Int J Clin Exp Med 2017;10(9):13482-13490
www.ijcem.com /ISSN:1940-5901/IJCEM0055332

Original Article

Human ovarian tissue xenografted in aseptically wounded mice to conserve follicles

Canqiao Luo[1*], Lingli Long[4*], Xuehua Li[2], Qingyun Mai[5], Bing Cai[5], Shuhui Zheng[4], Han Qiu[6], Yuxia Xu[4], Lei Chen[3], Yubin Li[5]

Departments of [1]Pathology, [2]Radiology, [3]Burns, [4]Translation Medicine Center, [5]The Reproductive Center, The First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan 2nd Road, Guangzhou 510080, China; [6]Faculty of Forensic Medicine, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China. [*]Equal contributors.

Received February 22, 2017; Accepted August 6, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: The aim of this research is to study the response of human ovarian follicles that were xenografted into different SCID mice. Human ovarian cortical pieces were transplanted to the subcutaneous space of intact, or castrated, or aseptically wounded male or female NOD-SCID mice. Grafts were collected after 1, 4, 8, and 10 weeks. The proportions of growing follicles and the reconstruction conditions of human ovarian tissue after transplantation were determined with the use of HE (hematoxylin and eosin) staining. The expression levels of Ki-67 in human ovarian grafts were determined by immunohistochemistry. One week after subcutaneous ovarian tissue transplantation, there was a significantly higher proportion of growing follicles in the aseptically wounded male mice group than any other group. Ten weeks later, the proportions of growing follicles in ovarian grafts were increased significantly ($P<0.05$). In the ovarian grafts of the wounded male mice group, it is shown that all stages of healthy follicles could be found, the boundaries of the follicles had angiogenesis, and Ki-67 mainly expressed in granulose cells of all stages of follicles. Therefore, the xenotransplant of human ovarian tissue into subcutaneous tissue of aseptically wounded male mice is a promising technology to conserve human follicles.

Keywords: Ovary, tissues, ovarian follicle, transplantation, heterologous, ovarian reserve

Introduction

As to the women with premature ovarian failure, xenotransplantation of cryopreserved ovarian tissue fragments can be considered as a method for them to preserve fertility. At 1906 a study reported the first successful case of ovary transplantation [1], but research on it still proceeded slowly. Recently, with the development of assisted reproduction means including cryopreservation technology, tumor diagnosis and treatment technology, which involves the reserve of human follicles, the study of ovary transplantation has been rekindled [2].

Ischemia reperfusion induces the loss of a large number of follicles in the early stage of transplantation [3]. In addition, angiogenesis restores vast perfusion around transplanted ovarian tissue. Non-physiological reconstructed blood circulation may also lead to excessive depletion of follicles [4]. Therefore, the greatest challenge in the development of ovary transplantation is to successfully reduce the damage of follicles.

Scholars have conducted massive xenotransplantation research by using severe combined immunodeficiency (SCID) mice to improve transplantation effect of human ovarian tissue.

This study aims to explore and improve the technology of human ovarian tissue xenotransplantation, and to explore the effects of the host itself and the local condition of host on xenotransplantation of ovarian tissue after thawing, which further establishes the technical foundation of ovarian tissue bank.

Materials and methods

Animals

Male or female intact NOD SCID mice (The reproductive Center, The First Affiliated Hospital

Table 1. Survival rates of the primordial follicles after 1 week of graft in 6 groups

Groups	Normal	Atresic	Total	Survival rate
Intact male group[1)]	26	31	57	45.6%
Castrated male group	32	40	72	44.4%
Aseptically wounded male group	31	17	48	64.6%
Intact female group	25	38	63	39.7%
Castrated female group	34	39	73	46.6%
Aseptically wounded female group	26	15	41	63.4%

1): Morphologically normal rates of the primordial follicles at the first week after transplantation among six groups were significantly different (χ^2=11.289, *P*=0.046).

of Sun Yat-sen University, Guangzhou, China) of 5~6 weeks old were hosts of human ovarian xenotransplantion. These animals were housed in an isolated positive-pressure room. Cages were filter topped, and animals had free access to gamma-irradiated food pellets and sterile water. The room used a 12:12 hour light-dark cycle.

Source and cryopreservation of human ovarian tissue

After approval from the ethics committee of The First Affiliated Hospital of Sun Yat-sen University and informed consent before surgical operation cutting benign tumor, nine patients (from 22 to 29 years old) donated ovarian tissue. The ovarian cortical tissue (the thickness approximately 1 mm) was frozen according to the protocol adapted from Newton et al. [5]. Briefly, slices of ovarian cortex were equilibrated for 30 minutes in cryoprotective medium containing 1.5 M DMSO. Cryovials containing ovarian pieces were placed in an automated freezer previously set to 0°C and cooled at -2°C per minute to -7°C. After seeding, cooling continued at -0.3°C per minute to -40°C, and then the cooling rate was increased to -10°C per minute down to -140°C. Finally, vials were plunged in liquid nitrogen and stored at -196°C until use. frozen ovarian tissue in cryovial was put into 25°C water to thaw. Thawed pieces of tissue were washed twice in α-MEM (Gibco, BRL) medium supplemented with 5% human serum albumin (HSA, SAGE IVF, USA). They were then cut into smaller pieces, which are approximately 3 mm with 1 mm of thickness. Our work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments.

Experimental design

The influences of the host mice sex and the special condition on follicular development of human ovarian grafts were evaluated. Twenty-four male mice were randomly assigned into one of the following treatments groups: (1) intact male host (n=8), (2) castrated male host (n=8), and (3) aseptically wounded male mice (n=8). Also twenty-four female mice were randomly assigned into one of the following treatments groups: (1) intact female host (n=8), (2) castrated female host (n=8), and (3) aseptically wounded female mice (n=8). Each mouse received eight ovarian pieces. Grafts were respectively recovered at the first week, fourth week, eighth week and tenth week after xenotransplantation, after euthanasia by CO_2 toxicity. Two mice were killed at certain week after xenotransplantation among each group.

Surgery

Surgical procedures were carried out under a laminar flow hood under aseptic conditions. Mice were anesthetized using chloral hydrate by intraperitoneal injection. Dorsal areas of mice were shaved, and the skin was aseptically cleaned immediately before surgery. A small dorsal skin incision allowed access to the subcutaneous space. Ovarian cortical tissue slices were placed under the skin on bilateral shoulder and lumbar subcutaneous area of each mouse (two slices on the right shoulder, two slices on the left side; two slices on the right lumbar subcutaneous area, two slices on the left side). Castration was performed 4 days before transplantation of ovarian tissue. The aseptically wounded (Φ=1.5 cm) was performed 2 days before transplantation in the subcutaneous space using a syringe. The mice had intramuscular injection of 100 iu gentamicin once a day for three days after all surgical procedures.

Histological and immunohistochemical examination of ovarian tissue

Recovered grafts were inspected and fixed in 10% formalin solution for 12-24 hours before

Table 2. Follicle growth rate at different week after transplantation in 6 groups (Growing follicle/Total survival follicle; n1/n2, %)

Groups	First Week	4th Week	8th Week	10th Week	χ^2	*P*
Intact male group	15/41 (36.6)	14/34 (41.2)	17/33 (51.2)	23/28 (82.1)	15.586	0.001
Castrated male group	19/51 (37.3)	18/44 (43.2)	21/41 (53.7)	23/29 (79.3)	14.708	0.002
Aseptically wounded male group	14/39 (35.9)	14/36 (38.9)	12/25 (48.0)	26/33 (78.8)	15.853	0.001
Intact female group	18/52 (34.6)	24/58 (41.4)	24/45 (53.3)	22/27 (81.5)	17.372	0.001
Castrated female group	19/50 (38.0)	17/40 (42.5)	27/38 (71.1)	36/45 (80.0)	23.597	<0.001
Aseptically wounded female group	17/43 (39.5)	19/46 (41.3)	23/36 (63.9)	16/21 (76.2)	11.714	0.008
χ^2	0.292	0.108	5.791	0.339		
P	0.998	0.999	0.327	0.997		

being stored in gradient ethanol solution (respectively 0% ethanol watering, 80% ethanol fixed 3-6 hours, 90% ethanol, 95% ethanol, anhydrous ethanol I, anhydrous alcohol II) and in xylene I, xylene II until they were embedded in paraffin (1-3 days later), in which Xylene enables organizations to xylene transparent. Then immerse it into 60-62°C paraffin liquid for 60 minutes, embedding tissue. Serial sections (4 μm) were made from each embedded ovarian graft.

Every tenth section was stained with hematoxylin and eosin (HE) for follicle morphological analysis, whereas random sections were processed for detection of proliferating cell nuclear antigen Ki-67 by immunohistochemistry. Endogenous peroxidase activity was inhibited by incubating the tissues with 3% hydrogen peroxide for 10 minutes. To block nonspecific binding, sections were incubated with nonimmune goat serum (Sigma, St. Louis, MO) for 10 minutes at 25°C. Sections were sequentially incubated as follows: primary antibody (anti-Ki-67 monoclonal antibody, ready-to-use DACO N-SERIES; DAKO Corporation, Carpinteria, CA), biotin-labeled secondary antibody (mouse anti-human), streptavidin conjugated to horseradish peroxidase, and 3, 3-diaminobenzidine (DAB) chromogen solution. Finally, specimens were counterstained with hematoxylin (Gill II formula).

Follicular assessment

Follicles were counted and classified as either resting (primordial) or growing (primary, secondary, and antral) follicles. Primordial follicles had one layer of flat cells, or a combination of flat and cuboidal pregranulosa cells. Among the growing follicles, primary follicles had one layer of cuboidal granulosa cells, secondary follicles had two or more layers of cuboidal cells, and antral follicles presented a fluid filled antral cavity. To avoid counting follicles more than once, growing follicles were only counted in the section where the nucleolus was clearly visible. Resting follicles were counted even in absence of a nucleolus when a distinguishable nuclear membrane was present.

Statistical study

We analyzed our data using the SPSS11.0 statistics software. We observed the growth of follicles using HE staining and compared growth rate of follicles (Growing follicle rate, GFR)=(primary follicles and secondary follicles)/(primordial follicles and primary follicles and secondary follicles)×100% (Note: owing to less antral follicle count, we ignored it.) between groups using χ^2 test analysis, with $P<0.05$ considered statistically significant.

Results

In our study, 384 ovarian tissues were transplanted heterologously into 64 SCID mice. We finally obtained 352 ovarian grafts (91.7%) from 48 mice in the best condition, and lost 32 ovarian grafts (9.3%).

Survival rates of primordial follicles in different groups

Table 1 shows the survival rates of primordial follicles after 1 week of the transplantation into each group. Our data indicated that the aseptically wounded male group had the highest survival rate (64.6%), while the intact female group had the lowest survival rate (39.7%). There was no significant difference between the intact male group and the intact female group, the

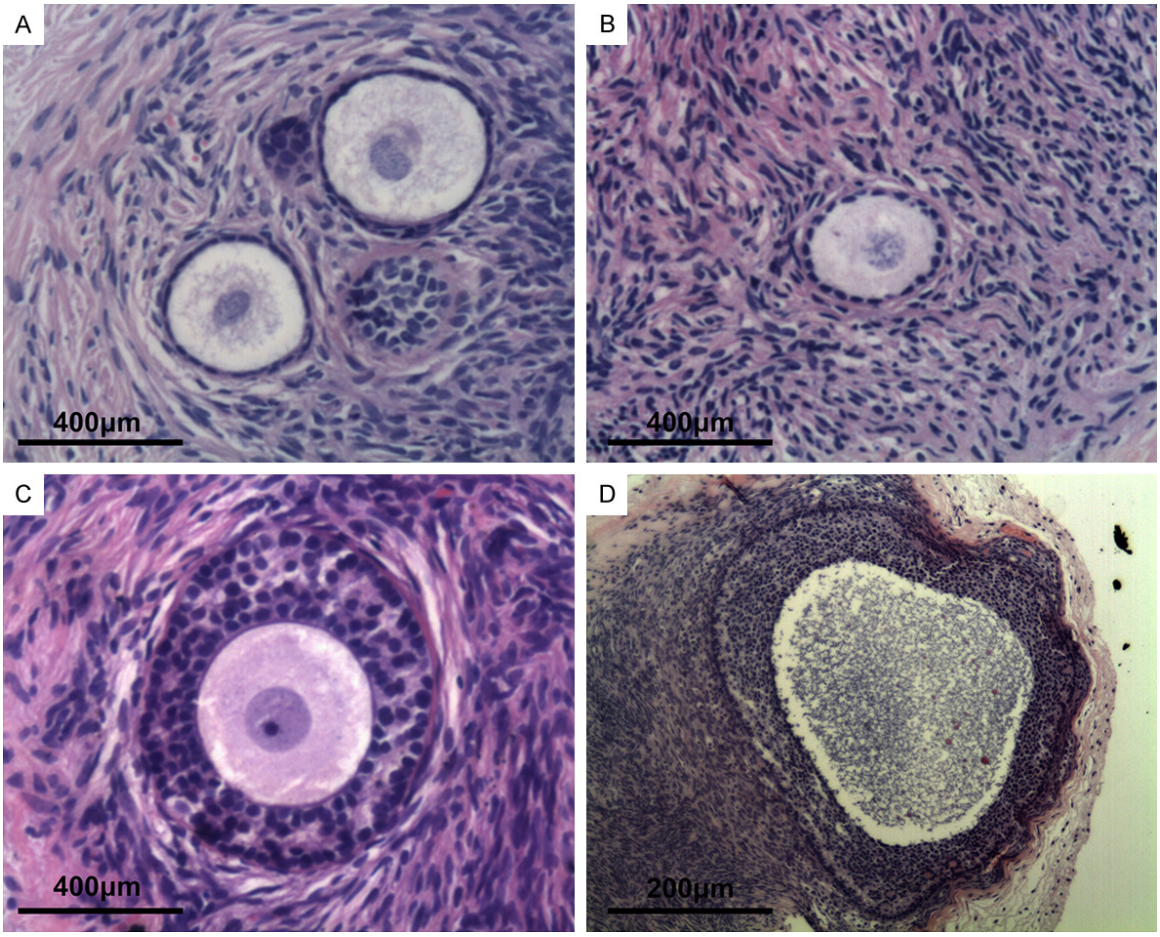

Figure 1. Four stages of healthy follicles in granulation tissue of aseptically wounded male mice 10 week after transplantation. A-C: Primordial follicle, primary follicle and secondary follicle (HE, ×400); D: Antral follicle (HE, ×200).

castrated male group and the castrated female group, and between the aseptically wounded male group and the aseptically wounded female group (χ^2=0.431, 0.066 and 0.013, *P*=0.512, 0.797 and 0.909 respectively). And the differences of primordial follicles between the intact male group and the castrated male group, the intact female group and the castrated female group were also not statistically significant (χ^2=0.018 and 0.654, *P*=0.894 and 0.419). These results suggested that neither the gender of host or whether being castrated or not had significant effect on survival rates of primordial follicles at 1 week of transplantation. Moreover, survival rates of primordial follicles between the three male mice groups had no statistical significance (χ^2=5.405, P=0.067), but the survival rate of the aseptically wounded male group was the highest (64.6%). Similarly, survival rates between the three female mice groups also had no statistical significance (χ^2=5.708, P=0.058), but the survival rate of the aseptically wounded female group was also the highest (63.4%). Therefore, we consider that the aseptically wounded group may have the best condition for the survival of primordial follicles.

Growth rates of follicles in different groups

Our research continued to discuss the follicle growth rate among these six groups from week 1 to week 10 (**Table 2**). Our results demonstrated that the follicle growth rate in all groups increased fast from week 1 to week 10 (P<0.05 between different weeks), and it reached 80% approximately in all groups at week 10. There was no significant difference between any pair of groups from week 1 to week 10.

Development of healthy follicles after xenotransplantation

Figure 1 shows the pictures of healthy primordial follicles, primary follicles, secondary folli-

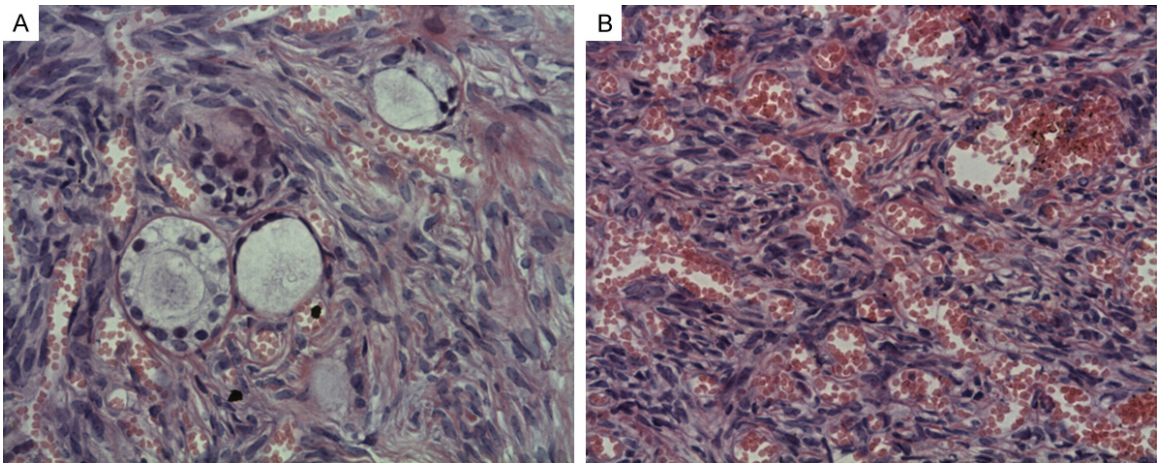

Figure 2. Wound-induced granulation tissue on ovarian grafts (7 days after transplantation). A: Wound-induced blood vessel invasion adjacent to follicles of 7 day ovarian graft. B: Angiogenic granulation tissue in the ovarian graft.

cles and antral follicles in the ovarian grafts 10 weeks after transplantation of ovarian tissue pieces into the wounded sites of SCID mice. We found that all types of healthy follicles could be obtained from aseptically wounded group 10 weeks after transplantation.

Effects of xenotransplantation into granulation tissue on revascularization of ovarian grafts

The ovarial tissues were transplanted heterologously into wounded sites of SCID male mice. Obvious wound-induced granulation tissue and neovascularization were detected inside the grafts 7 days after transplantation (**Figure 2**). Blood vessel invasion to the boundaries of the follicles was found (**Figure 2A**).

The expression of Ki-67 gene in ovarian graft

The expression level of Ki-67 represents the level of cell proliferation. The result indicated 10 weeks after transplantation, in the ovarian graft, Ki-67 mainly expressed in granulose cells of all stages of follicles (oocytes, primodial follicles, secondary follicles and antral follicles, **Figure 3A-D**). This result suggested that frozen-thawed ovarian tissues still have the ability to proliferate after being transplanted into wounded sites of SCID male mice.

Discussion

Many studies have succeeded in using frozen-thawed human ovarian tissue to solve reproduction-related problems. So far, more than 40 cases of live birth through autotransplantation of human ovarian tissue have been reported [6]. The objective of our study is to find out the best way to achieve the xenotransplant of ovarian tissues, and to observe different stages of follicles, graft revascularization and cell proliferation process.

Human ovarian tissue xenografted subcutaneously into SCID can be influenced by the host environment. Previous studies have shown that the gender of mice can influence the final ovarian graft. Weissman's [7] study showed that compared to the female mice hosts, more antral follicles could be gotten if tissue was transplantated in male mice hosts, which may be caused by different level of androgen, blood supply and energy supply in different hosts. Strauss [8] also found that DHEA could augment production of progesterone in a woman with low ovarian reserve being transplanted with cryopreserved ovarian tissue. However, our study showed that the gender of the host could not affect the survival rate of primordial follicles and the growth rate of follicles in ovarian grafts. This may caused by different xenotransplantation time. Our study only focus on the early period after xenotransplantation, but Weismann's study was a long-term observation. Therefore, we think that for a short-term xenotransplantation, the gender of the host is not an influence factor.

Castration is to get rid of the negative feedback of the gonad function in the mice, elevate

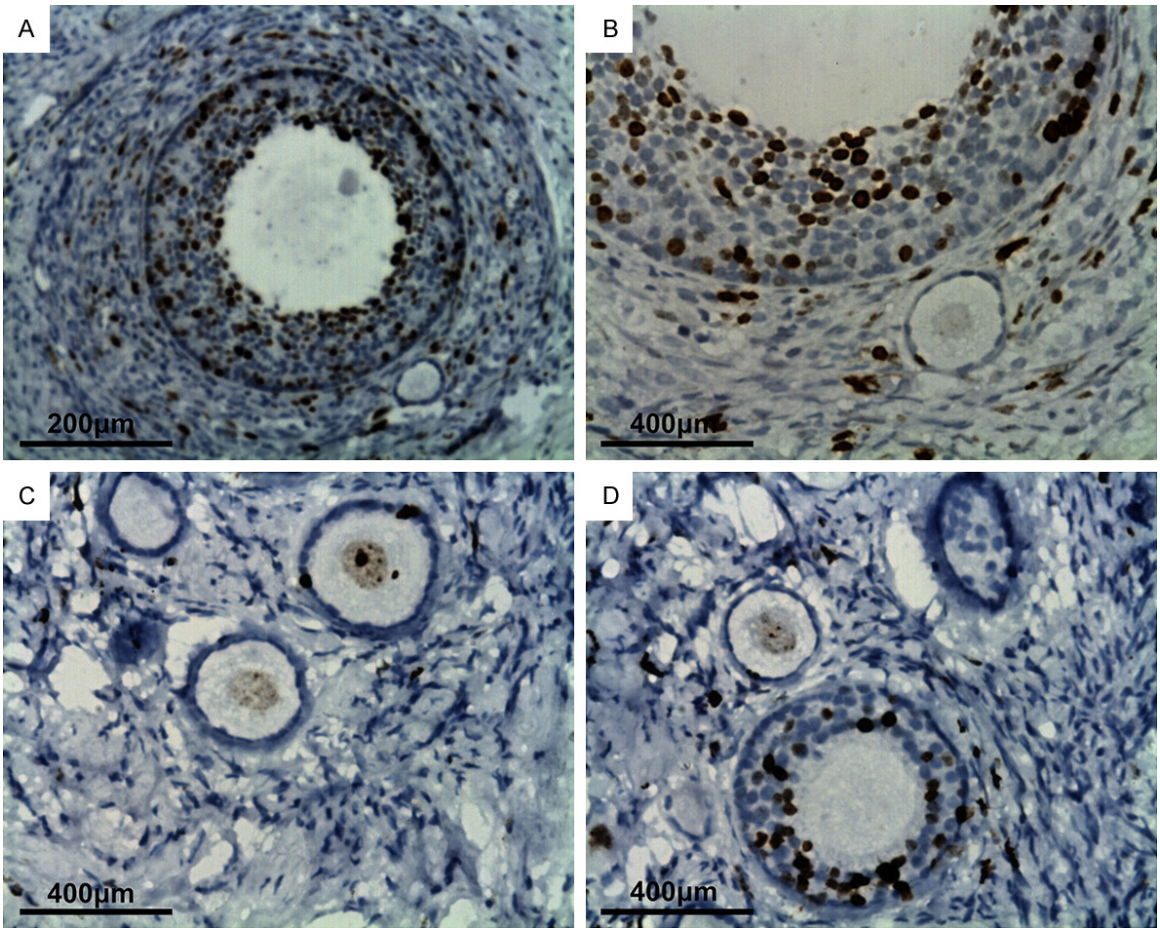

Figure 3. Expression level of Ki-67 in follicles of ovarian graft 10 weeks after transplantation (A: ×200 B-D: ×400). (A) Granulosa cells and theca cells in antral follicle granulosa cells show positive expression of Ki-67; (B) Granulosa cells and stroma tissue in a primordial follicle show positive expression of Ki-67. (C) Weakly positive expression of Ki-67 in the oocytes; (D) Granulosa cells in secondary follicles show positive express Ki-67.

gonadotropin, and promote the growth of follicles. Some researchers showed that castration can promote the development of follicles in tissue after transplantation [9]. However, other research showed the opposite conclusion [10]. Our study also demonstrates that castration failed to affect the survival rate of primordial follicles at 1 weeks after transplantation, as well as the growth rate of follicles at 1, 4, 8 and 10 weeks after transplantation. The physiological level of FSH helps maintaining follicular growth after transplantation to the stage of antral follicle [11]; in addition, after the blood circulation is re-established, the secretion of estrogen and progesterone shows a negative feedback effect [10], which counteracts the efficiency of castration. To confirm whether castration is necessary for transplantation of human ovarian tissues requires further study. Malaria's [4] research showed that the long-time stimulation of FSH may do harm to anatomical and normal follicles, resulting in the rapid depletion of ovarian reserve after transplantation. According to the results from previous and our study, we suspect the role of castration in accelerating follicular depletion. This may be discussed in the future.

Recently, more and more research have paid attention to improving transplantation efficiency of human ovarian tissue. Andersen [12] reported that after ovarian tissue was transplanted, the function of the ovary could be maintained for 7 years. But ischemia reperfusion injury of ovarian grafts is still the most difficult problem for ovary transplantation, rather than damage brought by the frozen-thawed process. Generally the primordial follicles lose

50%~60% at the early stage of transplantation [13]. Reperfusion injury usually occurs after vascular remodeling, but there is no effective measure to prevent it. Therefore how to build up an ovarian tissue microcirculation, and reduce the time of ovarian tissue ischemia are greatest challenges for ovary transplantation. Kim [14] used vitamin C to extend hypoxia tolerance of tissue in vitro. Zhang et al. showed that L-carnitine inhibited follicle apoptosis, and increased follicular survival and function of ovarian graft [15]. Some studies proved that in granulation tissue, vascular related factors (VEGF, EGF *etc*.) could promote angiogenesis. Israely [16] reported that granulation tissue around ovarian grafts could reduce mortality of follicle. As to the first successful live birth after autotransplantation of cryopreserved ovarian tissue reported by Donnez, two laparoscopic operations were performed before transplantation, making local environment similar to the granulation tissue in the transplantation area [2]. In our study we administrated the local aseptic trauma with needle two days before transplantation, and tried to make the aseptic inflammation similar to the granulation tissue to reduce death of follicle. The results showed that the survival rate of primordial follicles in the two aseptically wounded groups was significantly higher than that of other groups during the most important angiogenesis period (at first week); and the growth rate of follicles increased at eighth weeks after transplantation in the two aseptically wounded groups. Of course, due to the fact that SCID mice lack specific immune function, their ability to repair the wound may be different from normal healthy animals. Therefore, the administration of aseptic wound has an advantage of simple operation, and it is a good model for ovary transplantation.

In order to find out an effective way of transplantation, scientists have performed a series of research. Some thought that due to the extremely rich blood supply, transplantation effect in renal sac should be better than that in subcutaneous [17]. However, others believed that subcutaneous tissue also has rich blood supply and larger space, and it should be much more suitable for human follicular growth [10]. In 2014, Stern [18] reported the first case of delivery of twins following heterotopic graft in a patient who had previously undergone bilateral oopherectomy for a granulosa cell tumor. Frozen-thawed ovarian tissue was transplanted to the anterior abdominal wall in this case. Our study showed that antral follicles could be obtained subcutaneous xenotransplantation of human ovarian tissue successfully. This suggested that subcutaneous transplantation is one of effective methods of ovarian tissue transplantation, and can provide enough blood supply for maintaining complete growth of follicles.

Due to the change of environment, growth of ovarian grafts shows obvious difference from the growth of ovarian tissue in situ. Under normal physiological condition, there is no direct vascular supply around primordial follicles. The metabolic level of primordial follicles is low. Nutrient supply and material exchange depend mainly on the tissue fluid through distant osmosis [19]. One week after transplantation, the largest physiological change of tissue was that lots of blood vessels freely spread throughout the stroma and a number of primordial follicles began to growth. In this study, about 80% of the follicles had entered the growing stage by 10 weeks after transplantation, which was similar to previous studies. The change led to the rapid depletion of follicles. There were also some accelerating depletion phenomena in the autotransplantation process of human ovary. Therefore, we believe that angiogenesis can induce depletion of the follicles.

This study shows that after subcutaneous transplantation, all types of growth follicles can express Ki-67 and initiate proliferation process. Ki-67 antigen is a kind of cell nuclear antigen related to cell proliferation, coupling with DNA replication phase, and expresses in mitotic G1 and G2 and M and S, especially expresses highest in G2 and M, but not expresses in G0 [20]. Therefore, Ki-67 is often used to detect proliferation of granulosa cell. Recent studies inferred that follicles in the early stage lack the signals and materials that apoptosis procedure activation requires, and the atresia is actually a form of necrosis [21]. There were widely expressed Ki-67 in tissue after transplantation, and its expression was closely associated with the follicular growth and stroma reconstruction [22]. Many reported studies have demonstrated that xenografted ovarian tissue from human can produce antral follicles that contain mature (MII) oocytes [23]. In our study, the constant expression of Ki-67 reflected reconstruction activity of the tissue.

In summary, we established the method of human ovarian tissue subcutaneous xenotransplantation, and explored different status that affects the xenotransplantation. Fertility preservation in women with cancer has become an important clinical issue [24]. Through our method, antral follicles and growth follicles can be effectively gained. It is a quite useful technology for future reproductive research. We will continue to explore how to reduce follicle depletion and create more suitable environment for transplanted ovaries.

Acknowledgements

We thank Weiping Yu (University of Texas at Austin, America) for her comments on the manuscript. The work was supported by The National Natural Science Foundation of China (grant no. 81100470), The Natural Science Foundation of Guangdong Province, China (grant no. 2014A030313182), The Natural Science Foundation of Guangdong Province, China (grant no. 2014A030313105) and National Innovation Funding, China (grant no. 54101-31910004), Fundamental Research Funds for the Central Universities, Sun Yat-sen University (15ykjc10c) to L.C.

Disclosure of conflict of interest

None.

Address correspondence to: Yubin Li, The Reproductive Center of The First Affiliated Hospital, Sun Yat-sen University, 58# Zhongshan 2nd Road, Guangzhou 510080, China. Tel: +86- 18929553388; Fax: +86-021-64085875; E-mail: liyubin97200@163.com; Lei Chen, Department of Burns, The First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan 2nd Road, Guangzhou 510080, China. Tel: +86-13751766829; Fax: +86-021-64085875; E-mail: chenleigzburns@163.com

References

[1] Carrel A and Guthrie CC. Technique de la transplantation homeplastique de l'ovarie. C R Seances Soc Biol 1906; 6: 466-468.

[2] Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B and van Langendonckt A. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 2004; 364: 1405-1410.

[3] Labied S, Delforge Y, Munaut C, Blacher S, Colige A, Delcombel R, Henry L, Fransolet M, Jouan C, Perrier d'Hauterive S, Noel A, Nisolle M and Foidart JM. Isoform 111 of vascular endothelial growth factor (VEGF111) improves angiogenesis of ovarian tissue xenotransplantation. Transplantation 2013; 95: 426-433.

[4] Maltaris T, Beckmann MW, Mueller A, Hoffmann I, Kohl J and Dittrich R. Significant loss of primordial follicles after prolonged gonadotropin stimulation in xenografts of cryopreserved human ovarian tissue in severe combined immunodeficient mice. Fertil Steril 2007; 87: 195-197.

[5] Li YB, Zhou CQ, Yang GF, Wang Q and Dong Y. Modified vitrification method for cryopreservation of human ovarian tissues. Chin Med J (Engl) 2007; 120: 110-114.

[6] Donnez J and Dolmans MM. Transplantation of ovarian tissue. Best Pract Res Clin Obstet Gynaecol 2014; 28: 1188-1197.

[7] Weissman A, Gotlieb L, Colgan T, Jurisicova A, Greenblatt EM and Casper RF. Preliminary experience with subcutaneous human ovarian cortex transplantation in the NOD-SCID mouse. Biol Reprod 1999; 60: 1462-1467.

[8] Strauss S, Greve T, Ernst E, Fraidakis M, Grudzinskas JG and Andersen CY. Administration of DHEA augments progesterone production in a woman with low ovarian reserve being transplanted with cryopreserved ovarian tissue. J Assist Reprod Genet 2014; 31: 645-649.

[9] Maltaris T, Koelbl H, Fischl F, Seufert R, Schmidt M, Kohl J, Beckmann MW, Binder H, Hoffmann I, Mueller A and Dittrich R. Xenotransplantation of human ovarian tissue pieces in gonadotropin-stimulated SCID mice: the effect of ovariectomy. Anticancer Res 2006; 26: 4171-4176.

[10] Hernandez-Fonseca H, Bosch P, Sirisathien S, Wininger JD, Massey JB and Brackett BG. Effect of site of transplantation on follicular development of human ovarian tissue transplanted into intact or castrated immunodeficient mice. Fertil Steril 2004; 81 Suppl 1: 888-892.

[11] Lee DM, Yeoman RR, Battaglia DE, Stouffer RL, Zelinski-Wooten MB, Fanton JW and Wolf DP. Live birth after ovarian tissue transplant. Nature 2004; 428: 137-138.

[12] Andersen CY, Silber SJ, Bergholdt SH, Jorgensen JS and Ernst E. Long-term duration of function of ovarian tissue transplants: case reports. Reprod Biomed Online 2012; 25: 128-132.

[13] Donnez J, Martinez-Madrid B, Jadoul P, Van Langendonckt A, Demylle D and Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. Hum Reprod Update 2006; 12: 519-535.

[14] Kim SS, Yang HW, Kang HG, Lee HH, Lee HC, Ko DS and Gosden RG. Quantitative assessment of ischemic tissue damage in ovarian cortical tissue with or without antioxidant

(ascorbic acid) treatment. Fertil Steril 2004; 82: 679-685.
[15] Zhang Q, Wang SM, Yao PB, Zhang L, Zhang YJ, Chen RX, Fu Y and Zhang JM. Effects of L-carnitine on follicular survival and graft function following autotransplantation of cryopreserved-thawed ovarian tissues. Cryobiology 2015; 71: 135-140.
[16] Israely T, Nevo N, Harmelin A, Neeman M and Tsafriri A. Reducing ischaemic damage in rodent ovarian xenografts transplanted into granulation tissue. Hum Reprod 2006; 21: 1368-1379.
[17] Abir R, Orvieto R, Raanani H, Feldberg D, Nitke S and Fisch B. Parameters affecting successful transplantation of frozen-thawed human fetal ovaries into immunodeficient mice. Fertil Steril 2003; 80: 421-428.
[18] Stern CJ, Gook D, Hale LG, Agresta F, Oldham J, Rozen G and Jobling T. Delivery of twins following heterotopic grafting of frozen-thawed ovarian tissue. Hum Reprod 2014; 29: 1828.
[19] Baird DT, Campbell B, de Souza C and Telfer E. Long-term ovarian function in sheep after ovariectomy and autotransplantation of cryopreserved cortical strips. Eur J Obstet Gynecol Reprod Biol 2004; 113 Suppl 1: S55-59.
[20] Gerdes J, Schwab U, Lemke H and Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983; 31: 13-20.
[21] Jin S, Lei L, Shea LD, Zelinski MB, Stouffer RL and Woodruff TK. Markers of growth and development in primate primordial follicles are preserved after slow cryopreservation. Fertil Steril 2010; 93: 2627-2632.
[22] Goldman S and Shalev E. MMPS and TIMPS in ovarian physiology and pathophysiology. Front Biosci 2004; 9: 2474-2483.
[23] Dittrich R, Lotz L, Fehm T, Krussel J, von Wolff M, Toth B, van der Ven H, Schuring AN, Wurfel W, Hoffmann I and Beckmann MW. Xenotransplantation of cryopreserved human ovarian tissue--a systematic review of MII oocyte maturation and discussion of it as a realistic option for restoring fertility after cancer treatment. Fertil Steril 2015; 103: 1557-1565.
[24] De Vos M, Smitz J and Woodruff TK. Fertility preservation in women with cancer. Lancet 2014; 384: 1302-1310.