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Morphology and survival of cryopreserved-thawed ovarian tissues after heterotopic autotransplantation

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Abstract ***Objective:*** The aim of our study was to observe the survival and morphological changes of thawed ovarian tissues after heterotopic transplantation. ***Methods:*** Twenty SPF-SD female rats (5–6 weeks old) were equally randomized into the control group and experimental group. In control group, the freshly isolated ovaries were fixed in formalin. In experimental group, the freshly isolated ovaries were vitrified immediately and cut into thin slices. After stored in liquid nitrogen for 21 days, the tissues of experimental group were rapidly thawed and transplanted into back muscles of rats for 2 or 4 weeks, respectively. After that, all rats in experimental group were sacrificed and the ovarian tissues were collected and fixed in 4% formaldehyde solution. Then the ovarian tissues were stained with HE and observed under the light confocal microscope. ***Results:*** With the naked eyes, there was no specific alteration except the size reduction with color changing. Under microscopy, we found normal cortex and medulla in the ovary, and the primordial follicles and follicles in various stages were observed in the cortex. The normal oocytes in ovarian tissues of experimental group were significant decreased than in the control group. ***Conclusion:*** The ovarian tissues survive well in experimental group and there is no significant difference in the proportion of follicles between different times (2 and 4 weeks) after grafting. Our results suggest that thawed ovarian tissues could survive after heterotopic transplantation into back muscles of rat models and maintain their morphology and function.

Key words ovarian tissue; morphology; heterotopic transplantation; thawing

Cancer treatments such as chemotherapy, radiation and/or bone marrow transplantation can deplete follicles in the ovary leading to premature ovarian failure, infertility and long term health risks associated with menopause in female cancer patients [1]. Therefore to preserve fertility numerous attempts including autotransplantation, xenografting and follicular culture have been suggested as experimental strategies for restoring ovarian functions in female cancer survivors to make their own cryopreserved-thawed ovarian tissue [2]. Many orthotopic and heterotopic transplantation techniques have been described for fresh and cryopreserved-thawed ovarian cortical strips while many other animal experiments has been suggested that primordial follicles survive with normal ultrastructure by grafting the fresh or cryopreserved-thawed ovarian cortical strips both orthotopically or heterotopically autotransplantion [2, 3]. It has been reported by various studies that autotransplantaion of whole fresh or cryopreserved ovaries with vascular anastomosis is technically feasible and that once the ovarian graft tissues re-vascularized by the surrounding graft site, more follicular development at early stages of ovarian transplantation start to occur which maintain survival, morphology and endocrine function of graft after heterotopic autotransplantation [4–8]. Anna *et al* have demonstrated that autotransplantation of fresh ovaries to deep inferior epigastric vessels provides good short-term results [1], however, they have been performed a further detailed research for knowing the fertilizing potential of oocytes and vascularization of the whole ovary with microvacular anastomosis after freezing/thawing and heterotopic autptransplantation and finally demonstrated that hetertopic autotrasnplantation of the whole frozen/thawed ovary allows for development of preovulatory follicles, oocyte growth and restoration of vascularization and cellular function. Besides, in order to improve the oocyte survival and averting the ice formation in graft tissues, vitrification is considered as an alternative technique however the mixtures of cryoprotectants have less toxicity and are more effective than single-agent cryopreservation [9]. More than 50 years ago, only 5% of ovarian follicles survived after freezing-thawing process compared with fresh ovarian tissues with primordial follicles [10]. Now the animal experiments suggest that 87% of primordial follicles survive with normal morphology following vitrification process and that the

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cryopreservation does not affect the long-term survivability of ovarian tissues, while the fertility and endocrine function can be preserved by graft of cryopreserved ovarian tissues only [11]. In our previous study, we have demonstrated that cryopreservation of ovarian tissues by vitrification method has some detrimental effects on the follicle morphology but does not have negative impact on their survival [3]. However, long-term survival and morphological changes of these cryopreserved-thawed ovarian tissues after heterotopic autotransplantaion have not been performed in detail. Therefore, the objective of this study was to evaluate the survival and morphology in cryopreserved-thawed ovarian tissues after autologous transplantation.

Materials and methods

Animals

Twenty SPF-SD female rats (5–6 weeks, weighing 200–250 g) were purchased from the Hunan SJA Laboratory Animal Co., Ltd, China. All animals were cared for according to the International Guiding Principle for Biomedical Research Involving Animals. The experimental protocol was approved by the Animal Ethics Committee of Wuhan University, Wuhan, China. Animals were adapted to standard housing conditions with a room temperature of 22 ± 2 ℃, and light regimen L:D=12:12 h, with access to food and water *ad libtum*.

Experimental design

All rats were equally randomized into the control group and experimental group. In control group, the freshly isolated ovaries were fixed in formalin. In experimental group, the freshly isolated ovaries were vitrified immediately and then transplanted into back muscles of rats.

Model establishment and treatment

The rats were anesthetized by 10% Chloral Hydrate (0.3 mL/100 g) i.p injection. All the following surgical procedures were performed aseptically. A single median longitudinal skin incision was made on the lumbar portion to expose the subcutaneous tissue over the ovary. A small incision was made on the fascia and muscles immediately above the ovary, thereby exteriorizing the reproductive tract, to isolate and resects the ovary. In the end of surgery, rats were injected with 16000 IU of penicillin in 1 mL 0.9% NaCl by intraperitoneal.

Cryopreservation of ovarian tissue

The recovered ovaries were transported immediately in cell culture dish (Corning) containing 2 mL HTF medium (M&C Gene Technology, Ltd., China). Then ovarian cortex was processed at room temperature (25 ℃) and cut into small sections (between 2 mm × 2 mm × 3 mm × 3 mm in size). Ovarian cortical sections were transferred into cell culture dishes in stepwise manners, containing solution 1 (15% EG + 15% DMSO + 0.5 M sucrose with 10 mL HTF-HEPES) remained for 30–45 s, then transferred in solution 2 (7.5% EG + 7.5% DMSO) remained for 3 min and finally transferred in solution 3 (12% HTF-HEPES) remained for 3 min at room temperature. The ovarian tissues were then transferred in cryogenic vials and plunged into liquid nitrogen for storage.

Thawing procedure

After storage in liquid nitrogen for 21 days, the cryopreserved ovarian tissues were thawed rapidly (–100 ℃/min) by agitating in warm water (35 ℃) for 2 to 3 min and washed in a stepwise manner to minimize osmotic damages (1.0 M sucrose + 10 mL HTF-HEPES for one min, 0.5M sucrose + 10 mL HTF-HEPES for 3 min and 12% HTF-HEPES for 3 min) at room temperature before transplantation.

Transplantation procedure

All rats in group II were anesthetized by 10% chloral hydrate (0.3 mL/100 g) i.p injection. All the following surgical procedures were performed aseptically. The skin and muscle of back and frozen-thawed ovarian tissues were immediately inserted inside the muscle gap (made with ophthalmologic tweezers), then the incision was closed with stitches of nylon sutures. In the end of surgery, rats were injected with 16 000 IU of penicillin in 1 mL 0.9% NaCl by intraperitoneal. From the first day after transplantation, the vaginal cytology was observed every morning to monitor the resumption of cyclic ovarian activity after transplantation.

Rats killing and tissue sampling

In control group, the freshly isolated ovaries were fixed in formalin. In experimental group, the freshly isolated ovaries were vitrified immediately and cut into thin slices. After stored in liquid nitrogen for 21 days, the tissues of experimental group were rapidly thawed and transplanted into back muscles of rats for 2 or 4 weeks, respectively. After that, all rats in experimental group were sacrificed and the ovarian tissues were collected and fixed in 4% formaldehyde solution. Finally, the transplanted ovarian tissues were observed under the light confocal microscope after HE staining.

Morphological observation

The small pieces of cryopreserved cortical ovarian tissue from the nitrogen gas were recovered. The gross appearances of cortical ovarian tissues were recorded before they were fixed in freshly prepared 4% formaldehyde solution. Then these tissues were embedded in paraffin

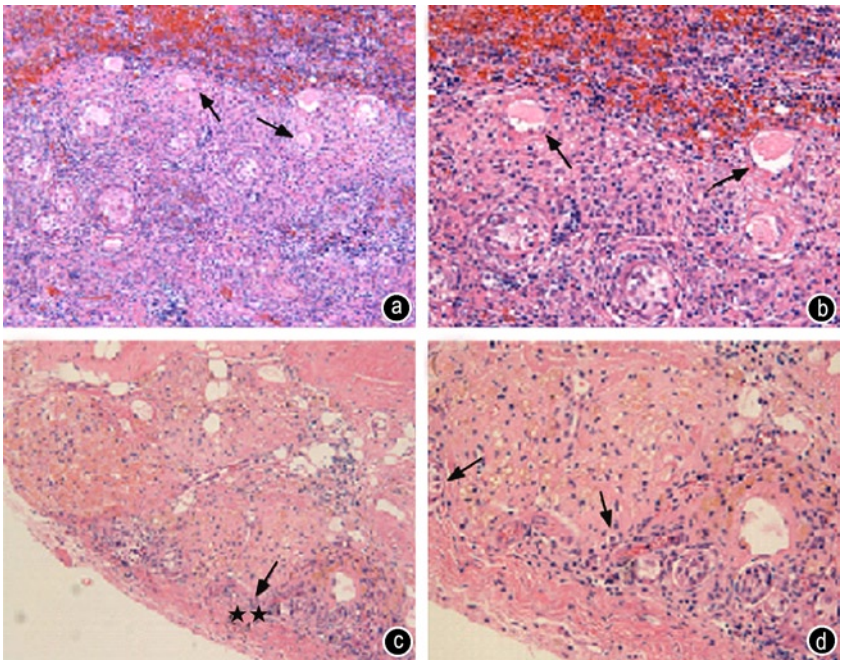

Fig. 1 Histological examination of rat ovarian tissues after transplantation. (a) Two weeks after transplantation. Primordial follicles (arrows) (HE staining × 200); (b) Two weeks after transplantation. Primordial follicles (arrows) (HE staining × 400); (c) Four weeks after transplantation. The grafts (arrow) attach to the host muscle (asterisk) (HE staining × 200); (d) Four weeks after transplantation. Primordial follicles (arrows) (HE staining × 400)

wax, serially sectioned at 3 μm and stained with HE. All sectioned were examined according to the following criteria: (1) identify the structure of the ovarian cortex and medulla; (2) presence of primordial follicles at different developmental stages; (3) observe the size, number and morphological changes of follicles.

Results

The ovarian tissues were removed from the transplant group after 2 and 4 weeks of transplantation, respectively. Then the tissues were stained by HE and the morphological changes were observed under the light microscope.

After 2 weeks of transplantation, we noticed that no significant reduction in the number of ovarian tissue pieces with the naked eye. The volume of most of the ovarian tissues turned to be smaller than the fresh control group, with a gray-red color and the ovarian tissues were closely connected with the muscle tissues. The volume of a small number of ovarian tissue pieces were significantly reduced, with a dim color. Observing under the light microscope, we found that there are visible ovarian tissues with abundant blood supply with normal cortex and medulla. More primordial follicles and ovarian follicles at different developmental stages can be seen in cortex, meanwhile, the normal development of secondary follicles, visible oocytes, oocyte morphology with round nucleus and tightly packed cells were also observed. But some of the ovarian tissues have obvious connective tissues, loss of cortical and medullary anatomical structure, although a small size and number of primordial follicles are visible with more disordered arrangement while the gap was increased between the ovarian tissues and the surrounding cells (Fig. 1a and 1b).

After 4 weeks of transplantation, we found significant reduction in the number of ovarian tissues with a thin layer of connective tissues than in the fresh control group, with pink color and the ovarian tissues were closely connected with the muscle tissues. Ovarian tissues with abundant blood supply and few connective tissues were observed under light microscope, but it can still identify the structure of the ovarian cortex and medulla. More primordial follicles and ovarian follicles at different developmental stages in cortex can be seen. Significantly reduction in the number of follicles than normal ovarian tissues were detected, but no significant difference in the density of oocyte morphology was observed (Fig. 1c and 1d).

Discussion

It had been reported that the precocious ovarian failure in women with cancer therapy can only be prevented by cryopreservation of cortex slices from one ovary before treatment. Primordial follicles have been reported to have tolerance to vitrification and warming injuries compared to other stages due to their special characteristics: (1) they represent 90% of the entire follicular population in the ovary; (2) they have a relatively inactive metabolic rate; (3) their small size (–20 μm diameter); (4) a low number of granulosa cells around the small oocyte; and (5) an absence of the zona pellucida and peripheral cortical granules [12]. And other certain physical factors may include: damage from the growth of ice crystals,

pressure changes, the formation of gas bubbles, chilling injury, osmotic swelling and shrinkage but these imagine as largely ignored factors for oocyte damage after cryopreservation.

It was seem that some degree of regained ovarian function in most attempts of human ovarian cortex transplantation, with the function of grafts persisting occasionally up to 4–5 years. The limited survival time of the graft may be attributed to the restricted amount of tissue that is transplanted, uneven follicular distribution as well as suboptimal cryopreservation or thawing protocols. The major factor behind the short life-span of the graft seems to be injuries during the extended time (several days) of warm ischemia after autotransplantation. There exist several experimental studies that have evaluated the detrimental effects of ischemia on the ovarian cortex. Thus the period of warm ischemia before neovascularisation is essential for follicular survival and the major follicular loss takes place after grafting rather than after freezing, which is in line with results of several studies using animal models.

Neovascularisation of a transplanted ovary is a process which takes at least 2–3 days, as indicated by comprehensive studies of human ovarian tissue transplanted onto the chick chorioallantoic membrane and reports of avascular transplantation of rat ovaries. However, although vascularization seems to be completed within less than five days, the local oxygen pressure in the tissue continues to increase for a period of 10 days after xenotransplantation of human ovarian tissue [13, 14]. In one study it had been demonstrated that fertility of ovariectomized non-pubertal recipient can be restored by grafting immature ovaries capable of ovulation for any age of recipient or graft site, and explained by the high density of primordial follicles in ovarian cortex which were better withstand in cryopreservation than more developed follicles [15].

The comparative study by Bedaiway *et al*, using the sheep model to examine the outcome after cryopreservation method of controlled-rate slow freezing with 1.5M DMSO of cortical strips and whole ovary. It was found that the follicular viability was around 80% in both groups, with evaluation performed by Trypan blue test [16]. In another study, by using human ovarian tissue, primordial follicle count were compared between the study groups as well as apoptosis that was also similar with fresh ovarian control tissue [17]. Evaluation after thawing of the whole human ovary showed adequate follicular survival and absence of apoptosis after cryopreservation. Compare to the other results, some researches show that the ovarian stroma is more sensitive than the follicles to cryoinjury during ovarian cortex cryopreservation, histological evaluation of stroma after whole human ovary cryopreservation did not reveal any abnormalities [14, 16].

After heterotopic autotransplantation, the ovarian tissues survive well in both fresh and cryopreserved transplantation groups and this revealed comparable proportion of follicles in each developmental stage in 1, 5, 8 or 10 month duration after grafting. This study explained that heterotopic autologus ovarian transplantation in rat model is surviving after cryopreservation process and maintain the function for more than 10 months as well. Moreover choosing of ovarian tissues with abundant primordial follicles and developmentally dormant is suggested a well suited for tissue storage in nitrogen gas [14].

The vitrification method has shown the higher percentage of morphologically normal follicles, the better ultrastructural preservation of primordial follicles compared with conventional vitrification and slow freezing methods [16]. A similar study by Deng *et al* evaluated the consequence of vitrification on primordial follicles in ovarian grafts before transplantation. They found that in cryopreserved ovarian tissues before transplantation, the proportion of follicles at each stage of follicunogenesis was not significantly different from fresh ovarian tissues. Also they did not find any significant difference in the mean number of recovered grafts between the fresh and cryopreserved transplantation groups at 5, 8 or 10 months duration after transplantation. The surviving grafts contained follicles at all stages of folliculogenesis and corpora lutea. The proportion of each stage follicles in both types of grafts was comparable with that in control group. All that study results indicated that the follicles in heterotopic grafts could develop further [12].

In our study, we observed the survival status and morphological alteration in cryopreserved ovarian tissues after transplantation at different duration of 2 weeks and 4 weeks respectively. With the naked eye, morphologically no specific alteration observed except the size reduction with color changing. While the microscopic observation found normal follicular architecture (normal cortex and medulla) with presence of intact primordial follicle in cortex as well as presence of different follicular development stages in some recovered ovarian tissues. These results may indicate the facts that the cryopreservation do not have any obvious detrimental effect on transplanted ovarian tissues or may be least damaging effects after using cryoprotectants such as EG and DMSO for vitrification-warming.

In addition, Deng *et al* observed that the percentage of morphologically normal tertiary follicles in cryopreserved ovarian tissues was lower than in fresh tissues before grafting [12]. Similarly, in our study we observed the loss of the cortical and medullary configuration with small number of visible disordered primordial follicles in some transplanted ovarian tissues. The reason of cortical and medullary configuration deterioration may be due to the certain factors such as unequal size of ovarian tissues, certain environmental factors or may not have developed

the blood anastomosis after transplantation, while the cause of presence of less disordered primordial follicles in graft may be due to the fact that at time of grafting there may be presence of few larger size of follicles which were easily destroyed by adequate dehydration at transplantation site.

Salehnia *et al* revealed that ovaries of mice seemed to be sufficiently small to reduce the devitrification effects [18]. On the other hand, Sugimoto *et al* observed that rat ovarian follicles survived after vitrification and transplantation with a decreased number of small and large follicles [19]. The result is in agreement with the present experiment in which after transplantation the number of intact follicles had decreased markedly in some of the ovarian grafts.

Conclusion

Our study demonstrated that the ovarian tissues survive well in cryopreserved transplantation group and this revealed comparable proportion of follicles in each developmental stage at different durations (2 and 4 weeks) after grafting into back muscles. This explained out that heterotopic autologus ovarian transplantations into back muscles in rat model can survive after cryopreservation process and maintain their morphology and function as well. Moreover, the cryopreservation do not have any obvious detrimental effect on transplanted ovarian tissues after using cryoprotectants such as EG and DMSO for vitrification-warming while the unequal size of ovarian tissues, certain environmental factors and development of blood anastomosis are considered the main factors for maintaining the survival and morphology of transplanted ovarian tissues into back muscles in rat model. Further research for alternatives such as whole ovary or isolated follicles transplantation in humans is required to observe their endocrine functions and survivability maintenance for long-term duration at large scale.

References

1. Grazul-Bilska AT, Banerjee J, Yazici I, *et al*. Morphology and function of cryopreserved whole ovine ovaries after heterotopic autotrasnplantation. Reprod Biol Endocrinol, 2008, 6: 16.
2. Bedaiwy MA, Falcone T. Harvesting and autotransplatation of vascularized ovarian grafts: approaches and techniques. Reprod Biomed Online, 2007,14: 360–371.
3. Boldt J, Cline D, McLaughlin D, *et al*. Human oocyte cryppreservation as an adjunct to IVF-embryo transfer cycles. Hum Reprod, 2003, 18: 1250–1255.
4. Jafarey R, Yang J. Morphological ultrastructural changes in rat ovarian tissues after cryopreservation. Chinese-German J Clin Oncol, 2012, 11: 527–532.
5. Falcone T, Attaran M, Bedaiwy MA, *et al*. Ovarain function preservation in the cancer patient. Fertil Steril, 2004, 81: 243–257.
6. Gosden RG, Baird DT, Wade JC, *et al*. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196 degrees C. Hum Reprod, 1994, 9: 597–603.
7. Jeremias E, Bedaiwy MA, Gurunluoglu R, *et al*. Hetertopic autotransplantation of the ovary with microvascular anastomosis: a novel surgical technique. Fertil Steril, 2002, 77: 1278–1282.
8. Goding JR, McCracken JA, Baird DT. The study of ovarian function in the ewe by means of a vascular autotrasnplantation technique. J Endocrinol, 1967, 39: 37–52.
9. Arav A, Revel A, Nathan Y, *et al*. Oocyte recovery, embryo development and ovarian function after cryopreservation and transplantation of whole sheep ovary. Hum Reprod, 2005, 20: 3554–3559.
10. Cancer and pregnancy. Methods for fertility preservation [EB/OL]. Accessed 24th May 2010. http://www.cancerinpregnancy.org/node/61
11. Green SH, Smith AU, Zuckerman S. The number of oocytes in ovarian autografts after freezing and thawing. J Endocrinol, 1956, 13: 330–334.
12. Donnez J, Dolmans MM, Demylle D, *et al*. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet, 2004, 364: 1405–1410.
13. Deng X, Zheng H, Yu X, *et al*. Cryopreserved ovarian tissues can maintain a long-term function after heterotopic autotrasnplantation in rat. Reproduction, 2009, 138: 519–525.
14. Milenkovic M. Experimental studies on ovarian cryopreservation and transplantation. Geson Hylte Goteborg, Sweden. 2011, 1–118.
15. Damous LL, Silva SM, Lopes RA, *et al*. Study on the vaginal smear of rats submitted to autologus ovarian transplant: Impact of remlote ischemic preconditioning. Acta Cir Bra, 2009, 24: 387–392.
16. Shaw JM, Oranratnachai A, Trounson AO. Fundamental cryobiology of mammalian oocytes and ovarian tissue. Theriogenology, 2000, 53: 59–72.
17. Yang YH, Cox SL, Jenkin G, *et al*. Graft site and gonadotrophin stimulation influences the number and quality of oocytes from murine ovarian tissue grafts. Reproduction, 2006, 131: 851–859.
18. Bedaiwy MA, Jeremias E, Gurunluoglu R, *et al.* Restoration of ovarian function after autotransplantation of intact frozen-thawed sheep ovaries with microvascular anastomosis. Fertil Steril, 2003, 79: 594–602.
19. Kim SS. Fertility preservation in female cancer patients: current developments and future directions. Fertil Steril, 2006, 85: 1–11.