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Original Research

The effect of VEGF and Ang-1 on cryopreserved human ovarian grafts in severe combined immunodeficient mice

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Summary

This study investigates the independent and combined effect of vascular epithelial growth factor (VEGF) and angiopoietin 1 (Ang-1) on the follicle survival and vasculogenesis of cryopreserved human ovarian grafts in severe combined immunodeficient (SCID) mice. Cryopreserved human ovarian tissue was transplanted into the thigh of SCID mice with treatment of VEGF, Ang-1, or combination at the site of transplantation. Number of follicles, morphology change, follicle apoptosis, micro-vessel density (MVD), and expression of follicle development related genes were assessed in each transplanted ovarian group of SCID mice. VEGF and Ang-1 treatments increased functional follicles and MVD value, reduced the level of follicle apoptosis, and modulated expression of ovarian development related genes and FSH level. Synergistic effect of VEGF and Ang-1 on follicle survival was significantly stronger than treatment alone. In conclusion, treatment of VEGF and Ang-1 significantly improved the survival of follicles and vasculogenesis of transplanted human ovarian tissue in SCID mice.

Key words: VEGF; Ang-1; Cryopreservation; Ovarian,; Hetero-transplantation; Survival of follicles; Vasculogenesis.

Introduction

The incidence of malignant neoplasms in young patients is generally increasing in recent years. Development of new chemotherapy and radiotherapy successfully eliminates cancer cells; meanwhile, it also damages the germ cells causing infertility in young female patients. Studies have demonstrated that cryopreservation and autotransplantation of ovarian tissue may recover the fertility of patients after radiotherapy/chemotherapy [1]. However, there are still some issues needed to be resolved. The major problem is the loss of follicles from freezing/thawing procedure and ischemic and hypoxic injury. Research has found that ischemia and hypoxia cause damages to loss of follicles even greater than that of the effect from freezing/thawing procedure [2]. Therefore, regeneration of blood supply is one of the key points to reduce the loss of follicles in transplanted ovarian tissue.

Two regulation pathways are specifically mentioned in the development of vasculogenesis in endothelial cells, including the vascular epithelial growth factor (VEGF) and angiopoietin 1 (Ang-1) along with their receptors flt-1 and Tie, respectively. VEGF is the most effective vascular growth factor in the process of vasculogenesis. Treatment of VEGF increases the survival of functional follicles, improves oocyte quality, and promotes new vascular growth in transplanted mouse ovarian tissues [2]. The regulation of Ang-1 system in vasculogenesis is gradually revealed in recent years and has been reported to play important roles in several ischemic diseases [3-5]. This study used slow freezing method for preserving human ovarian tissue and transplanted into severe combined immunodeficient (SCID) mice model to investigate whether treatment of VEGF and Ang-1 improved the survival of follicles and vasculogenesis in the transplanted ovarian tissue.

Material and Methods

Patient recruitment and sample collection: Twenty patients who had to have an ovarian resection surgery were recruited in this study. All of the participants were under age 40 with regular ovarian functions. Informed consents were received from all of the participants. This study was approved by the Institutional Review Board of Shenzhen People's Hospital (IRB number: 2014KY027).

Mice: This study used female BALB/c nude mice from the Guangdong Medical Science Experiment Center. Mice were all under 6-8 weeks of age, and the average weight was 18.6 grams. All of the mice were supplied with sterile water and rodent pellets *ad libitum* in the SPF laboratory animal room of the experiment animal center.

Cryopreservation protocol: Ovarian tissue was repeatedly washed by RPMI-1640 medium containing with penicillin and streptomycin to remove necrotic tissue and blood. Ovarian cortex was dissected from medulla and cut into 15 slices (2×2×1 mm) and kept in the cryovial with 1 mL of cryoprotectant for each patient.

After stored in the 4°C refrigerator, cryovials containing ovarian tissue slices were ready for the freezing procedure as described in the following statements. (1) Starting at 4°C, and to cool at 2°C/minute to -7°C, (2) equilibrated for 10 minutes, (3) contin-

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Table 1. — *Morphology distribution of different level of follicles in ovarian tissues with VEGF or Ang-1 treatment.*

Group	Primordial follicle		Primary follicle		Secondary follicle	
	Normal (%)	Abnormal	Normal (%)	Abnormal	Normal (%)	Abnormal
Blank	4 (22)	14	1 (25)	3	0	0
Ang-1(L)	6 (35)	11	2 (33)	4	0	0
Ang-1(H)	5 (36)	9	3 (60)	2	0 (0)	1
VEGF	7 (58)	5	3 (60)	2	1 (50)	1
VEGF+Ang-1(H)	6 (46)	7	4 (57)	3	2 (66)	1

ued to cool at 0.3°C/minute to -40°C, (4) cooled at a faster rate of 10°C/minutes to -140°C, and (5) transferred to liquid nitrogen flask for storage.

Transplantation procedure: Surgery was conducted under anesthesia with 2% of pentobarbital sodium. During surgery, the incision site was cleaned by alcohol and covered with sterile towel. Mice ovaries were removed before transplantation. Human ovarian tissue slices were carefully thawed and dehydrated for transplantation. Three frozen/thawed pieces of human ovarian tissue were placed in one side of the thigh muscle tissue of each SCID mice.

Treatment of VEGF and Ang-1: Transplanted SCID mice were randomly assigned into five groups, including the saline group, VEGF group (1 ng/uL), high concentration of Ang-1 group (0.2 ug/uL), low concentration of Ang-1 group (0.02 ug/uL), and the combination of VEGF and Ang-1(H) groups. Mice were subcutaneous injected with 10 uL of assigned treatment at the site of transplantation for consecutive five days from the day of surgery.

Evaluation of morphology and number of follicles: Seven days after ovarian transplantation, mice were sacrificed through anesthesia overdose. The transplanted ovarian tissue was fixed in 4% of paraformaldehyde and embedded by paraffin. Samples were serially sectioned (5 um), and every fifth section was stained with hematoxylin and eosin (H&E) and examined for morphological, structure and number of follicles microscopically. Marking of the follicle nuclear was used for evaluating number of follicles to avoid over-counting. Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay was conducted for the detection of cell apoptosis according to the manufacturer's protocol. The rate of follicle apoptosis was represented as number of apoptotic follicles divided by total number of follicles times 100%.

CD34 detection and micro-vessel density (MVD) evaluation: CD34 is a marker for vascular endothelial cells, which can be used for evaluating the density of micro-vessels through immunohistochemical S-P assay. The CD34 positive micro-vessel was shown in brown color under the microscope. The inner cavity of vessels greater than eight red blood cells was neglected.

Quantitative real-time PCR: PCR primers for follicle development related genes are listed as follows: human VEGF: F-5'ACA ACA AAT GTG AAT GCA GAC CA-3', R-5'GAG GCT CCA GGG CAT TAG AC-3'; human Ang-1: f-5'GAA CAC GAT GGC AAC TGT CG-3', R-5'TCT CAA GTT TTT GCA GCC ACT G-3'; human β-microglobulin: F-5'GAG AAA ATC TGG CAC CAC ACC T-3', R-5'GCA CAG CCT GGA TAG CAA CGT A-3'; human Kitlg: F-5'CGC TGC GGA TCC TTA TGA AGA AGA CA-3', R-5'CGA AAG TAA CAG TGT TGA CTC GAG CCA CAA-3'; human Figla: F-5'TCG TCC ACT GAA AAC CTC CAG-3', R-5'TTC TTA TCC GCT CAC GCT CC-3'; human H1foo: F-5'TGA GAC AGT ACA GGA GAC CAA AGT G-3', R-5'AGA CAA TAA AAG CTT CAG AAT CGC C-3'; human AMH: F-5'CGC CTG GTG GTC CTA CAC-3', R-5'GAA CCT CAG CGA GGG TGT T-3'. The cycling condition is 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 45 seconds, for 45 cycles. Ct values were used for gene expression analysis.

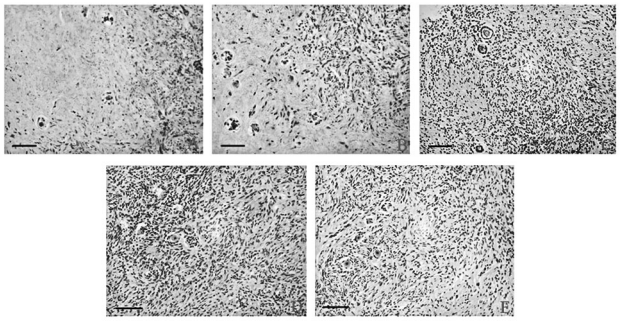

Figure 1. — H&E staining of each transplanted ovarian tissue with VEGF or Ang-1 treatment. Mice with transplantation received (A) saline buffer, (B) Ang-1 (L), (C) Ang-1(H), (D) VEGF, and (E) VEGF + Ang-1 (H) at the site of transplantation for five consecutive days from the beginning of surgery. After seven days of follow up, transplanted ovarian tissues were stained for functional follicle observation. Scale bar equals 100 μm.

Enzyme-linked immunosorbent assay (ELISA): The 50 uL of each sample was added into the respective wells of microtiter plate. The plate was covered with adhesive film and incubated at 37°C for 30 minutes. Plate was further washed five times with 300 uL of wash buffer and excess solution was removed by tapping the inverted plate on a paper towel. HRP conjugate, substrate solution, and stop solution were added into each well according to the manufacturer's protocol. The optical density was measured with a photometer at 450/650 nm within 15 minutes.

Statistical analysis: Values are expressed as mean ± SD from at least four independent experiments. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc comparisons. Two-tailed *p* values of less than 0.05 were considered as statistically significant. SPSS 17.0 was used for statistical analysis.

Results

The follicles in the sample of ovarian tissue were mostly primordial and a few primary follicles, with very little second follicles. The morphology distribution of each level of follicles is categorized in Table 1. Results showed that VEGF and Ang-1 treatments significantly increased the level of functional follicles and decreased the number of abnormal follicles in three different types of follicle groups. Among the treatment groups, VEGF had obviously better effect in promoting normal follicle development than Ang-1(L) and Ang-1(H) (Figures 1A-E).

Table 2. — *Quantification of CD34 level in each transplanted ovarian tissue.*

Group	Blank	Ang-1(L)	Ang-1(H)	VEGF	VEGF+Ang-1(H)
O.D.value	65.31 ± 2.47	75.16 ± 4.29	82.5 ± 4.74	83.82 ± 5.17	98.32 ± 3.14

O.D.: Optical density

Table 3. — *MVD value in each group of transplanted ovarian tissue*

Group	Blank	Ang-1(L)	Ang-1(H)	VEGF	VEGF+Ang-1(H)
MVD value	4.1 ± 1.3	6.7 ± 1.9	9.4 ± 2.7	10.3 ± 2.1	14.3 ± 3.2

MVD: micro-vessel density.

Table 4. — *Quantification of follicle apoptosis in each group of transplanted ovarian tissue.*

Group	Blank	Ang-1(L)	Ang-1(H)	VEGF	VEGF+Ang-1(H)
Apoptosis (%)	25.3 ± 2.4	15.1 ± 5.2	16.5 ± 5.7	13.8 ± 1.7	8.9 ± 3.1

Table 5. — *The FSH level in each group of transplanted ovarian tissue*

Group	Blank	Ang-1(L)	Ang-1(H)	VEGF	VEGF+Ang-1(H)
O.D. value	13.93 ± 1.47	18.38 ± 2.29	21.89 ± 2.17	22.33 ± 2.74	28.23 ± 3.41

O.D. = optical density.

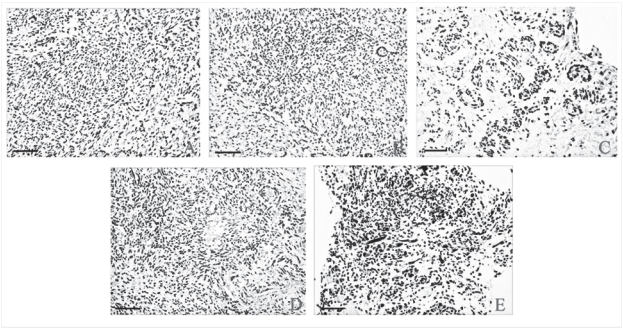

Figure 2. — Level of CD34 in each group of transplanted ovarian tissue. Mice with transplantation received (A) saline buffer, (B) Ang-1 (L), (C) Ang-1(H), (D) VEGF, and (E) VEGF + Ang-1 (H) at the site of transplantation for five consecutive days from the beginning of surgery. After seven days of follow up, the expression of CD34 of transplanted ovarian tissues were stained for evaluating the level of MVD. Scale bar equals 100 μm.

The immunohistochemical S-P staining of CD34 was used as the marker for micro-vessel density evaluation (Figure 2). Level of CD34 was obviously incremented after treatment of VEGF or Ang-1, and VEGF had better effect than Ang-1(L) and Ang-1(H) (Table 2). It is noteworthy that there was a synergistic effect of the combination of VEGF and Ang-1(H) in elevating level of CD34, which showed better efficacy than that of used alone.

The CD34 positive micro-vessels were recorded for evaluating the MVD value. Microvessels with functional morphology were abundant around primordial follicle colonies in ovarian tissue (Figure 2). As shown in previous results, VEGF treatment had more MVD value than Ang-1(L) and Ang-1(H), and a significant synergistic effect of MVD value was observed in the combination of VEGF and Ang-1(H) group (Table 3).

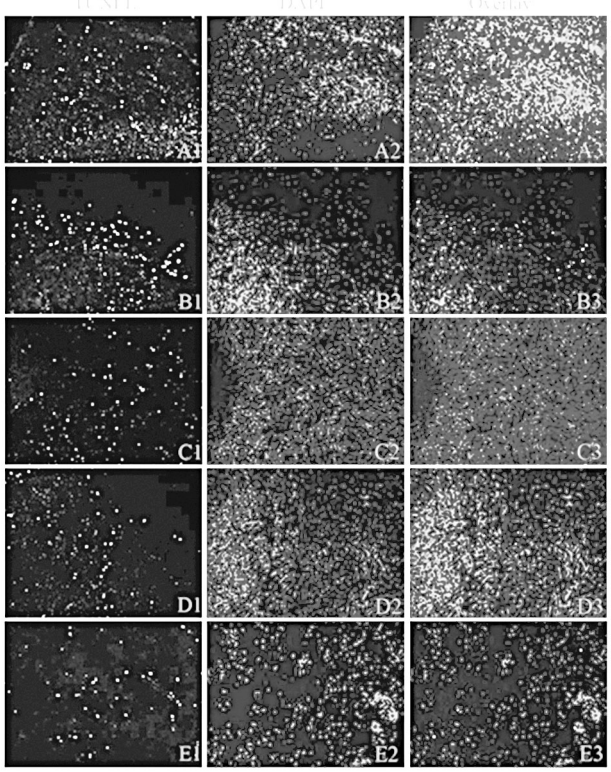

Figure 3. — Follicle apoptosis in each group of transplanted ovarian tissue. Mice with transplantation received (A) saline buffer, (B) Ang-1 (L), (C) Ang-1(H), (D) VEGF, and (E) VEGF + Ang-1 (H) at the site of transplantation for five consecutive days from the beginning of surgery. After seven days of follow up, transplanted ovarian tissues were stained with TUNEL for evaluating the level of apoptosis. Scale bar equals 100 μm

The authors used TUNEL assay for investigating the DNA damage of oocytes under treatment of VEGF or Ang-

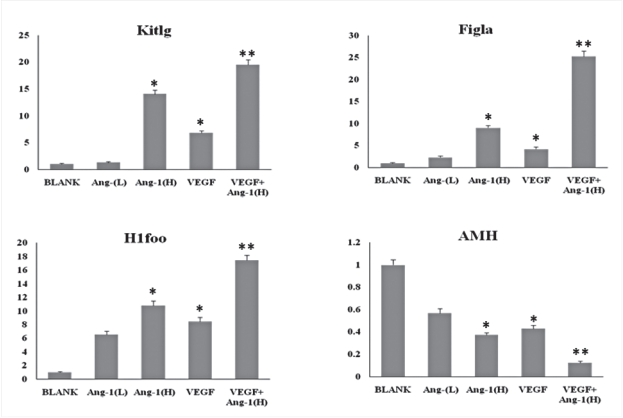

Figure 4. — The gene expression of follicle development related genes. For the Kitlg, Figla, and H1foo genes, the expression was significantly higher after treatment of VEGF, Ang-1 or both, and for AMH gene, expression was obviously lower in groups with the treatment of VEGF, Ang-1 or both. *$p < 0.05$, **$p < 0.01$.

1 in transplanted ovarian tissue. The nuclear of apoptotic oocytes were marked in light green color (Figure 3). All of the treatment groups showed significantly lower apoptosis level than the control group, and VEGF had better effect than Ang-1(L) or Ang-1(H) ($p < 0.05$). Furthermore, the combination of VEGF and Ang-1(H) had the lowest level of apoptosis than any other groups (Table 4).

Four follicle development genes were selected for evaluating the effect of VEGF and Ang-1 treatment in transplanted ovarian tissue. In general, all of the Ang-1 and VEGF treatment groups exhibited higher expression for Kitlg, Figla. and H1foo, and had lower expression for AMH gene in follicles of transplanted ovarian tissue. The combination of VEGF and Ang-1(H) showed significant synergistic effect with the strongest effect in all four genes, which was consistent with previous results. Interestingly, the Ang-1(H) group showed stronger effect than VEGF and Ang-1(L), which was a little different than previous effect pattern (Figure 4).

The protein level of FSH in transplanted ovarian tissue was detected through ELISA assay. Results showed that after VEGF or Ang-1 treatment, the level of FSH was significantly increased in all four groups when compared to the control group. As shown in previous results, combination of VEGF and Ang-1(H) exhibited higher influence in FSH level than that of worked alone in transplanted ovarian tissue ($p < 0.05$) (Table 5).

Discussion

There are several ways for preserving ovarian function, including drug intervention, hormone replacement, oocyte cryopreservation, embryo cryopreservation, and ovarian cryopreservation [6]. GnRH is the most used drug for protecting ovarian function through decreasing the sensitivity to damage of radiotherapy; however, it is difficult to preserve the primordial follicles [7] and fertility [8]. Hormone replacement therapy can be used for substituting the endocrine function of ovarian, but has a limited effect on fertility improvement; moreover, it may increase the risk of relapse and metastasis for hormone dependent tumors [9]. Cryopreservation of oocyte has very little success rate [10], which greatly restricts its use in clinical applications. Embryo cryopreservation is a relatively mature technology [11], but is only suitable for patients with spouse. In addition, cryopreservation of oocytes and embryos require longer ovulation cycle, which may delay the treatment for cancer patients [12]. Moreover, cryopreservation of both oocytes and embryos lacks the potential to maintain the endocrine function of ovarian for patients. Unlike the aforementioned methods, cryopreservation of ovarian tissue encompasses patients of all ages and marriage status and does not need to postpone the treatment period, which is generally appropriate for every female cancer patient seeking fertility recovery. The abundant primordial follicles and immature oocytes in the ovarian cortex have a higher tolerance to freezing and thawing procedures [13]. Furthermore, the autotransplantation of ovarian tissue after treatment does not involve moral issues. Therefore, cryopreservation and transplantation of ovarian tissue becomes one of the most effective ways to preserve ovarian endocrine function and fertility for cancer patients [14].

Cryopreservation and transplantation of ovarian tissue has become the hot spot for the area of reproductive medicine in the world with observable achievements. So far there are more than 90 cases from the transplanted ovarian tissue worldwide [1]. These cases sufficiently demonstrate the feasibility for ovarian cryopreservation and transplantation as a successful way to preserve the fertility for female cancer patients. However, the interruption of ischemic and ischemia reperfusion injuries, causing follicle loss and affecting the recovery of ovarian hormones and fertility after transplantation, still urgently require a solution. Studies have found that different selections of cryoprotectants and freezing methods causes about 7% to 22% of follicle damage rate after cryopreservation, and the ischemia and hypoxia after transplantation results in 60% to 80% of follicle loss rate, which is greatly higher than the damage from freezing [15-17]. Therefore, a feasible method to reconstruct blood supply for reducing the follicle loss is the most critical issue in ovarian cryopreservation and transplantation.

VEGF and Ang cytokines and their receptors, Flt-1, and Tie, have been found to co-regulate the process of vasculogenesis through variant synergistic effects. VEGF is a kind of highly bioactive functional dimeric glycoprotein with strong ability to promote proliferation of vascular endothelial cells. VEGF plays a central role in modulating vascular growth and permeability, which makes it one of the most effective vascular growth factors. It has been

found that exogenous treatment of VEGF significantly increases the survival rate of grafts and promotes angiogenesis in transplanted ovarian tissue of SCID mice [16]. VEGF and its receptor are not only widely expressed in animal ovaries but also highly involved in important biological processes, such as follicle development, selection, maturation, ovulation, and corpus luteum functions [18], which might be helpful for the follicle development in transplanted ovarian tissue. Injection of VEGF to the ovarian tissue of mice increases the vascular network and reduces cell apoptosis [19]. A study investigated the vasculogenesis around the cortex in 48 hours after ovarian tissue transplantation indicates a ten-fold higher expression of VEGF188, which implies that VEGF188 may regulate early vasculogenesis in transplanted ovaries in rabbits [5]. Incubation of thawed human ovarian tissue in medium containing VEGF for two hours significantly reduces the level of cell apoptosis after transplantation into mice [4]. In this study, the authors identified that treatment of VEGF increases the development of functional follicles, raises the MVD value, and reduces follicle apoptosis. Thus, VEGF treatment does have a great potential for promoting hormone growth and fertility recovery in transplanted ovaries.

Ang-1 is a glycoprotein that comprises 498 amino acids with a molecular weight of 70 KD, which is expressed in vascular smooth muscle cells or other vascular peripheral cells [20]. The major biological functions of Ang-1 include: (1) promoting the budding, migration, chemotaxis, and aggregation of endothelial cells to form primordial tubular structures, (2) inhibiting the apoptosis of endothelial cells, (3) maintaining and stabilizing the blood vessels, and (4) anti-inflammation [21]. Ang-1 and its receptor Tie has been reported for their critical function in ischemic diseases. In Ang-1 transgenic rabbits, the new development of collateral vessels in the ischemic lower limb tissues is significantly higher than that of in control groups [22]. Treatment with Ang-1 gene therapy enhances proliferation and reperfusion of arteries in originally ischemic myocardium [18]. Moreover, treatment with 200 ng/mL of Ang-1 significantly increases the survival rate of injured myocardium [5]. Based on the present authors' knowledge, this is the first study that applies Ang-1 to the cryopreservation and transplantation of ovarian tissue. In this study, treatment of Ang-1 showed benefits to the development of functional follicles, the increase of MVD value, and the reduction of follicle apoptosis. Therefore, Ang-1 treatment was able to protect the transplanted ovaries from the damage of ischemia and hypoxia, as the effect of VEGF observed in this study.

A synergistic effect of VEGF and Ang-1 has been found in the process of vasculogenesis. VEGF regulates the conformation of primordial vascular network in the early phase of vasculogenesis, and Ang-1 contributes to the reconstruction and maturation of the vascular network, with spatial structure formation in the following phase of vasculogenesis [23]. When the effect of VEGF exists, treatment of Ang further increases the diameter of capillaries, promotes proliferation and migration of endothelial cells, and stimulates the budding of neovascularization [24]. It has been found in the autotransplantation of adipose tissue that Ang could work with VEGF to accelerate the reconstruction of blood supply of grafts to shorten the phase of ischemia and hypoxia, which reduces necrosis and increases the survival rate of adipose grafts [18]. The present results demonstrated that the combination effect of VEGF and Ang-1(H) worked much better together than by themselves, which provides a good reference for strengthening the functions of transplanted ovarian tissue.

It has been reported that Kitlg, Figla, H1foo, and AMH genes are highly associated with female reproductive function and pathological changes in ovarian tissue. Kitlg gene effectively promotes the functional development of ovarian tissue [25]. Figla and H1foo genes are also highly beneficial in facilitating follicle development in ovarian tissue [26]. Expression of AMH is negatively correlated with the number of follicles, which is a good evaluation for follicle development [27]. In the present study, the authors identified that the treatment of both VEGF and Ang-1 significantly increased the expression of Kitlg, Figla, and H1foo, and decreased the expression level of AMH in follicles of transplanted ovarian tissue. Moreover, the level of FSH was also significantly elevated in groups with VEGF or Ang-1 treatment in transplanted ovarian tissue. These results suggested that both VEGF and Ang-1 were able to protect the endocrine function, increase the survival rate of follicles, and facilitate the fertility recovery in transplanted ovarian tissue.

Conclusion

This study identified that VEGF and Ang-1 promoted the reconstruction of vasculogenesis and shortened the period of blood supply, which reduced the damage of ischemia and hypoxia and preserved more number of functional follicles in transplanted ovarian tissue. The present results provided valuable information for future technical development and clinical application for the fertility recovery of transplanted human ovarian tissue.

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