Effects of cooling rates and ice-seeding temperatures on the cryopreservation of whole ovaries

Jian-Min Zhang · Yan Sheng · Yong-Zhi Cao ·
Hong-Yan Wang · Zi-Jiang Chen

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Abstract

Purpose The aim of this study was to detect the effects of different cooling rates and different ice-seeding temperatures on the cryopreservation of whole ovaries.

Methods Cow whole ovaries were slowly frozen using different protocols with different cooling rates and different ice-seeding temperatures. Follicular viability was assessed using the trypan blue test; the percentage of morphologically normal primordial follicles and the follicular densities of grafts were measured.

Results Protocol IIb was most effective protocol. Protocol Ib was more effective than protocol Ia and protocol Ic, and protocol IIIb was more effective than protocol IIIa and protocol IIIc.

Conclusions Protocol IIb (the cooling rate was 0.2°C/min, and the ice-seeding temperature was −5°C) was appropriate for slow freezing of cow whole ovaries.

Keywords Cooling rate · Cryopreservation · Ice-seeding temperature · Whole ovaries

Introduction

Patients requiring chemotherapy and/or radiotherapy for cancer or other benign pathologies are likely to develop premature ovarian failure and loss of fertility because of these potentially gonadotoxic treatments [1, 2]. Surgical removal of ovarian tissue, followed by cryopreservation and autotransplantation, offers the possibility of circumventing gonadotoxic injury.

Human ovarian cortical tissue strips have been cryopreserved, thawed, and autotransplanted to several sites with successful reproductive function [3–6]. However, a cessation of the function is seen in most cases within 6–9 months after retransplantation [7]. Studies have shown that the key factor responsible for follicular survival is the postgrafting ischemia, which resulted in significant follicular loss and affected long-term graft functionality [8–10].

In theory, the cryopreservation of intact ovary followed by autologous transplantation using microvascular anastomosis, could achieve an immediate blood supply to maximize graft survival. Spontaneous pregnancy after transplantation of an intact frozen-thawed ewe ovary with microvascular anastomosis shows that this technique is promising [11]. Unfortunately, follicular loss after vascular transplantation of the whole frozen-thawed ovaries is as severe as after transplantation of frozen-thawed ovarian tissue [11, 12]. The protocol of cryopreservation of whole ovaries should be improved.

Cryopreserving a large-sized intact ovary is problematic because of the difficulty of adequate diffusion of cryoprotective agents into large tissue masses. Another challenge of freezing whole ovaries is related to heat transfer in such a large-sized organ [13]. Imperfect freezing technologies for bulky organs can be a big barrier to whole ovary transplantation.

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Capsule Protocol IIb (the cooling rate was 0.2°C/min, and the ice-seeding temperature was −5°C) was appropriate for slow freezing of cow whole ovaries.

J.-M. Zhang · Y. Sheng · Y.-Z. Cao · H.-Y. Wang · Z.-J. Chen (✉)
Shandong Provincial Key Laboratory of Reproductive Medicine,
Center for Reproductive Medicine, Provincial Hospital, Shandong
University,
324 Jingwu Road,
Jinan, China 250021
e-mail: chen zijiang@hotmail.com

The factors affecting the efficiency of slow freezing include the cooling rate and the ice-seeding temperature. Previous studies have shown that the survival of oocytes, morulae, and ovarian tissue after freezing and thawing is affected by the cooling rate and the ice-seeding temperature during cryopreservation [14–16]. To our knowledge, few studies have reported the optimal cooling rate and ice-seeding temperature for slow freezing whole ovaries. The present experiment was therefore designed to perform a comparative investigation of several different protocols using different cooling rates and ice-seeding temperatures and to improve the protocol for slow freezing whole ovaries.

In this study, we chose a cow model for the following reasons: (1) cow ovaries are comparable in size to human ovaries; (2) only one or two follicles mature during each cycle as the maturation of multiple follicles greatly influences the ovarian volume, and therefore influences the outcome of the freezing process (in pigs, multiple follicles mature during each cycle); and (3) cows have monthly cycles [17].

Materials and methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

This study was approved by the Animal Research Ethical Committee of the Shandong University. We collected ovaries with ovarian pedicles from on average 5-year-old cows at the Shandong Hypor Liuhe Breeding Farm. All the selected ovaries were in follicular phase and the ovarian vessels were identified and cut so as to be as long as possible. The ovaries were perfused with heparinized (100 IU ml^{-1}) Ringer's solution immediately after removal from cows, and the ovaries and vessels were transported to the laboratory at 10°C to minimize ischemia.

Fifty ovaries were randomly assigned to the control groups and the experimental groups. Five ovaries were in each group. Based on the cooling rate, the protocols were assigned into protocol I, protocol II, and protocol III.

Protocol I: the cooling rate was 2°C/min . Protocol II: the cooling rate was 0.5°C/min . Protocol III: the cooling rate was 0.1°C/min .

Based on the ice-seeding temperature, the protocols were assigned into protocol a, protocol b, and protocol c.

Protocol a: the ice-seeding temperature was -2°C . Protocol b: the ice-seeding temperature was -5°C . Protocol c: the ice-seeding temperature was -8°C . Therefore, the experimental groups were as follows: group Ia, group Ib, group Ic; group IIa, group IIb, group IIc; group IIIa, group IIIb, and group IIIc.

Cryopreservation of the ovaries

The ovaries were perfused via the ovarian artery with the cryoprotective mixture for 30 min, and the flow rate was 2.5 ml/min . The cryoprotective mixture contained modified phosphate-buffered saline (DPBS) medium, 1.5 mmol/L dimethyl sulfoxide (DMSO) and 15% heated-inactivated fetal calf serum (FCS) (Sijiqing Co., Hangzhou, China). After perfusion, the ovary was transferred to 15 mL cryovials (Corning Coaster Corporation, Cambridge, MA, USA) containing the cryoprotective mixture for controlled freezing using a Planer cryochamber. Cooling began at $+4^\circ\text{C}$ and continued at $2^\circ\text{C per minute}$ until ice nucleation was induced at different temperature (-2°C , -5°C , and -8°C). The temperature was then reduced at different cooling rates (2°C/min , 0.5°C/min , and 0.1°C/min) until -40°C and subsequently at $10^\circ\text{C per minute}$ until -140°C before the cryovials were plunged into liquid nitrogen.

Thawing of ovary

One week later, the vials were removed from the Dewar and held in air at room temperature for 2 min before plunging in a water bath at 37°C for another 2 min with gentle shaking. To remove the cryoprotectant, the ovaries were bathed and perfused in three steps of 10 min each at room temperature with a reversed sucrose concentration gradient (1.0 M , 0.25 M and 0 M sucrose) in Leibovitz L-15 medium to prevent osmotic injury. The perfusion rate was 2.5 ml/min .

Evaluation of follicular viability

The cryopreserved thawed ovaries were evaluated for follicular viability in terms of structural integrity of plasma membrane by the trypan blue exclusion test. Ovarian fragments were thinly sectioned in Leibovitz L-15 medium supplemented with 1 mg/mL (200 IU/ml) type 1 collagenase, incubated at 37°C for 2 h and pipetted every 30 min. Collagenase activity was inhibited by 50% FCS. The suspension was filtered through a $70\text{-}\mu\text{m}$ nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and centrifuged at $400g$ for 5 min. The precipitate was diluted with $50 \mu\text{l}$ of Leibovitz L-15 medium and kept in a water bath at 37°C . Twenty microliters of 0.4% trypan blue was added to the suspension containing the follicles, deposited on a glass slide and examined under an inverted microscope. For each fragment, 100 small intact follicles were examined; the partially or completely denuded oocytes were excluded. Both the number of stained cells and the total number of cells were counted. The percentages of viable follicles were determined by calculating the percentage of unstained cells.

Fig. 1 Ovarian follicle viability in each group

Histological examination

Ovaries were fixed in Bouin's solution for light microscopic evaluation. Serial 5- μ m sections were prepared; every 10th section of each ovary was mounted on a glass slide, and stained with hematoxylin and eosin.

Follicular morphology was examined by microscope (magnification, $\times 400$). For each ovary, 100 primordial follicles were counted in sections where the oocyte nucleus was visible, and their morphology was recorded. Normal follicles had a complete layer of flattened pregranulosa cells, oocytes with cytoplasm, and a normal nucleus. Abnormal follicles were classified as follows: pyknotic nucleus, and both nuclear and cytoplasmic damage.

Transplant into nude mice

Female nude mice (SCID) were used in this study. The mice were housed in groups of three per cage, maintained at 28°C under controlled sterile conditions, with a 12-h light/dark cycle and free access to an autoclaved pelleted diet and water.

The mice were anesthetized by intraperitoneal injection of ketamine (75 mg/kg). The kidney was exteriorized through a dorsal-horizontal incision. A small hole was torn in the kidney

capsule using fine watchmaker's forceps under aseptic conditions. Two pieces of cortex tissue were cut from each whole ovary in each group, and the cortex tissue was cut into 1 $\times$ 1 $\times$ 1 mm slices. Ovarian slices were inserted under the kidney capsule through the small hole. Both kidneys of each mouse received grafts, and four grafts were transplanted to each mouse. Finally, the body wall incisions and skin were closed. The transplantation process was performed at room temperature. At 30 days after surgery, the whole grafts were removed for histological evaluation. Measurement of the follicular density was performed. The whole ovarian graft was analyzed field by field by the light microscopy with a 40 $\times$ objective. The follicular densities were estimated as previously reported [18]. The volume of the ovarian tissue analyzed (V) was calculated by the formula: $V(\text{mm}^3) = S \times 0.06$, where S corresponds to the sum of all the sections areas (mm^2) and 0.06 mm was considered to be the thickness of the tissue analyzed. For each slice, the follicular density was expressed as the number of follicles per mm^3 of ovarian tissue.

Statistical analysis

Follicle viability and the percentage of morphologically normal primordial follicles were compared using χ^2

Fig. 2 Follicle considered to be alive after trypan blue staining (Bar=10 μ m)

Fig. 3 Follicle with a blue oocyte considered to be dead (Bar=10 μ m)

Fig. 4 The percentage of morphologically normal primordial follicles in each group

analysis. The follicular density was compared by analysis of variance (ANOVA). Values were considered significant when $P < 0.05$. SAS version 8.1 software (SAS Institute, Cary, NC, USA) was used for all statistical analysis.

Results

Ovarian follicle viability

Figures 1, 2 and 3 show the follicle viability in each group. The follicle viability in the fresh control group ($89.2\% \pm 5.3\%$) was significantly higher than those in all experimental groups ($P < 0.05$). The follicle viability in group IIb ($76.9\% \pm 3.5\%$) was the significantly highest in all experimental groups ($P < 0.05$). With regard to group I, the follicle viability in group Ib ($63.3\% \pm 4.9\%$) was significantly higher than those in group Ia ($52.6\% \pm 4.1\%$) and group Ic ($54.1\% \pm 4.5\%$) ($P < 0.05$). With regard to group III, and the follicle viability in group IIIb ($65.2\% \pm 4.5\%$) was significantly higher than those in group IIIa ($54.6 \pm 3.6\%$) and group IIIc ($56.1 \pm 3.7\%$) ($P < 0.05$).

Fig. 5 Morphologically normal primordial follicle: spherical follicle, spherical oocyte, and a normal nucleus (→) (Bar=10 μ m)

Histological examination of primordial follicles

Figures 4, 5 and 6 show the percentage of morphologically normal primordial follicles in each group. The percentage of morphologically normal primordial follicles in fresh control group ($91.5\% \pm 8.3\%$) was significantly higher than those in all experimental groups ($P < 0.05$). The percentage of morphologically normal primordial follicles in group IIb ($80.2\% \pm 7.1\%$) was the highest in all experimental groups ($P < 0.05$). With regard to group I, the percentage of morphologically normal primordial follicles in group Ib ($67.3\% \pm 5.6\%$) was significantly higher than those in group Ia ($55.2\% \pm 5.2\%$) and group Ic ($58.5 \pm 5.4\%$) ($P < 0.05$). With regard to group III, the percentage of morphologically normal primordial follicles in group IIIb ($69.2\% \pm 5.3\%$) was significantly higher than those in group IIIa ($57.6\% \pm 3.5\%$) and group IIIc ($59.5\% \pm 4.5\%$) ($P < 0.05$).

Follicular density

The follicular density of the ovarian grafts in each group is shown in Figs. 7 and 8. The follicular density of the ovarian grafts in fresh control group (1.31 ± 0.21) was

Fig. 6 Morphologically abnormal primordial follicle showing the shrinkage of the oocyte and a pyknotic nucleus (→) (Bar=10 μ m)

Fig. 7 The follicular density of the grafts in each group

significantly higher than those in all the experimental groups ($P < 0.05$). The follicular density of the ovarian grafts in group IIb (0.94 ± 0.23) was the highest in the all experimental groups ($P < 0.05$). With regard to group I, the follicular density of the ovarian grafts in group Ib (0.82 ± 0.19) was significantly higher than those in group Ia (0.69 ± 0.16) and group Ic (0.72 ± 0.15) ($P < 0.05$). With regard to group III, the follicular density of the ovarian grafts in group IIIb (0.79 ± 0.15) was significantly higher than those in group IIIa (0.65 ± 0.09) and group IIIc (0.63 ± 0.14).

Discussion

The emerging technology of ovarian cryopreservation followed by transplantation can be a useful option for fertility preservation in cancer patients. Although transplantation of frozen-thawed ovarian tissue has been successful for restoring fertility and ovarian function, the clinical efficacy and practicability are still uncertain [8–10]. Due to the limited life span of ovarian tissue grafts, whole ovary transplantation by vascular anastomosis is being explored as an alternative. The advantages and disadvantages of whole ovary transplantation are showed in Table 1. Trans-

plantation of the whole ovaries by vascular anastomosis has been performed in experimental animals [11, 19, 20]. Moreover, fertility in these animal transplantation models was evidenced by the delivery of offspring by the experimental rats and ewes [11, 21]. Despite of these encouraging results, however, a dramatic depletion in follicular density was observed in transplanted frozen-thawed grafts. Imhof et al. reported a massive follicular depletion, with less than 8% follicles surviving in 18 months after grafting [11]. Other authors reported only a 6% follicular survival rate and the depletion of the entire follicular population after grafting [12]. Thus, further efforts are needed to be made to perfect the technology of whole ovary cryopreservation and transplantation.

The major cause of freezing injury is the growth of ice crystals. During controlled freezing, intracellular water will have time to flow out by osmosis, and ice crystals will then form extracellularly. The formation of ice raises the concentration of solutes. Increased extracellular osmosis causes the cells to become dehydrated. Thus, the injury caused by crystallization can be minimized or avoided.

Cryopreservation of an entire organ is much more complicated than ovarian cortical strips or suspended cells (oocyte) or group of cells (embryo). The large volume of tissue and the diversity of cell types influence the efficiency

Fig. 8 The follicle in the graft (←), the kidney tissue (→) (Bar=10 μm)**Table 1** Advantages and disadvantages of whole ovary transplantation

Advantages	Disadvantages
Immediate blood supply to the graft	Difficulties of organ cryopreservation
Minimizing tissue ischaemia	Surgical complexities and longer surgical time
Possible long-term ovarian function	Ischaemia-reperfusion injury
	Thromboembolism
	Increased morbidity and mortality
	Risk of reintroduction of cancer cells (especially with minimal residual disease in ovarian medulla)

of the cryopreservation of whole ovaries. The optimal cooling rate for slow freezing whole ovaries has not been reported.

Histological examination is an important tool to evaluate the protocol of ovary cryopreservation. Follicle morphology is usually analyzed through several criteria: (1) eosinophilia and vacuolations, which are the most commonly used criteria for oocyte cytoplasm; and (2) contraction and clumping of nucleus chromatin material, which are usually considered signs of atresia [22–24]. However, in our study, we noticed that: (1) oocyte cytoplasm staining frequently varied from one slice to another, independent of any follicular damage; and (2) oocyte cytoplasm vacuolations were also observed on fresh ovarian tissue. Previous studies have also reported that the vacuolization of cytoplasm in oocytes appeared in both the fresh and the frozen-thawed groups analyzed by HE staining [25, 26]. It seems that this phenomenon occurs during the fixation and the stain procedures. As a consequence, we have chosen to evaluate oocyte morphology considering only the chromatin material aspect. Because the trypan blue test provides a means to estimate the follicular viability after cryopreservation, it has been used as a tool to assess the efficiency of the cryopreservation procedure [27–29].

In this study, follicle viability, the percentage of morphologically normal primordial follicles, and the follicular density of the ovarian grafts in group IIb were the highest in all the experimental groups. During the process of freezing whole ovaries, if the cooling rate is fast, intracellular water will have no time to flow out, and the cells, especially those in the deepest parts of the ovary will be unable to maintain equilibrium, and ice crystals will form intracellularly. Intracellular freezing generally causes great damage to living cells, and thus the cooling rate must be slow enough to overcome the obstacle of difficult heat transfer and equilibrium due to the long distance between the inner cells and the ovarian surface. However, slowly cooled cells suffer damage due to long exposure to high electrolyte concentrations, excessive cell dehydration, and mechanical effects of the external ice. Thus the cooling rates should be fast enough to minimize the long exposure of ovaries to deleterious freezing conditions, but slow enough to avoid the damaging effects of intracellular ice formation. This may be the reason that protocol IIb was more effective than the other protocols in slow freezing of whole ovaries.

Ice-seeding is a process that induces ice nucleation using cold tweezers or a cold rod. Ice-seeding is essential for the frozen storage of oocytes, embryos and ovarian tissue because it enhances the dehydration of the cells by extracellular ice crystallization and leads to fewer internal ice crystallization. Trad et al. reported that the extracellular ice-seeding temperature significantly affects intracellular ice formation and cell

viability during the frozen storage of human oocytes [14]. Nakamura et al. also reported that the ice-seeding temperature significantly affected yeast cell viability [30]. However, as far as we know, there are no published data on the detailed effects of the ice-seeding temperature on the survival of frozen whole ovaries. In this study, the protocol in which ice-seeding was -5°C was more effective than the protocol in which ice-seeding was -2°C or -8°C . These findings may improve the freezing procedures of whole ovaries.

In summary, protocol IIb (the cooling rate is $0.2^{\circ}\text{C}/\text{min}$, and the ice-seeding temperature is -5°C) is suitable for slow freezing of cow whole ovaries. Yet, we should make further efforts to explore the optimal protocol for slow freezing of human whole ovaries, thereby restoring the endocrine and reproductive functions of female patients with cancer.

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Conflict of interest statement The authors declare that there are no conflicts of interest.

References

1. Apperley JF, Reddy N. Mechanism and management of treatment-related gonadal failure in recipients of high dose chemoradiotherapy. *Blood Rev.* 1995;9:93–116.
2. Baker TG. Radiosensitivity of mammalian oocytes with particular reference to the human female. *Am J Obstet Gynecol.* 1971;110:746–61.
3. Oktay K, Karlikay G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *N Engl J Med.* 2000;342:1919.
4. Oktay K, Buyuk E. Fertility preservation in women undergoing cancer treatment. *Lancet.* 2004;363:1830.
5. Radford JA, Lieberman BA, Brison DR, Smith AR, Critchlow JD, Russell SA, et al. Orthotopic reimplantation of cryopreserved ovarian cortical strips after high-dose chemotherapy for Hodgkin's lymphoma. *Lancet.* 2001;357:1172–5.
6. Tryde Schmidt KL, Yding Andersen C, Starup J, Loft A, Byskov AG, Nyboe Andersen A. Orthotopic autotransplantation of cryopreserved ovarian tissue to a woman cured of cancer-follicular growth, steroid production and oocyte retrieval. *Reprod Biomed Online.* 2004;8:448–53.
7. Schmidt KL, Andersen CY, Loft A, Byskov AG, Ernst E, Andersen AN. Follow-up of ovarian function post-chemotherapy following ovarian cryopreservation and transplantation. *Hum Reprod.* 2005;20:3539–46.
8. Nugent D, Newton H, Gosden RG, Rutherford AJ. Investigation of follicle survival after human heterotopic grafting. *Hum Reprod.* 1998;13:23–4.
9. Liu J, Van der Elst J, Van den Broecke R, Dhont M. Early massive follicle loss and apoptosis in heterotopically grafted newborn mouse ovaries. *Hum Reprod.* 2002;17:605–11.
10. Aubard Y, Piver P, Cogni Y, Vermeaux V, Poulin N, Driancourt MA. Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. *Hum Reprod.* 1999;14:2149–54.

11. Imhof M, Bergmeier H, Lipovac M, Rudas M, Hofstetter G, Huber J. Orthotopic microvascular reanastomosis of whole cryopreserved ovine ovaries resulting in pregnancy and live birth. *Fertil Steril*. 2006;85(1 Suppl):1208–15.
12. Courbiere B, Caquant L, Mazoyer C, Franck M, Lornage J, Salle B. Difficulties improving ovarian functional recovery by microvascular transplantation and whole ovary vitrification. *Fertil Steril*. 2009;91:2697–706.
13. Martinez-Madrid B, Dolmans MM, Van Langendonck A, Defrere S, Donnez J. Freeze-thawing intact human ovary with its vascular pedicle with a passive cooling device. *Fertil Steril*. 2004;82:1390–4.
14. Trad FS, Toner M, Biggers JD. Effects of cryoprotectants and ice-seeding temperature on intracellular freezing and survival of human oocytes. *Hum Reprod*. 1998;14:1569–77.
15. Gook DA, Edgar DH, Stern C. Effect of cooling and dehydration regimen on the histological appearance of human ovarian cortex following cryopreservation in 1,2-propanediol. *Hum Reprod*. 1999;14:2061–8.
16. Miyamoto H, Ishibashi T. Effects of the temperature of ice-seeding on survival of frozen-and-thawed mouse morulae. *Experientia*. 1981;37:187–8.
17. Gerritse R, Beerendonk CCM, Tijink MSL, Heetkamp A, Kremer JAM, Braat DDM, et al. Optimal perfusion of an intact ovary as a prerequisite for successful ovarian cryopreservation. *Hum Reprod*. 2008;23:329–35.
18. Nisolle M, Casanas-Roux FQuJ, Motta P, Donnez J. Histologic and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. *Fertil Steril*. 2000;74:122–9.
19. Chen CH, Chen SG, Wu GJ, Wang J, Yu CP, Liu JY. Autologous heterotopic transplantation of intact rabbit ovary after frozen banking at -196°C . *Fertil Steril*. 2006;86(4 Suppl):1059–66.
20. Yin H, Wang X, Kim SS, Chen H, Tan SL, Gosden RG. Transplantation of intact rat gonads using vascular anastomosis: effects of cryopreservation, ischemia and genotype. *Hum Reprod*. 2003;18:1165–72.
21. Wang X, Chen H, Yin H, Kim SS, Lin Tan S, Gosden RG. Fertility after intact ovary transplantation. *Nature*. 2002;415:385.
22. Fabbri R, Venturoli SD, Errico A, Iannascoli C, Gabusi E, Valeri B, et al. Ovarian tissue banking and fertility preservation in cancer patients: histological and immunohistochemical evaluation. *Gynecol Oncol*. 2002;89:259–66.
23. Neto V, Buff S, Lornage J, Bottolier B, Guérin P, Joly T. Effects of different freezing parameters on the morphology and viability of preantral follicles after cryopreservation of doe rabbit ovarian tissue. *Fertil Steril*. 2008;89:1348–56.
24. Sadeu JC, Cortvindt R, Ron-EI R, Kasterstein E, Smitz J. Morphological and ultrastructural evaluation of cultured frozen-thawed human fetal ovarian tissue. *Fertil Steril*. 2006;85:1130–5.
25. Perdrix A, Macé B, Milazzo JP, Liard-Zmuda A, Baron M, Rives N. Ovarian tissue thawing: a comparison of two conditions. *Fertil Steril*. 2010;93:307–10.
26. Wang Y. Reply: human ovarian tissue: vitrification versus conventional freezing. *Hum Reprod*. 2009;24:1768–9.
27. Fauque P, Amor AB, Joanne C, Agnani G, Bresson JL, Roux C. Use of trypan blue staining to assess the quality of ovarian cryopreservation. *Fertil Steril*. 2007;87:1200–7.
28. Courbiere B, Massardier J, Salle B, Mazoyer C, Guerin JF, Lornage J. Follicular viability and histological assessment after cryopreservation of whole sheep ovaries with vascular pedicle by vitrification. *Fertil Steril*. 2005;84(2 Suppl):1065–71.
29. Courbiere B, Odagescu V, Baudot A, Massardier J, Mazoyer C, Salle B, et al. Cryopreservation of the ovary by vitrification a an alternative to slow-cooling protocols. *Fertil Steril*. 2006;86(4 Suppl):1243–51.
30. Nakamura T, Takagi H, Shima J. Effects of ice-seeding temperature and intracellular trehalose contents on survival of frozen *saccharomyces cerevisiae* cells. *Cryobiology*. 2009;58:170–4.