RESEARCH ARTICLE

Follicle-stimulating hormone intervention promotes neovascularization of human ovarian transplants during vitrification cryopreservation

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Cryopreservation of human ovarian by vitrification is an effective method to reserve fertility in females suffering from cancer and being treated with chemoradiotherapy. However, after vascular anastomosis transplantation, the lifespan of human ovarian grafts is decreased by ischemia and hypoxia. Our previous study showed that follicle stimulating hormone (FSH) played a key role in anti-apoptosis and promoted the construction of "blood bed" in ovarian grafts with avascular anastomosis transplantation in mice. This research aimed to evaluate whether it could improve anti-freeze injury and promote angiogenesis and avascular anastomosis transplantation by adding 0.3 IU/mL FSH to the solution during human ovarian cortical fragments cryopreserved by vitrification. The results revealed that, compared to no-FSH intervention group (N-FSH), more primordial, primary, and secondary follicles were retained within grafts, and atretic follicles were remarkably decreased in FSH intervention group (FSH) during the process of pre-culturing fresh and vitrification human ovarian cortical fragments. Compared to FSH group of fresh ovarian fragments, the peak time of neonatal endothelial cells was slower for 1-2 days in angiogenesis and revascularization in FSH intervention group of vitrification ovarian fragments (FSH-VG), but it was similar in FSH-VG group and N-FSH group. Furthermore, compared to fresh ovarian fragments, the number of neovascularization endothelial cells was decreased in vitrification ovarian fragments. In all, morphology, angiogenesis, and revascularization of human ovarian grafts were improved by FSH during vitrification and maintained follicles survival after transplantation. Both grafts and recipient vessels participated in blood flow reconstruction in ovarian at the same time, and the peak time of neonatal endothelial cells was improved by FSH intervention.

Keywords: follicle stimulating hormone (FSH); ovarian vitrification; human ovarian grafts; angiogenesis; vascularization.

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Introduction

Increased cancer rate has severely decreased female lifespan. With the advancement of diagnosis and anticancer treatment technologies, the survival rate of patients with malignant tumors has been significantly enhanced. However, the risk of premature ovarian failure

due to anticancer treatment has become the main reason affecting the life quality of women surviving from cancer [1]. Furthermore, the rate of young women developing cancer has increased. As of today, more than 90% of children and adolescent cancer patients can be cured through radiotherapy and chemotherapy [2], but these treatments severely deplete ovarian reserve, which may trigger infertility or premature menopause. Therefore, fertility preservation of cancer patients has become a new medical field to guarantee the life quality of female cancer patients.

The vitrification and transplantation of ovarian tissue have become an effective method of preserving female fertility [3, 4]. Currently, a rapidly growing number of live births from vitrified/thawed human transplantation ovarian suggests this clinical success [5]. Even though a great number of babies has been born by human transplantation ovarian cryopreserved by vitrification, several drawbacks limit the clinical application of this method, which include osmotic stress during vitrification and freezing-thawing processes, follicle loss due to freezing injury, and ischemia/reperfusion during transplantation processes. Especially, ischemia/reperfusion depletes most primordial follicles (PrF) [6]. Furthermore, following orthotopic transplantation of freeze-thawed ovarian tissue in *in vitro* fertilization process, women were found to have high numbers of empty follicles and abnormal and immature oocytes [7]. Therefore, revascularization and vascular remodeling of transplantation ovarian are probably the most critical factor in successful transplantation because it determines follicle survival in transplantation ovarian. It is well known that the follicle stimulating hormone (FSH) plays critical roles in the development and growth of ovarian follicles. FSH deficiency triggers ovarian atresia [8]. In addition, FSH regulates ovarian angiogenesis and expression of vascular endothelial growth factors (VEGFs) [9]. FSH and activin A play key roles in ovarian follicle activation and development through Akt and MAPK pathways *in vitro* [10]. Our previous research revealed that FSH intervention during ovarian cryopreservation process by vitrification could enhance ovarian follicle survival rate and in revascularization by avascular anastomotic transplantation in mice. Previous research also showed that 1-hour FSH intervention before cryopreservation could improve follicle survival and enhance the damage resistance of ovarian cryopreservation. It was found that *in vitro* co-culture with FSH for 2 hours after thawing could contribute to the generation of angiogenic factors and accelerate blood flow reconstruction in transplanted ovarian [11].

Whether FSH intervention may increase human ovarian graft revascularization in human ovarian cortical fragments during vitrification is still unknown. This research aimed to investigate whether FSH could improve anti-freeze injury and promote angiogenesis and avascular anastomosis transplantation by adding 0.3 IU/mL FSH to the solution during human ovarian cortical fragments cryopreservation by vitrification. The results of this study would provide more scientific bases for the technology of human ovarian cortical fragment cryopreservation by vitrification and transplantation and serve future human applications.

Materials and methods

Experimental animals

10-week-old, female, severe combined immune deficient (SCID) mice (Beijing HFK Bio-Technology Co. Ltd., Beijing, China) were adopted as receptors without any rejection reaction. The mice were maintained at 24 ± 2°C with a light control of 12 h light and 12 h darkness. All animals had free access to water and food. The experimental protocol was approved by the Ethic Committee for Animal Care and Experiments (Ningxia Medical University, Yinchuan, Ningxia, China). All surgical procedures on animals were performed under 0.5% sodium pentobarbital anesthesia at the dosage of 0.1 mL/10g body weight.

Human ovaries collection

The experimental procedures were approved by Ethics Committee of Ningxia Medical University (Yinchuan, Ningxia, China). Two ovaries were obtained from a 24-year-old and a 31-year-old woman, which were the remaining of ovarian biopsy for gynecologic non-neoplastic disease, with patients signed informed consent for the specimens to be used for scientific research. The ovarian tissues were brought to the laboratory within 1 hour of the procedures at 37°C, and then cut into small fragments with the volume of 2.5 × 2.5 × 1 mm^3 [7]. Fresh ovarian fragments were cultured in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 12% human serum albumin (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) in a Sanyo MCO175 incubator (Sanyo, Moriguchi, Osaka, Japan) at 37°C with 5% CO_2 for 3 hours and then divided into two groups of no-FSH (N-FSH) and with 0.3 IU/mL FSH (FSH) (Merck Serono (Beijing) Pharmaceutical R&D Co. Ltd., Beijing, China). Similarly, the vitrification ovarian fragments were divided into two groups of no FSH intervention (VG) and 0.3 IU/mL FSH intervention (FSH-VG). The ovarian cortical fragments of these four groups were transplanted into the back muscles of experimental animals. *In vitro* FSH intervention was also applied for fresh ovary transplantation for 3 hours.

Ovarian transplantation

The four human ovarian cortical fragment groups were adopted as transplantation donors. Before transplantation, the 10-week-old recipient female mice underwent oophorectomies, and human ovarian cortical fragments were xenotransplanted as described by Tahaei *et al*. [12]. Briefly, mice were anesthetized with 4% sodium pentobarbital (0.2 mL/20 g) and disinfected with 75% alcohol on the back skin to prepare the surgical area. Vertical incision of about 1 - 2 cm long were made in the middle of the back, and small pockets were made with a micro instrument in the muscle layers of the back spine. Fresh and vitrification ovarian fragments were subsequently placed into small pockets. To avoid the effects of various internal environments on grafts, ovarian samples obtained from N-FSH and VG groups were transplanted into the left side, while those obtained from FSH and FSH-VG groups were transplanted into the right side. For each group, at least 24 mice were used as recipients to abrogate the effects arising from individual differences and only one ovarian fragment was transplanted under each of the left and right back muscles of each mouse. The peritoneal layer and muscle incisions were then closed with 7/0 surgical absorbable sutures and skins were closed with 5/0 surgical sutures. Following surgery, the mice were returned to clean cages, kept warm, closely observed, and returned to the animal room after awakening. Transplanted ovarian were removed to count ovarian follicle and perform immunohistochemistry tests after anesthetizing mice on days 3, 4, 5, and 6 after transplantation.

Vitrification procedure

Base medium, culture medium, freezing solution and warming solution were prepared according to our previous research with slight modifications [11]. All FSH groups contained 0.3 IU/mL FSH. Briefly, after pre-incubation in nutrient solution at 37°C with 5% CO_2 for 1 hour, human ovarian cortical fragments were pre-equilibrated for 15 min in pre-equilibration solution containing DMEM/F12, 12% HSA, 1.5 mol/L ethylene glycol (EG). The osmotic equilibrium was reached after 6 min incubation with DMEM/F12, 12% HSA, 5.5 mol/L EG, 0.5 mol/L sucrose, and 30% Ficoll as vitrification solution. The ovarian fragments were then cryopreserved in liquid nitrogen for at least 3 days. The ovarian fragments were thawed by immediately immersing into 0.5, 0.25, and 0.125 M sucrose solutions at 37°C for 10 min, respectively, followed by post-culturing at 37°C with 5% CO_2 in culture medium for 2 hours.

Ovarian count

Ovarian follicle counting methods were applied according to previous reports [13, 14]. Briefly, paraffin-embedded ovarian grafts were cut into samples with 5 µm thickness. One section was selected at 10 intervals with a total of 20 sections

being stained using hematoxylin and eosin (H&E). Under microscopy, five high power fields (400X) were adopted for each section and follicle number per high power field (HPF) was counted according to normal follicular morphology. Only the oocyte nuclei were counted to ensure that no antral anterior follicles were double counted. Follicle quality was generally assessed based on basement membrane integrity, cell density of well-arranged granulosa cell layers, oocyte integrity, and presence or absence of nucleosomes (oocytes or granulosa cells without nuclear concentration). Follicles were classified as normal or abnormal according to these criteria. The percentages of primordial follicles (PrF), primary follicles (PF), secondary follicles (SF), and atretic follicles (AF) in the total number of follicles (TF) were calculated. The total number of follicles was considered as the sum of normal and atretic follicles.

Immunohistochemical staining

After dehydration, antigen retrieval was conducted in citrate buffer (pH 6.0) by treating the samples twice in a microwave oven at 100℃ for 13 min followed by washing the slides three times with PBS. To quench endogenous peroxidase activity, the obtained sections were pretreated with 0.3% H_2O_2 in methanol. After washing with PBS, the sections were incubated with 10% goat serum at 37℃ for 30 min. After blocking, sections were incubated overnight at 4℃ with Anti-mouse CD31, CD34, Anti-human CD31, CD34 antibodies (Abcam, Cambridge, UK) diluted with PBS at ratios of 1:200, 1:200, 1:300, and 1:150, respectively. After washing with PBS and incubated with biotinylated anti-rabbit or mouse IgG antibodies (IgG/HRP) (Beijing 4A Biotech Co. Ltd., Beijing, China) for 1 hour at 37℃, the sections were washed 3 times with PBS and incubated with HRP-labeled streptavidin (SA-HRP) for 30 min at 37℃. The positive reactions were visualized with diaminobenzidine (DAB)-peroxidase substrate for 30 s and the nuclei were counterstained with hematoxylin. Appropriate negative slides were run in parallel without adding primary antibody. Images of slides were captured using a Motic BA400 digital microscope (Motic, Wetzlar, Hesse, Germany). Platelet endothelial cell adhesion molecule-1 (CD31) was applied as marker to evaluate neovascularization endothelial cells in ovarian grafts and recombinant cluster of differentiation 34 (CD34) was employed as marker to investigate new mature vascularization endothelial cells in ovarian grafts [15]. CD31 and CD34 were applied for the detection of angiogenesis in human ovary grafts and mouse hosts.

Microvessel counts

Microvessels were counted according to the method described in a previous report with minor modifications [16]. Briefly, all blood vessels were highlighted by staining endothelial cells with factors CD31 and CD34 (differentiation 31 and 34 cluster) through standard immunohistochemistry. Individual microvessels were counted at 400X power field. The criterion of microvessel adjustment contained cluster of brown staining endothelial or endothelial cells, which could be distinctly isolated from adjacent microvessels. Other connective tissues were regarded as countable and single microvessel. However, vessel lumen could not be defined as microvessel structure and red blood cells could not be defined as microvessels. Three individual investigators performed the counting.

Statistical analysis

SPSS 21.0 (IBM, Armonk, NY, USA) was employed for statistical analysis of this study. The data were recorded as mean ± standard error and analyzed through ANOVA followed by Fisher's least significant different test (Fisher LSD). *P* value less than 0.05 was defined as statistically significant difference.

Results

The morphology of ovarian grafts from fresh ovarian fragment group

Fresh ovarian fragments of N-FSH and FSH groups were untransplanted on day 0. Ovarian morphology on day 0 and ovarian grafts on days 3, 4, 5, and 6 were checked by H&E staining

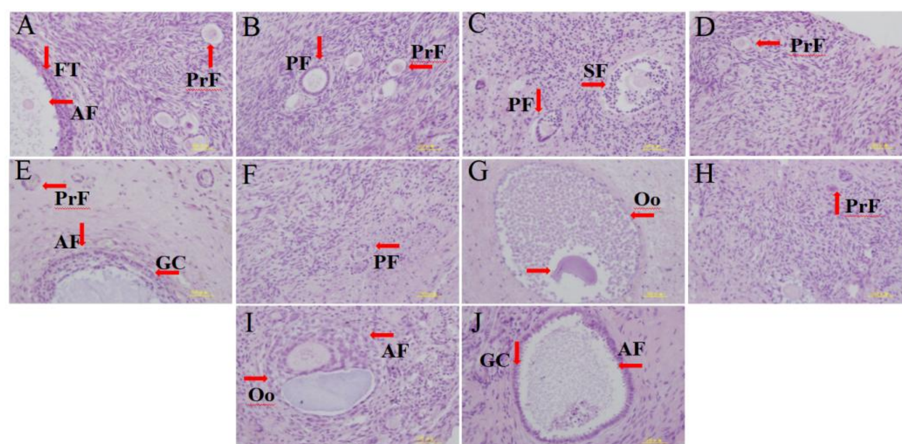

Figure 1. Morphological observation of ovarian grafts from fresh ovarian fragments.

Table 1. The number of ovarian follicles of ovarian grafts (fresh ovarian transplantation, 6.25 mm^3/ follicles).

Group	PrF	PF	SF	AF	TF
N-FSH (control)	10.67 ± 2.52	8.00 ± 2.00	4.33 ± 1.53	11.00 ± 3.61	34.33 ± 10.07
FSH (control)	14.3 ± 2.08*	10.0 ± 2.00	6.67 ± 2.52	6.33 ± 1.53*	37.33 ± 8.02
N-FSH (3 d)	9.00 ± 2.00	6.00 ± 2.00	3.00 ± 2.00	12.00 ± 3.00	29.67 ± 9.02
FSH (3 d)	9.67 ± 2.52	7.67 ± 2.52	4.00 ± 3.00	8.00 ± 2.00*	29.33 ± 10.02
N-FSH (4 d)	8.00 ± 2.00	5.67 ± 3.06	2.67 ± 1.53	14.00 ± 3.61	30.00 ± 10.54
FSH (4 d)	8.67 ± 1.53	6.33 ± 2.52	3.33 ± 2.08	11.67 ± 3.52	30.33 ± 9.54
N-FSH (5 d)	7.33 ± 2.52	4.67 ± 1.53	2.67 ± 1.53	16.00 ± 2.65	30.67 ± 8.08
FSH (5 d)	8.00 ± 2.00	5.67 ± 1.53	4.00 ± 2.00	12.6 ± 2.52*	30.33 ± 8.02
N-FSH (6 d)	7.00 ± 2.00	5.67 ± 2.52	3.00 ± 1.00	14.00 ± 3.46	29.67 ± 8.74
FSH (6 d)	7.67 ± 2.52	6.33 ± 2.08	3.67 ± 1.53	11.33 ± 3.06	29.00 ± 9.17

Note: * indicated significant difference compared with N-FSH ($P < 0.05$).

(Figure 1). Compared with the ovarian fragments of untransplantation control group, the morphology of transplantation ovarian on days 3, 4, 5, and 6 were scattered and the numbers of stromal cells and ovarian follicles were reduced. These results suggested that transplantation damaged ovarian morphological integrity. However, compared with N-FSH intervention group, ovarian graft morphology was more compact, ovarian follicle structure was more intact, and more stromal cells were detected in FSH intervention group. These findings revealed that FSH intervention increased ovarian graft morphological integrity. Furthermore, ovarian follicles were counted, and the findings revealed that, compared with untransplantation ovarian group, the numbers of PrF, PF, and SF in transplantation ovarian group were decreased (Tables 1). However, compared with N-FSH control group, the quantities of PrF, PF, and SF were improved, and atretic follicles were significantly less in FSH intervention group than in N-FSH intervention control group ($P < 0.05$).

The morphology of ovarian grafts from vitrification ovarian fragment group

Vitrification ovarian transplantation fragments with no FSH intervention (VG) and vitrification ovarian transplantation fragments with FSH intervention (FSH-VG) groups were untransplanted on day 0. Ovarian morphology on day 0 and ovarian grafts on days 3, 4, 5, and 6 were checked by H&E staining (Figure 2). Compared with untransplantation control group ovarian fragments, transplantation ovarian morphology on days 3, 4, 5, and 6 was scattered,

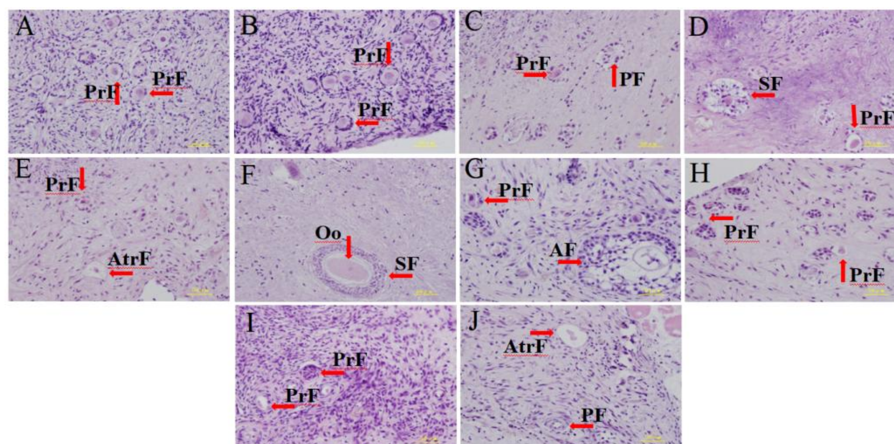

Figure 2. Morphological observation of ovarian grafts from vitrification ovarian fragments.

Table 2. The number of ovarian follicles of ovarian grafts (vitrification ovarian transplantation, 6.25 mm^3/ follicles).

Group	PrF	PF	SF	AF	TF
VG (control)	14.33 ± 2.08	11.67 ± 1.53	8.67 ± 1.53	7.67 ± 1.53	42.33 ± 6.66
FSH-VG (control)	17.0 ± 2.65*	13.33 ± 3.06	9.00 ± 2.00	6.33 ± 1.53	45.67 ± 9.07
VG (3 d)	13.00 ± 2.00	10.00 ± 1.00	9.00 ± 2.00	10.33 ± 2.52	42.33 ± 7.51
FSH-VG (3 d)	14.33 ± 1.53	11.00 ± 2.00	9.00 ± 2.00	12.00 ± 2.00	46.33 ± 7.51
VG (4 d)	12.00 ± 3.00	9.00 ± 2.00	8.33 ± 2.52	12.00 ± 2.00	41.00 ± 10.00
FSH-VG (4 d)	12.67 ± 2.52	8.33 ± 2.52	7.00 ± 2.00	8.00 ± 2.00*	36.00 ± 9.00
VG (5 d)	10.00 ± 2.00	6.33 ± 2.52	6.33 ± 2.52	14.67 ± 2.52	37.33 ± 9.50
FSH-VG (5 d)	12.00 ± 2.00	9.00 ± 2.00*	8.00 ± 2.00	9.67 ± 2.52*	38.67 ± 8.50
VG (6 d)	8.67 ± 1.53	7.00 ± 2.00	6.00 ± 2.00	15.33 ± 2.08	37.00 ± 7.55
FSH-VG (6 d)	10.67 ± 2.52	9.33 ± 2.52	8.00 ± 3.00	12.00 ± 2.00	40.00 ± 10.00

Note: * indicated significant difference compared with VG ($P < 0.05$).

and the numbers of stromal cells and ovarian follicles decreased. These findings revealed that transplantation damaged ovarian morphological integrity. However, compared with VG control group, ovarian graft morphology in FSH-VG intervention group was more compact, ovarian follicle structure was more intact, and more stromal cells were detected in FSH intervention group. These results revealed that FSH intervention increased ovarian graft morphological integrity. In addition, the counting of ovarian follicles found that, compared with untransplantation ovarian group, PrF, PF, and SF numbers in transplantation ovarian group were decreased. However, compared with VG control group, PrF, PF, and SF numbers were increased in FSH-VG intervention group, and atretic follicles in FSH-VG intervention group was much lower than that in VG control group ($p < 0.05$) (Tables 2). Compared to fresh ovarian fragments, vitrification ovarian fragment morphology was obviously scattered. The numbers of stromal cells and ovarian follicles were remarkably decreased. These results suggested that vitrificated cryopreservation damaged ovarian morphological integrity. Therefore, FSH intervention was able to maintain ovarian graft structural integrity during *in vitro* culture and vitrification.

Revascularization of ovarian grafts by CD31 staining

Ovarian graft neovascularization endothelial cells were evaluated using CD31 immunohistochemistry. Microvessel density was measured in Anti-human CD31 and Anti-mouse CD31 labeled vessels. Fresh ovarian fragments of N-FSH and FSH were transplanted. Ovarian grafts

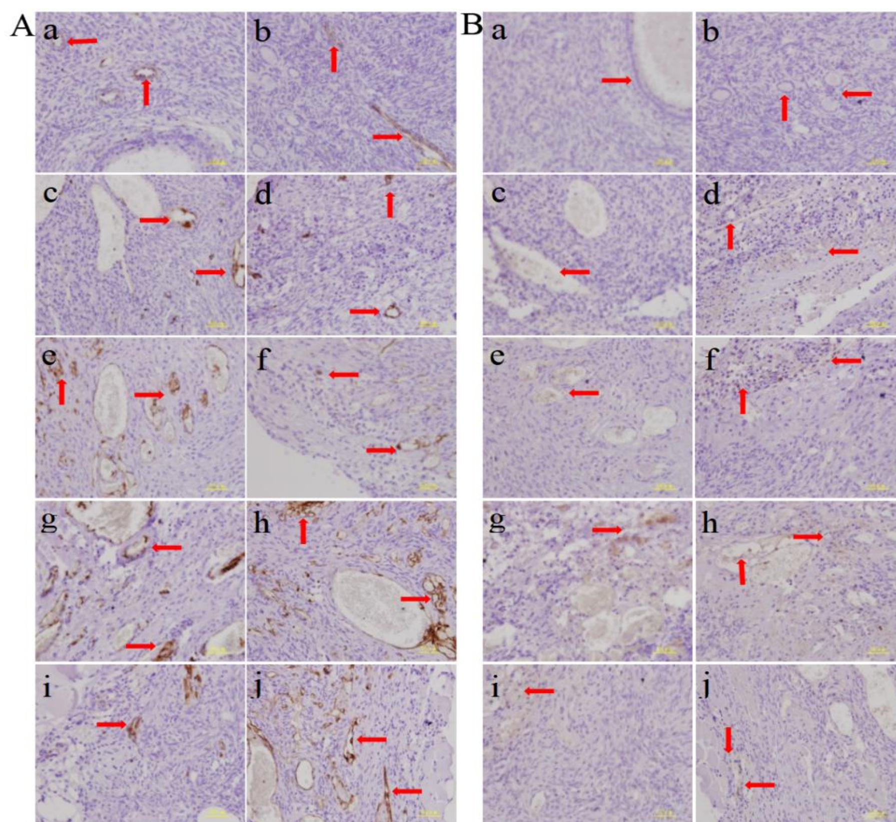

Figure 3. Revascularization of ovarian grafts obtained from fresh ovarian fragments by CD31.

Table 3. Microvessels in the ovarian grafts detected by CD31 staining (fresh ovarian, 6.25 mm^3/ follicles).

Groups	CD31 (human)	CD31 (mouse)
N-FSH (control)	32.67 ± 7.51	0.00 ± 0.00
FSH (control)	8.00 ± 2.64*	0.00 ± 0.00
N-FSH (3 d)	31.00 ± 5.57	3.67 ± 2.06
FSH (3 d)	4.00 ± 1.00*	8.34 ± 2.94*
N-FSH (4 d)	58.33 ± 5.51	5.00 ± 1.65
FSH (4 d)	104.67 ± 20.51*	5.67 ± 0.42
N-FSH (5 d)	45.00 ± 30.27	23.00 ± 2.61
FSH (5 d)	55.33 ± 29.57*	33.67 ± 4.00*
N-FSH (6 d)	121.67 ± 19.22	10.33 ± 1.08
FSH (6 d)	54.00 ± 20.08*	12.00 ± 3.21

Note: * indicated significant difference compared with N-FSH ($P < 0.05$).

of control and days 3, 4, 5, and 6 were stained with Anti-human CD31 (Figure 3A), and Anti-mouse CD31 (Figure 3B) antibodies and were applied to detect vessels with immunohistochemistry according to a method reported in a previous study [15]. The microvessels were counted and the results showed that, in fresh ovarian transplantation fragments, the numbers of Anti-human CD31 and Anti-mouse CD31 positive staining of FSH intervention group on day 3 were relatively small. The maximum number of Anti-human CD31 positive staining in FSH intervention group was observed on day 4, after which the number was decreased. However, corresponding maximum number in N-FSH intervention group was

witnessed on day 6. The numbers of Anti-mouse CD31 positive staining of FSH and N-FSH intervention groups both peaked on day 5, and then decreased. Anti-human CD31 staining results revealed that on day 4, the number of neovascularization endothelial cells in FSH intervention group was obviously higher than that in N-FSH intervention group ($P < 0.05$). However, Anti-mouse CD31 staining results revealed that neovascularization endothelial cell number in FSH intervention group on day 3 was significantly higher than that in N-FSH intervention group ($P < 0.05$) (Table 3). Hence, intervention of human ovarian cortical fragments with 0.3 IU/mL FSH during *in vitro* culturing accelerated ovarian graft revascularization. In vitrification ovarian fragments of VG and FSH-VG groups, ovarian grafts of control and days 3, 4, 5, and 6 were stained with Anti-human CD31 (Figure 4A) and Anti-mouse CD31 (Figure 4B) antibodies and were applied for the detection of vessels by immunohistochemistry according to a method reported in a previous study [15]. The results showed that, in vitrification ovarian transplantation fragments, the numbers of Anti-human CD31 and Anti-mouse CD31 positive staining of FSH-VG intervention group on day 3 were both increased, then decreased on days 4 to 5, and finally peaked on day 6. However, the corresponding number for VG control group peaked on days 3 and 5, then decreased suddenly. Anti-human CD31 staining results showed that the number of neovascularization endothelial cells in FSH-VG intervention group on day 3 was remarkably higher than that in VG control group ($P < 0.05$). However, Anti-mouse CD31 staining results presented that the number of neovascularization endothelial cells in FSH-VG intervention group on day 6 was much higher than that in VG control group ($P < 0.05$) (Table 4). Therefore, the intervention of human ovarian cortical fragments with FSH during vitrification accelerated ovarian graft revascularization. Compared to FSH group, the peak times of Anti-human CD31 and Anti-mouse CD31 positive staining neovascularization endothelial cells were slower for 1-2 days in revascularization in FSH-VG group, but it was similar in FSH-VG group and N-FSH group. The results indicated that vitrificated cryopreservation damaged ovarian graft revascularization, but FSH intervention improved the angiogenic activity of transplanted ovarian.

Angiogenesis and vascularization of ovarian grafts by CD34 staining

CD34 staining was applied to check new mature vascularization endothelial cells of ovarian grafts. The microvessel density was measured in Anti-human CD34 and Anti-mouse CD34 labeled vessels. The transplanted fresh ovarian fragments of N-FSH and FSH were shown in Figure 5. The ovarian grafts of control and days 3, 4, 5, and 6 were stained with Anti-human CD34 (Figure 5A) and Anti-mouse CD34 (Figure 5B) antibodies were applied to detect vessels by immunohistochemistry according to a method reported in a previous study [15]. The microvessels were counted in Table 5. In fresh ovarian transplantation fragments, the numbers of Anti-human CD34 and Anti-mouse CD34 positive staining of FSH intervention group peaked on day 4 and maintained at a high level, while N-FSH intervention group peaked on day 6. Anti-human CD34 staining results revealed that the number of new mature vascularization endothelial cells in FSH intervention group on day 5 was much higher than that in N-FSH intervention group ($P < 0.05$). However, Anti-mouse CD34 staining results revealed that the number of new mature vascularization endothelial cells in FSH intervention group on day 4 was obviously higher than that in N-FSH intervention group ($P < 0.05$). The findings illustrated that the number of CD34 positive staining vascular endothelial cells was maintained at high levels in fresh ovarian transplantation fragment group, which was because the ovarian did not experience cryopreservation and vascular endothelial cells were not damaged. Therefore, human ovarian cortical fragment intervention with FSH during *in vitro* culturing accelerated the angiogenesis and vascularization of ovarian grafts. Vitrification ovarian fragments of VG and FSH-VG were transplanted, and ovarian grafts of control and

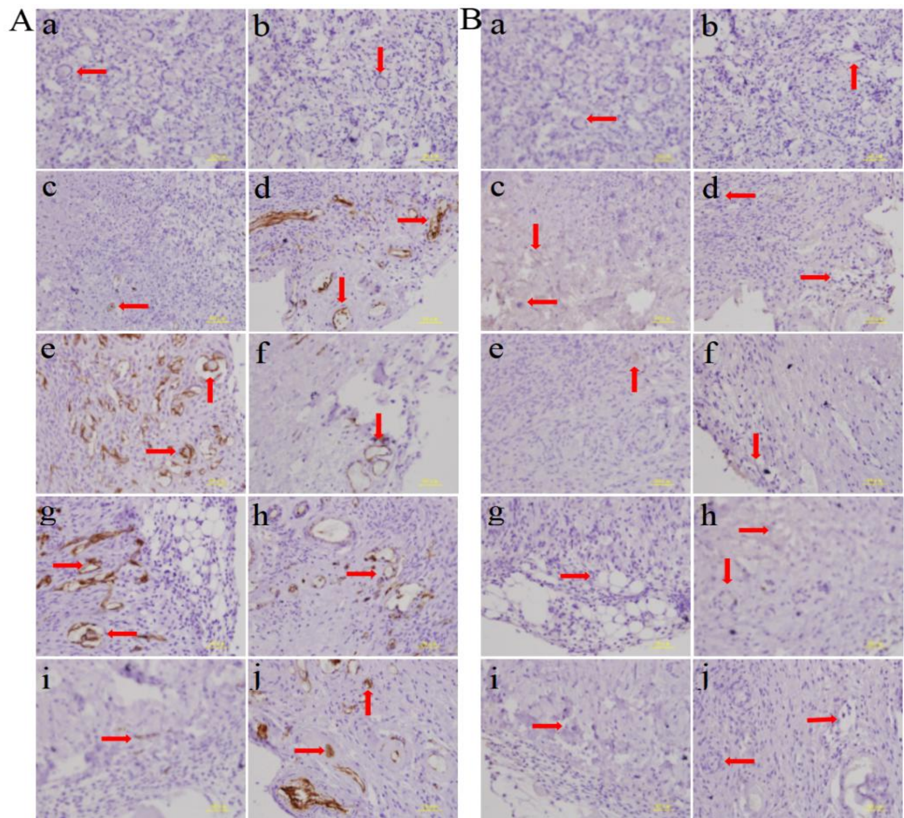

Figure 4. Revascularization of ovarian grafts obtained from vitrification ovarian fragments by CD31.

Table 4. Microvessels in the ovarian grafts detected by CD31 staining (vitrification ovarian, 6.25 mm^3/ follicles).

Groups	CD31 (human)	CD31 (mouse)
VG (control)	0.33 ± 1.31	0.00 ± 0.00
FSH-VG (control)	0.00 ± 0.00	0.00 ± 0.00
VG (3 d)	12.33 ± 2.52	15.67 ± 3.93
FSH-VG (3 d)	30.33 ± 7.77*	19.00 ± 8.00
VG (4 d)	29.00 ± 8.00	4.00 ± 0.00
FSH-VG (4 d)	27.67 ± 7.10	1.67 ± 1.08
VG (5 d)	54.00 ± 12.29	10.67 ± 2.06
FSH-VG (5 d)	18.00 ± 15.72*	11.33 ± 2.51
VG (6 d)	18.67 ± 4.04	8.33 ± 1.52
FSH-VG (6 d)	29.67 ± 12.53*	19.00 ± 12.00*

Note: * indicated significant difference compared with VG ($P < 0.05$).

days 3, 4, 5, and 6 were stained with Anti-human CD34 (Figure 6A) and Anti-mouse CD34 (Figure 6B) antibodies and applied for the detection of vessels by immunohistochemistry. The counts of microvessels found that, in vitrification ovarian transplantation fragments, the numbers of Anti-human CD34 and Anti-mouse CD34 positive staining of FSH-VG intervention group peaked on days 4 to 6 and maintained at a certain level, while VG control group peaked on days 4 to 5, then suddenly decreased. Anti-human CD34 staining results showed that the number of new mature vascularization endothelial cells in FSH-VG intervention group on day 5 was much higher than that in VG control group ($P < 0.05$). However, Anti-mouse CD34 staining results

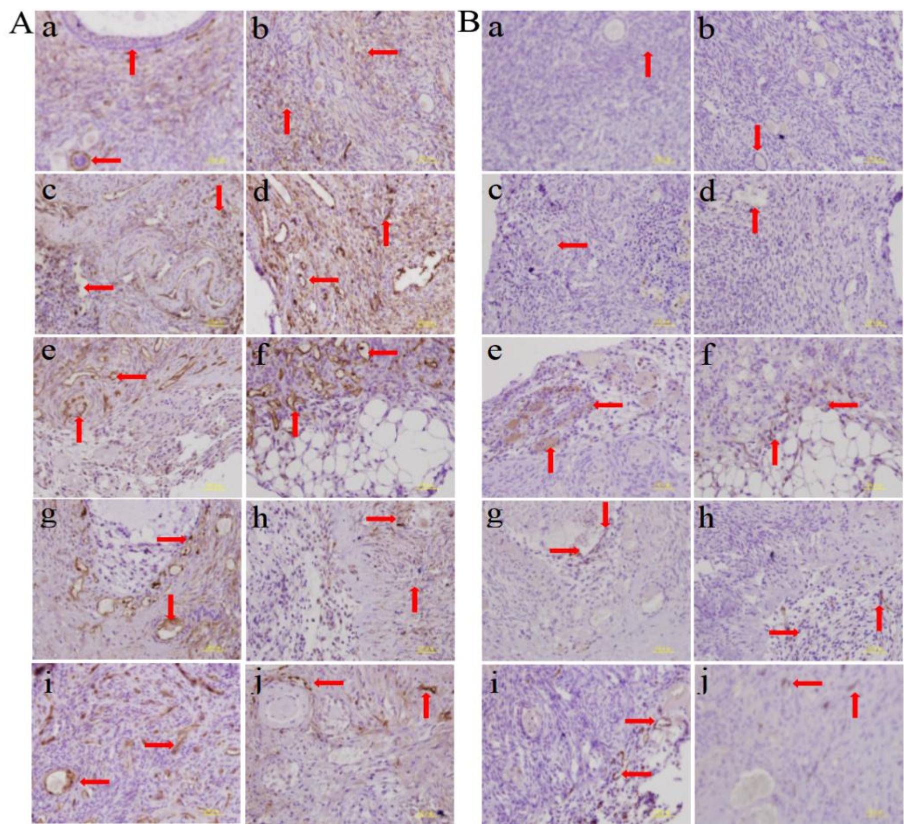

Figure 5. Angiogenesis and vascularization of ovarian grafts obtained from fresh ovarian fragments by CD34.

Table 5. Microvessels in the ovarian grafts detected by CD34 staining (fresh ovarian, 6.25 mm^3/follicles).

Groups	CD34 (human)	CD34 (mouse)
N-FSH (control)	133.67 ± 16.47	0.00 ± 0.00
FSH (control)	129.00 ± 26.79	0.00 ± 0.00
N-FSH (3 d)	150.00 ± 12.77	19.00 ± 7.97
FSH (3 d)	150.33 ± 8.08	20.33 ± 4.35
N-FSH (4 d)	162.67 ± 11.06	26.67 ± 9.01
FSH (4 d)	169.00 ± 13.75	36.00 ± 10.00*
N-FSH (5 d)	134.00 ± 16.70	25.67 ± 8.32
FSH (5 d)	163.00 ± 9.54*	29.33 ± 6.33
N-FSH (6 d)	161.33 ± 25.78	31.34 ± 5.98
FSH (6 d)	169.33 ± 19.55	37.00 ± 11.00

Note: * indicated significant difference compared with N-FSH ($P < 0.05$).

found that the number of new mature vascularization endothelial cells in FSH-VG intervention group on day 6 was significantly higher than that in VG control group ($P < 0.05$) (Table 6). The findings also revealed that in untransplantation group, FSH-VG intervention more effectively preserved vascular endothelial cells compared to that in VG control group.

Hence, human ovarian cortical fragment intervention with FSH during vitrification accelerated the angiogenesis and vascularization of ovarian grafts. Furthermore, CD34 positive expression was witnessed around the follicles and edges of grafts, indicating the formation of new blood vessels after human ovarian transplantation. Compared to FSH group, the

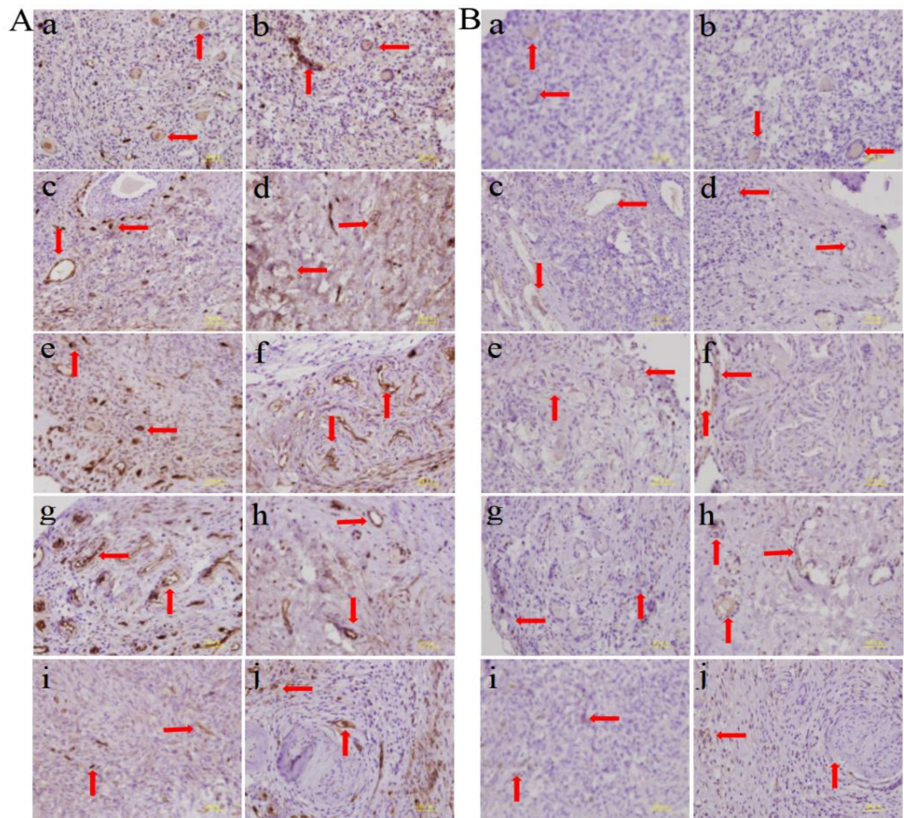

Figure 6. Angiogenesis and vascularization of ovarian grafts obtained from vitrification ovarian fragments by CD34.

Table 6. Microvessels in the ovarian grafts detected by CD34 staining (vitrification ovarian, 6.25 mm^3/follicles).

Groups	CD34(human)	CD34(mouse)
VG (control)	90.66 ± 91.01	00.00 ± 0.00
FSH-VG (control)	167.67 ± 13.39*	00.00 ± 0.00
VG (3 d)	164.33 ± 13.32	20.00 ± 0.00
FSH-VG (3 d)	197.67 ± 19.51*	18.00 ± 0.96
VG (4 d)	213.00 ± 43.56	21.66 ± 5.23
FSH-VG (4 d)	228.00 ± 38.74	31.00 ± 5.29
VG (5 d)	149.33 ± 29.57	29.33 ± 2.49
FSH-VG (5 d)	186.33 ± 43.25*	40.00 ± 2.35
VG (6 d)	188.00 ± 22.61	12.00 ± 6.75
FSH-VG (6 d)	197.00 ± 47.51	42.33 ± 10.36*

Note: * indicated significant difference compared with VG ($P < 0.05$).

peak times of Anti-human CD34 and Anti-mouse CD34 positive staining new mature vascularization endothelial cells were slower for 1-2 days in angiogenesis and vascularization in FSH-VG group, but it was similar in FSH-VG group and N-FSH group. The results indicated that vitrificated cryopreservation damaged the angiogenesis and vascularization of ovarian grafts, but FSH intervention improved the angiogenic activity of transplanted ovarian.

Discussion

FSH is a glycoprotein hormone secreted by pituitary gland, which regulates follicle

development and prevents follicle apoptosis. Also, pituitary hormone regulates periodic blood vessel changes in the ovarian during follicular development. FSH is a vital hormone for ovarian follicle development and is a marker hormone applied for the evaluation of ovarian graft functional recovery. Therefore, optimal FSH intervention can improve ovarian revascularization [17]. Although several successful cryopreservation and transplantation procedures of mouse ovaries have been reported in past decades, lack of progress in the cryopreservation and transplantation of human ovarian cortical fragments may be attributed to hard texture and relatively low number of ovarian follicles in human ovaries compared with those in mice. Our previous research suggested that FSH intervention improved the morphology and follicle survival of mouse and transplantation ovarian during ovarian cryopreserved by vitrification, and offspring were successfully born. Due to texture differences between mouse and human ovaries, such as more fiber and denser tissue in human ovarian cortex, it is not known whether the response to hormone level is different. Further, it is unknown whether FSH plays a vital role in vitrification and transplantation of human ovarian cortical fragments. To investigate this issue, FSH was applied in human ovarian cortical fragment vitrification in this study.

The results of this study showed that FSH intervention improved transplanted ovarian graft morphology in fresh and vitrification groups. The numbers of PrF, PF, SF, and TF were increased, and atretic follicles were decreased with FSH intervention during the process of pre-culturing fresh and vitrification human ovarian cortical fragments. These findings were consistent to our previous research on the vitrification and transplantation of mouse ovarian. Revascularization and follicles survival of mouse transplantation ovarian were increased with FSH intervention during cryopreserved by vitrification. Therefore, optimal FSH intervention during ovarian vitrification/thawing promoted the survival and anti-apoptosis of ovarian follicles. FSH was also found to play a vital role in the growth and development of ovarian follicles, especially in anti-apoptosis of ovarian granulosa cells [8]. The number of atresia follicles in FSH intervention transplantation group was less than that in N-FSH intervention group on days 4 and 5. These results might be related to the follicular protection of FSH and other FSH up-regulated molecules. These anti-apoptotic molecules contained basic fibroblast growth factor (bFGF) and VEGF that enhanced anti-ischemia-hypoxic that induced apoptosis, which might be one of the reasons why the number of atresia follicles was significantly reduced in FSH intervention group, and the accelerated blood perfusion of transplantation ovarian tissue by FSH intervention.

Ovarian follicle death occurring during early ovarian transplantation period was attributed to tissue ischemia and reperfusion injury [18, 19]. Following transplantation, hypoxia decreased ovarian transplants reproductive viability [20]. Hence, “blood bed” formation is essential for follicle survival in ovarian transplantation. Previous studies have revealed that intervention accelerated the vascularization and vascular remodeling of the grafts during ovarian vitrification and transplantation such as angiopoietin-2, VEGF, fibroblast growth factor (FGF), and melatonin [21]. Our previous findings from mouse revealed that angiogenesis-related factors, VEGF, angiopoietin-2, and Intergrin αvβ3, expression were significantly increased in FSH intervention group [11]. The neonatal endothelial cell markers of CD31 and CD34 antibodies were applied for the detection of angiogenesis. The results obtained from CD31 positive staining suggested that *in vitro* human ovarian cortical fragment intervention with 0.3 IU/mL FSH during culturing and vitrification accelerated ovarian graft revascularization. In vitrification ovarian transplantation fragment group, the numbers of Anti-human CD31 and Anti-mouse CD31 positive staining neovascularization endothelial cells in FSH-VG intervention group were increased on day 3, then decreased on days 4-5, and finally peaked on day

6, while that in VG control group peaked on days 3 and 5, then suddenly decreased. Such results might indicate that the cryopreservation agent and sharp change of temperature in vitrification cryopreservation might cause certain damage to ovarian tissue cells and such damage had a repair period after transplanting the ovary back into the body. CD34 positive staining results suggested that human ovarian cortical fragment intervention with FSH during *in vitro* culturing and vitrification accelerated ovarian graft angiogenesis and vascularization, which provided a favorable opportunity for the ovarian grafts to timely reconstruct blood flow. This rapid blood flow reconstruction was closely related to the effective protection of FSH in vascular endothelial cells (CD34) during freezing and thawing processes, and more preserved vascular endothelial cells effectively guaranteed neovascular budding. However, this result was due to FSH up-regulating the levels of pro-angiogenesis-related factors. Also, CD34 positive expression was observed around the follicles and edges of the grafts, indicating that new blood vessels formed after human ovary transplantation. Compared to FSH group, the peak times of Anti-human CD31/CD34 and Anti-mouse CD31/CD34 positive staining neonatal endothelial cells were slower for 1-2 days in angiogenesis and revascularization in FSH-VG group but were similar in FSH-VG and N-FSH groups, which indicated that vitrificated cryopreservation damaged angiogenesis and revascularization of ovarian grafts. However, FSH acted as a protective agent of hormone class could improve transplanted ovarian angiogenic activity. In addition, the reason for the number of neonatal endothelial cells in CD31 staining was decreased and maintained at a certain level after reaching the peak might be that the new blood vessels were transformed into mature blood vessels [22]. Also, CD34 remained at a high level after reaching the peak. These results indicated that neovascularization and mature blood vessels could basically satisfy ovary transplantation blood perfusion and maintain a certain balance.

Therefore, FSH intervention accelerated angiogenesis, and co-culturing with activated ovarian endothelial cells (ECs) promoted early follicular development and survival *in vitro* [23]. Previous research showed that mouse neovascularization and perfusion regions were located at the periphery of human transplanted ovarian fragments, whereas unperfused native human blood vessels were present within human transplanted ovarian fragments. Our findings revealed that vascularization of grafts was checked by Anti-mouse and Anti-human antibodies, and both grafts and recipient vessels participated in blood flow reconstruction in the ovaries. However, the main composition of neovascularization was human vascular endothelial cells. To observe the angiogenic effects produced by different interventions on grafts, ovarian cortical sections were transplanted with and without FSH interventions on both sides of the spine in the same recipient mouse to observe the outcomes of grafts revascularization in the same receptor environment. Neonatal endothelial cells began to generate in the receptor and ovarian grafts at the same time on day 3. However, the peak time of Anti-mouse CD31/CD34 positive staining neonatal endothelial cells in FSH intervention group was 1-2 days earlier than that in N-FSH intervention group. The results suggested that the angiogenic factors upregulated by FSH intervention on the grafts might accelerate neonatal endothelial cell generation in recipient tissue at graft site through the action of osmotic diffusion. The opposite growth of both sides shortened graft blood perfusion time and helped functional maintenance of transplanted ovarian. Therefore, ovarian graft reperfusion was initiated by human ovarian graft angiogenesis. The recipient site was also involved in microvascular reconstruction of transplanted ovarian. Some microvascular anastomoses were established between the host and transplanted ovarian vessels. Earlier host microangiogenesis and infiltration in FSH intervention group than that in non-intervention group may be due to the upregulated angiogenic factors by FSH intervention, which might act on host blood vessels through osmosis increasing the permeability of blood vessels and exudation of

tissue fluid. Higher amounts of upregulated vascular factors resulted in faster regeneration of host blood vessels. On the other hand, the angiogenesis of transplanted ovaries occurred at high speeds under the action of FSH. Therefore, graft blood perfusion recanalization time was shortened. The time of viable ovaries to function was advanced. The blood vessel regeneration was accelerated under the effect of locally increased estrogen [24, 25].

Our previous research suggested that FSH administration in the process of ovarian cryopreservation by vitrification improved mouse ovarian follicle survival rate in vitrification process and increased angiogenesis and revascularization by avascular transplantation through upregulating connexin 43, connexin37, VEGF/VEGF receptor 2, bFGF, and anti-apoptotic [11]. It is known that the number of target genes was increased by hypoxia-inducing factor (1α) including VEGF and its receptor genes, which participated in vascularization, vasoconstriction, and revascularization. In fact, neovascularization and ovarian graft morphology were better achieved by administering FSH after transplantation and increased ovarian follicle survival. Previous studies also found that FSH administration during mouse transplantation ovarian cryopreserved by vitrification could increase follicle survival and revascularization through upregulating the expression of ovarian survival factors and angiogenesis [11]. Taken together, we speculated that FSH intervention in ovarian cryopreservation by vitrification played a dual role in anti-apoptotic and stimulating angiogenesis. The faster blood flow reconstitution allowed more follicles in transplanted ovarian to survive and develop. In addition, our previous study suggested an increased neoangiogenesis in ovarian grafts with FSH intervention during the whole process of mouse ovarian vitrification, which might be attributed to increased expression of VEGF and VEGF receptors in vitrified/thawed ovarian with FSH intervention. Furthermore, ovarian intervention with FSH for 6 hours accelerated the revascularization of the whole transplanted ovarian tissue and could not cause ovarian over stimulation. The results of this study confirmed that FSH intervention could accelerate the angiogenesis and vascularization of ovarian grafts during ovarian cryopreservation by vitrification and preserve follicle reserve. Both grafts and recipient vessels participated in blood flow reconstruction in ovarian at the same time. Earlier peak times of neonatal endothelial cells were observed by FSH intervention. Angiogenic factors upregulated by FSH in human ovarian grafts also accelerated the angiogenesis of recipient tissues at graft site.

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