Reprod., 24, 1670–1683. [Medline] [CrossRef]

28) Rahimi, G., Isachenko, E., Sauer, H., Isachenko, V., Wartenberg, M., Hescheler, J., Mallmann, P. and Nawroth, F. (2003): Effect of different vitrification protocols for human ovarian tissue on reactive oxygen species and apoptosis. Reprod. Fertil. Dev., 15, 343–349. [Medline] [CrossRef]

29) Salehnia, M., Sheikhi, M., Pourbeiranvand, S. and Lundqvist, M. (2012): Apoptosis of human ovarian tissue is not increased by either vitrification or rapid cooling. Reprod. Biomed. Online, 25, 492–499. [Medline] [CrossRef]

30) Ishijima, T., Abe, Y. and Suzuki, H. (2009): Follicular loss of the cryopreserved canine ovary after xenotransplantation. J. Mamm. Ova Res., 26, 61–65. [CrossRef]

31) Newton, H., Aubard, Y., Rutherford, A., Sharma, V. and Gosden, R. (1996): Low temperature storage and grafting of human ovarian tissue. Hum. Reprod., 11, 1487–1491. [Medline] [CrossRef]

32) Candy, C.J., Wood, M.J. and Whittingham, D.G. (1997): Effect of cryoprotectants on the survival of follicles in frozen mouse ovaries. J. Reprod. Fertil., 110, 11–19. [Medline] [CrossRef]

33) Aubard, Y., Piver, P., Cogni, Y., Fermeaux, V., Poulin, N. and Driancourt, M.A. (1999): Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. Hum. Reprod., 14, 2149–2154. [Medline] [CrossRef]

34) Baird, D.T., Webb, R., Campbell, B.K., Harkness, L.M. and Gosden, R.G. (1999): Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at -196 C. Endocrinology, 140, 462–471. [Medline]

35) Oktay, K., Newton, H. and Gosden, R.G. (2000): Transplantation of cryopreserved human ovarian tissue results in

follicle growth initiation in SCID mice. Fertil. Steril., 73, 599–603. [Medline] [CrossRef]

36) Liu, J., Van der Elst, J., Van den Broecke, R. and Dhont, M. (2002): Early massive follicle loss and apoptosis in hetero-topically grafted newborn mouse ovaries. Hum. Reprod., 17, 605–611. [Medline] [CrossRef]

37) Maltaris, T., Dimmler, A., Müller, A., Binder, H., Hoff-mann, I., Kohl, J., Siebzehnrübl, E., Beckmann, M.W. and Dittrich, R. (2005): The use of an open-freezing system with self-seeding for cryopreservation of mouse ovarian tissue. Reprod. Domest. Anim., 40, 250–254. [Medline] [Cross-Ref]

38) Tsai, Y.C., Tzeng, C.R., Wang, C.W., Hsu, M.I., Tan, S.J. and Chen, C.H. (2014): Antiapoptotic agent sphingosine-1-phos-phate protects vitrified murine ovarian grafts. Reprod. Sci., 21, 236–243. [Medline] [CrossRef]

39) Nugent, D., Newton, H., Gallivan, L. and Gosden, R.G. (1998): Protective effect of vitamin E on ischaemia-reper-fusion injury in ovarian grafts. J. Reprod. Fertil., 114, 341–346. [Medline] [CrossRef]

40) Kim, S.S., Yang, H.W., Kang, H.G., Lee, H.H., Lee, H.C., Ko, D.S. and Gosden, R.G. (2004): Quantitative assessment of ischemic tissue damage in ovarian cortical tissue with or without antioxidant (ascorbic acid) treatment. Fertil. Steril., 82, 679–685. [Medline] [CrossRef]

41) Long, H.D., Zhou, C.G., Li, Y.B., Yao, S.Z., Zheng, M. and Wu, Y.P. (2007): Protective effect of reduced glutathione and uninastatin on cryopreserved human ovarian tissue after heterotopic transplantation in nod-scid mice. Fertil. Steril., 88, S337–S338. [CrossRef]

42) Hancke, K., Walker, E., Strauch, O., Göbel, H., Hanjalic-Beck, A. and Denschlag, D. (2009): Ovarian transplantation for fertility preservation in a sheep model: can follicle loss be prevented by antiapoptotic sphingosine-1-phosphate ad-ministration? Gynecol. Endocrinol., 25, 839–843. [Medline] [CrossRef]

43) Hayashi, S., Asano, T., Kakizaki, R. and Suzuki, H. (2012): Beneficial effect of hyperbaric oxygen therapy on the fol-licular survival in the mouse ovary after transplantation. J. Reprod. Dev., 58, 260–263. [Medline] [CrossRef]