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Angiopoietin-1 and -2 and vascular endothelial growth factor expression in ovarian grafts after cryopreservation using two methods

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Objective: The favored method of preserving fertility in young female cancer survivors is cryopreservation and autotransplantation of ovarian tissue. Reducing hypoxia until angiogenesis takes place is essential for the survival of transplanted ovarian tissue. The aim of this study was to investigate the role of angiopoietin-1 (Angpt-1), angiopoietin-2 (Angpt-2), and vascular endothelial growth factor (VEGF) in ovarian tissue grafts that were cryopreserved using two methods.

Methods: Ovarian tissues harvested from ICR mice were divided into three groups: group I (control), no cryopreservation; group II, vitrification in EFS (ethylene-glycol, ficoll, and sucrose solution)-40; and group III, slow freezing in dimethyl sulfoxide. We extracted mRNA for VEGF, Angpt-1, and Angpt-2 from ovarian tissue 1 week following cryopreservation and again 2 weeks after autotransplantation. We used reverse transcriptase-polymerase chain reaction to quantify the levels of VEGF, Angpt-1, and Angpt-2 in the tissue.

Results: Angpt-1 and Angpt-2 expression decreased after cryopreservation in groups II and III. After autotransplantation, Angpt-1 and Angpt-2 expression in ovarian tissue showed different trends. Angpt-1 expression in groups II and III was lower than in group I, but Angpt-2 in groups II and III showed no significant difference from group I. The vitrified ovarian tissues had higher expression of VEGF and Angpt-2 than the slow-frozen ovarian tissues, but the difference was not statistically significant.

Conclusion: Our results indicate that Angpt-2 may play an important role in ovarian tissue transplantation after cryopreservation although further studies are needed to understand its exact function.

Keywords: Angiopoietin-1; Angiopoietin-2; Autografts; Cryopreservation; Fertility preservation; Mice; Neovascularization; Ovary; Reverse transcriptase polymerase chain reaction; Vascular endothelial growth factors

Introduction

Remarkable advances in the diagnosis and treatment of cancer in women have greatly increased women's life expectancy. However, some female survivors suffer from fertility loss caused by chemotherapy and/or radiotherapy, thereby reducing their quality of life [1,2]. Methods for preserving pretreatment fertility in female cancer patients include ovarian tissue, oocyte, and embryo freezing and transplantation. Oocytes can be obtained by superovulation, but the number that can be retrieved in a single menstrual cycle is limited, this technique requires time after the cancer diagnosis; and using embryos has the disadvantage that they can only be generated from married women in Korea. Transplantation of cryopreserved ovarian tissue has been considered experimental, but more than 100 children have been born following this process [3,4]. The primary advantage of ovarian tissue freezing is that it can be used by adolescents and does not require a waiting time in patients who need cancer treatment [5]. Therefore, al-

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though ovarian tissue transplantation has drawbacks in clinical practice, it is considered the best method to preserve or restore fertility [6].

The most problematic part of the ovarian tissue freezing and transplantation is tissue necrosis that appears early following transplantation, which is thought to be a consequence of failure of angiogenesis. Thus, promoting angiogenesis may be a solution for minimizing the hypoxic damage to the ovary after transplantation [7,8]. Angiogenesis is the process in which new blood vessels develop from existing blood vessels; and it is essential for many physiological functions, such as embryogenesis and wound healing, as well as for pathological processes, such as the expansion of malignancies [9]. Angiogenic processes are regulated by the interaction between angiogenic growth factor complexes. Among them, vascular endothelial growth factor (VEGF), angiopoietin-1 (Angpt-1), and angiopoietin-2 (Angpt-2) are especially important. Of the various angiogenic factors, VEGF is particularly important because of its role in inducing vascular permeability and neovascularization. Recently, Angpt, which regulates new blood vessel formation and inflammatory processes along with VEGF, has attracted increasing attention [10,11].

Angpt-1 plays a role in new blood vessel maturation and stabilization, the inhibition of endothelial apoptosis, and the reduction of vascular permeability in a stable environment without hypoxia [10,12]. Angpt-2 is an antagonist of Angpt-1, and is known to enhance the plasticity, destabilization, and permeability of blood vessels at vascular remodeling sites. It exerts its function through cell matrix loosening and the destabilization of existing blood vessels, especially in ischemic/hypoxic environments, such as in transplanted or damaged tissues. Angpt-2 expression increases dramatically during vascular remodeling [11,13,14]. Angpt-2, in conjunction with VEGF, induces angiogenesis and the expression of matrix metalloproteases constitute the basement membrane in vessels [15]. Angpt-1 and -2 are known to regulate ovarian angiogenesis in ovarian follicles although the exact mechanism has not been elucidated [16].

We have already examined the damage caused by freezing conditions on angiogenic factors during cryopreservation of ovarian tissue and the expression of angiogenic factors after heterotopic autotransplantation [17,18]. The aim of this study was to investigate the expression of Angpt-1 and -2, which play a major role in reducing hypoxic damage in post-transplant ischemic processes, in mouse graft ovaries cryopreserved using two different freezing methods and following autotransplantation.

Methods

In this study, we conducted the freezing, thawing, and transplantation of ovarian tissue in accordance with the methods described in a prior study [18].

1. Animals and ovarian collection

Female ICR mice were purchased from Koatech (Pyeongtaek, Korea). The mice were treated in accordance with the standard guidelines for Laboratory Animal Care at the Animal Facility of Gyeongsang National University (GLA-090107-M0001).

ICR mice 5 to 6 weeks of age (n = 15) were divided into three groups and underwent bilateral oophorectomies under anesthesia. The harvested ovaries of the two test groups were subjected to cryopreservation, with one ovary of each pair reserved for later autotransplantation. The specimens were grouped as follows: group I (control), no cryopreservation; group II, vitrification in EFS (ethylene-glycol, ficoll, and sucrose solution)-40; and group III, slow freezing in dimethyl sulfoxide. Experimentation was conducted in two phases. First, angiogenic factors were evaluated in test ovaries (cryopreserved for 1 week) and in controls. Second, angiogenic factors were evaluated 2 weeks after autotransplantation of test ovaries. In the control group, non-cryopreserved ovaries were autotransplanted immediately following oophorectomies. After 1 week of cryopreservation, we measured VEGF, Angpt-1, and Angpt-2 levels and autotransplanted the specimens into the abdominal wall. We again measured the levels of these factors 2 weeks post-transplantation. We quantified the levels of these factors using reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 1).

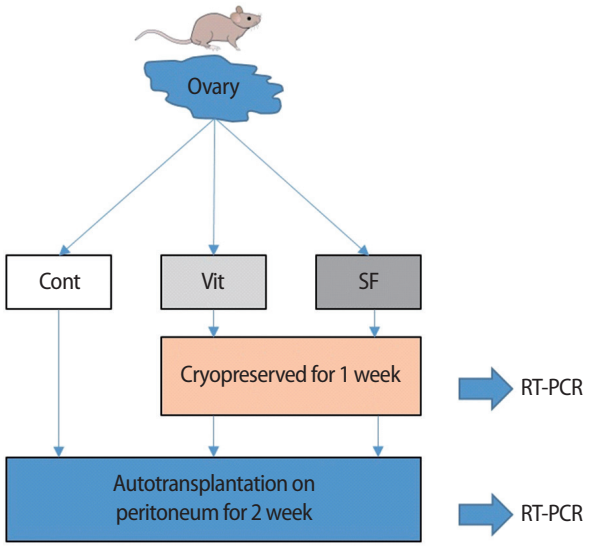

Figure 1. Flow diagram of experimental design. After 1 week of cryopreservation (vitrification [Vit] or slow freezing [SF]), a mouse ovary was analyzed for angiopoietin-1, -2, and VEGF; the other ovary was autotransplanted beneath the abdominal wall. After a 2-week autotransplantation period, angiopoietin-1, -2, and VEGF were assayed using RT-PCR. Cont, control; RT-PCR, reverse transcriptase-polymerase chain reaction; VEGF, vascular endothelial growth factor.

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2. Cryopreservation

1) Vitrification

Paired ovaries were immersed for 10 minutes in an equilibration solution (EG-20), consisting of 20% ethylene glycol (EG; Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's phosphate-buffered saline (DPBS; Gibco, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich). This was followed by a 2-minute immersion in vitrification solution (EFS-40), consisting of 40% EG (v/v), 18% ficoll (ficoll 70; average molecular weight 70,000 Da; Sigma-Aldrich), 0.5 mol/L sucrose, and 20% FBS in DPBS [19,20]. The specimens were then loaded onto an electron microscope grid (IGC400; Pelco International, Clovis, CA, USA) and immediately quick-frozen in liquid nitrogen. After 1 week, a three-step cryoprotectant dilution method was implemented for thawing. One ovary was used for RT-PCR, and the other ovary was reserved for autotransplantation.

2) Slow-freezing

Paired ovaries were transferred to cryovials containing a cryoprotective mixture (Leibovitz L-15 medium, 10% FBS, and 1.5 M dimethyl sulfoxide) for controlled freezing via planar cryochamber. Cooling began at 4°C and proceeded at a rate of 2°C/min (past induction of ice nucleation at −7°C) until reaching −35°C. Freezing was accelerated to 25°C/min, and the cryovials were plunged into liquid nitrogen once a threshold temperature (−140°C) was reached. After 1 week, the specimens were thawed at room temperature.

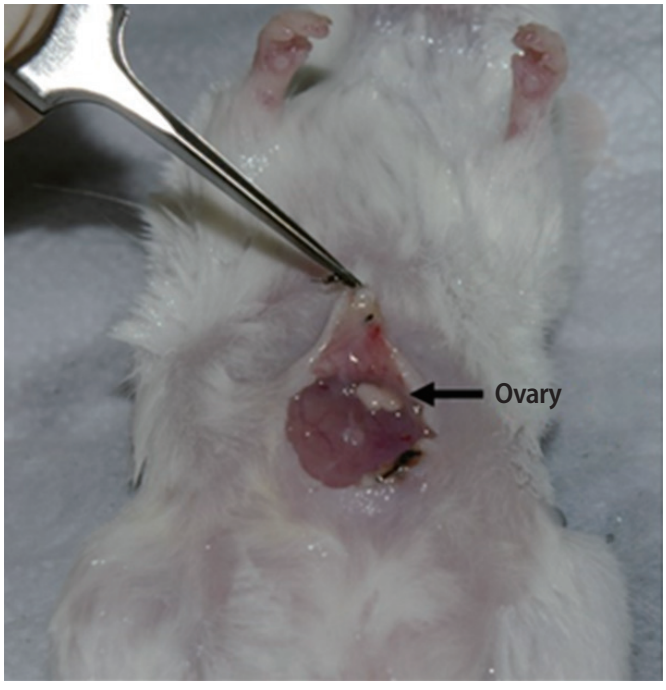

Figure 2. Autotransplanted mouse ovary beneath the abdominal wall (arrow) to be assayed after 2 weeks. The ovary was attached to the peritoneum by fine fibrous tissue.

3. Heterotopic autotransplantation and retrieval of ovarian tissue

One ovary of each harvested pair served as a heterotopic autotransplant, to be performed 1 week after cryopreservation. After inhalation anesthesia was administered, the animal's abdominal wall was carefully opened, creating space for the graft without breaching the peritoneum. The ovaries were affixed to the exposed peritoneum with sutures (Figure 2). Two weeks later, the mice were sacrificed, the ovaries were recovered, and RT-PCR was performed.

4. RNA extraction and RT-PCR

Total RNA was isolated from ovarian tissues with TRizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed using Super Script III RT (Invitrogen), random hexamers (50 pmol), and deoxynucleotide (2.5 mM) at 37°C for 20 minutes. Specific primer sequences for VEGF-A, Angpt-1, and Angpt-2 were obtained from the Gene Bank Database (Table 1). The PCR reactions were performed as follows: specimens were first heated to 94°C for 4 minutes, then subjected to 30 cycles of denaturation at 94°C for 40 seconds, annealed at 55°C for 40 seconds, extended at 72°C for 40 seconds, and then underwent a final elongation step at 72°C for 8 minutes. The PCR products were analyzed by 2% agarose gel electrophoresis. Expression of each mRNA species was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Relative quantitation of the target gene expression was performed using SigmaGel software (Sigma, St. Louis, MO, USA).

5. Statistical analysis

All data were expressed as mean ± standard error. We used PASW ver. 18.0 (SPSS Inc., Chicago, IL, USA). The nonparametric Kruskal-Wallis method was applied for comparisons using the Tukey test for post-hoc analysis. Significance was set at $p < 0.05$.

Table 1. Primer sequences for VEGF-A, Angpt-1, and Angpt-2

Gene symbol	Direction	Sequence (5′ to 3′)	Size (bp)
VEGF-A	Forward	GTGGACATCTTCCAGGAGTA	269
	Reverse	CCCTTTCCTCGAACTGATTT	
Angpt-1	Forward	CAGCACGAAGGATGCTGATA	98
	Reverse	TTAGATTGGAAGGGCCACAG	
Angpt-2	Forward	ACATGAAGAAGGAGATGGTG	149
	Reverse	CGTCTGGTTTAGTACTTGGG	
GAPDH	Forward	CTAAAGGGCATCCTGGGC	201
	Reverse	TTACTCCTTGGAGGCCATG	

VEGF-A, vascular endothelial growth factor-A; Angpt-1, angiopoietin-1; Angpt-2, angiopoietin-2; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

Results

1. Decreased expression of angiogenic factors after cryopreservation

In groups II and III, levels of VEGF, Angpt-1, and Angpt-2 mRNA were significantly lower than in the control group ($p < 0.05$) (Figure 3).

2. VEGF expression level recovery in autotransplanted ovarian tissue

VEGF expression was lower in groups II and III than in the control group. After autologous transplantation, VEGF expression was lower in groups II and III than in the control group, but the difference was not statistically significant. VEGF expression was higher in the vitrification group than in the slow-freezing group, but the difference was not statistically significant.

3. Different expression levels of Angpt-1 and Angpt-2 after autotransplantation

Angpt-1 and Angpt-2 expression was lower in groups II and III than in the control group. After autotransplantation, the expression of Angpt-1 and Angpt-2 in ovarian tissues showed opposite trends. The level of Angpt-1 expression remained significantly lower in groups II and III than in the control group, whereas the level of Angpt-2 expression in groups II and III was not significantly lower than in the control group (Figure 4).

Discussion

In cryopreservation and autotransplantation of ovarian tissue, reducing ischemic damage is essential for post-transplant tissue survival [7,8,21]. Some angiogenic factors are believed to upregulate neo-angiogenesis in transplanted ovarian tissue [22,23]. Angpt-1

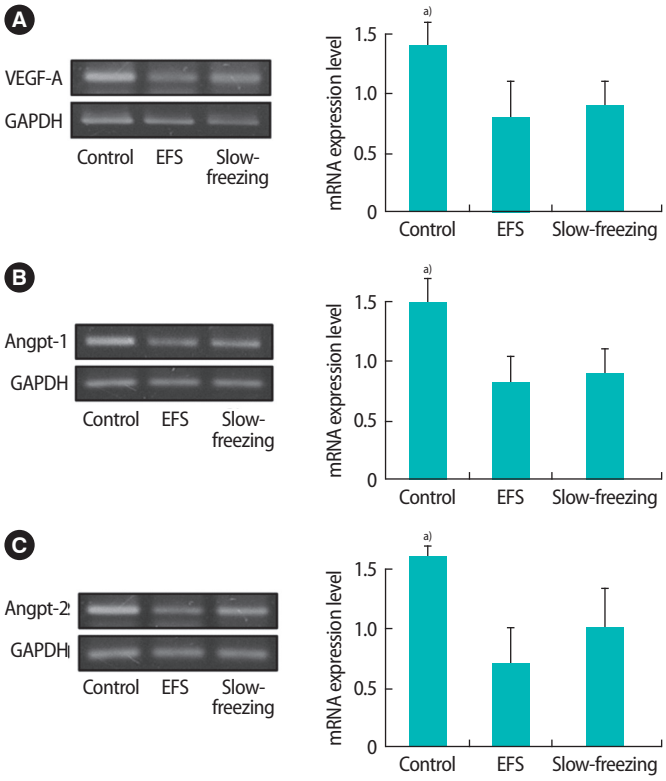

Figure 3. Reverse transcriptase-polymerase chain reaction analysis of vascular endothelial growth factor-A (VEGF-A; A), angiopoietin-1 (Angpt-1; B), and angiopoietin-2 (Angpt-2; C) mRNA levels after 1 week of vitrification or slow-freezing compared to the control group and quantitative analysis of each group. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal standard. Values are presented as mean ± standard error of three independent experiments. EFS, ethylene-glycol, ficoll, and sucrose solution. [a]$p < 0.05$ for the control vs. cryopreservation groups (EFS and slow-freezing).

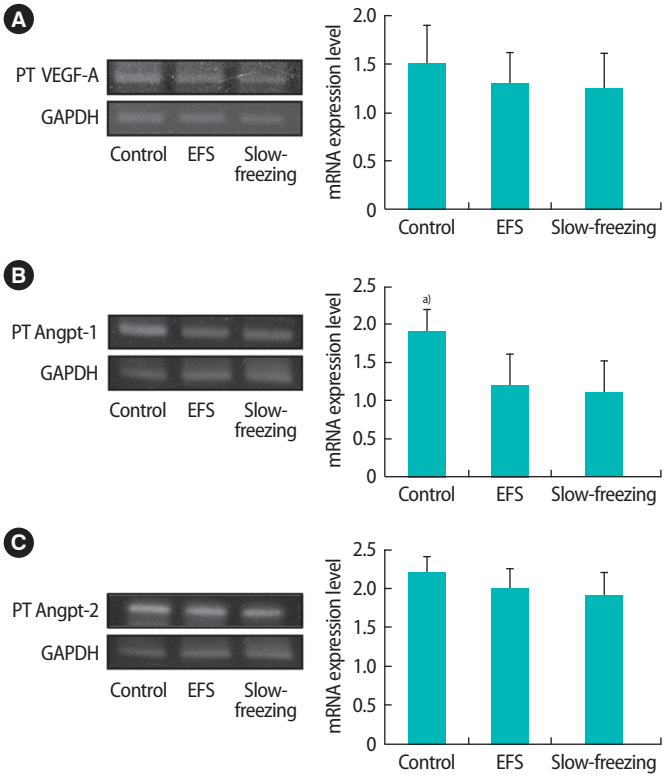

Figure 4. Reverse transcriptase-polymerase chain reaction analysis of post-transplant (PT) vascular endothelial growth factor-A (VEGF-A; A), angiopoietin-1 (Angpt-1; B), and angiopoietin-2 (Angpt-2; C) mRNA levels after 2 weeks of autotransplantation beneath the abdominal wall. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal standard. Values are presented as mean ± standard error of three independent experiments. EFS, ethylene-glycol, ficoll, and sucrose solution. [a]$p < 0.05$ for the control vs. cryopreservation groups (EFS and slow-freezing).

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and Angpt-2 play important roles in angiogenesis, albeit at different developmental stages. Angpt-1 is associated with vascular remodeling in the embryonic state, and Angpt-2 is associated with postnatal remodeling. In adults, Angpt-1 is expressed in a wide range of tissues at low concentrations and contributes to vascular maturation and the stabilization of mature blood vessels. Angpt-2 is expressed at sites where blood vessels are newly formed in normal or cancerous tissues, as well as in blood vessels of the ovaries and in ischemic areas. Angpt-1 is mainly expressed in pericytes and smooth muscle cells, while Angpt-2 is mainly expressed in vascular endothelial cells. In hypoxic conditions, Angpt-2 expression increases to be 15-fold greater than that of Angpt-1 [24,25].

In this study, cryopreservation reduced the expression of VEGF, Angpt-1, and Angpt-2 regardless of the freezing method. These results are consistent with those obtained in previous studies performed in our laboratory [18]. VEGF and Angpt-2 expression significantly increased in post-transplant ovarian tissues, whereas expression of Angpt-1 did not. These results suggest that VEGF and Angpt-2 are closely involved with hypoxia-induced neovascularization. VEGF and Angpt-2 expression, which were decreased by cryopreservation, may be restored after transplantation, promoting angiogenesis and thereby reducing ischemic damage to heterotrophic autotransplanted ovarian tissues. Youm et al. [26] investigated folliculogenesis, angiogenesis, follicle-stimulating hormone levels, and apoptosis in mouse ovarian tissue transplantation following pretreatment with Angpt-2 at different concentrations. They suggested that Angpt-2 pretreatment promoted intact folliculogenesis and angiogenesis. Kong et al. [27] evaluated the effect of Angpt-2 and VEGF on the improvement of xenotransplantation of bovine ovarian tissue, the enhancement of neovascularization, and the reduction of ischemic injury. They suggested that administering Angpt-2 and VEGF to the recipient prior to xenotransplantation resulted in increased angiogenesis and reduced ischemic injury.

Angpt-1 expression does not increase in autografted ovarian tissue after cryopreservation because Angpt-1 does not play a significant role in new blood vessel formation; instead, it contributes to the maturation and stabilization of blood vessels [28]. Hormozi et al. [29] reported that immediately after transplantation, a decrease in Angpt-1 promoted new angiogenesis and contributed to the maturation of new blood vessels. Angpt-1 expression in autografted ovarian tissue was higher in the control group than in the groups containing tissues that were cryopreserved. We believe that Angpt-1 mRNA expression in the control group, which was not impaired during freezing, may have been detected although we do not have a clear explanation for this observation.

The major limitation of our study is that we only used RT-PCR to quantify mRNA levels, instead of measuring protein expression by a Western blot, which would have yielded more accurate and reliable results. A strength of this study is that VEGF, Angpt-1, and Angpt-2 levels were measured in tissues frozen by two different methods and after the transplantation of thawed tissue.

Although the expression of these angiogenic factors was decreased by freezing, expression levels recovered after transplantation. Our results confirmed that VEGF and Angpt-2 function as neovascularizing factors. In particular, Angpt-2 appears to play an important role in the post-transplantation angiogenesis of cryopreserved ovarian tissue. Future studies should investigate whether pretreatment with angiogenic factors reduces post-transplant ischemic injury in ovarian tissue.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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