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Effect of Antifreeze Protein and Necrostatin on Ovarian Cryopreservation and Transplantation

난소동결과 이식에 있어서 항동결단백과 Necrostatin의 효과

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by
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ABSTRACT

INTRODUCTION: Ovarian cryopreservation and transplantation is one of the most promising options of fertility preservation for women have survived malignant disease. However, the survival rate of cryopreserved ovarian tissue after transplantation is still not sufficient for the application of this procedure in routine clinical practice. Possible causes of ovarian damage after cryopreservation and transplantation are cryodamage during cryopreservation and ischemic damage after transplantation. Cryoprotective and anti-necrotic agents are expected to reduce this damage. Antifreeze proteins (AFPs) are a class of polypeptides produced by animals such as antarctic fish; these proteins allow their survival in subzero environments. Several recent studies on the protective effect of AFP for cryopreservation of animal cells and organs have been reported. However there has been no study on AFP for ovarian tissue cryopreservation. Necrostatin-1 (Nec-1) is a receptor-interacting protein 1 kinase (RIP1) inhibitor and known to have inhibitory effect for necroptosis via RIP1. The purpose of this study was to investigate the effects of AFP and Nec-1 supplementation during ovarian tissue vitrification and transplantation.

METHODS: Ovaries from 4-week-old ICR mice were used for vitrification. Ovaries were vitrified using a two-step procedure involving exposure to equilibrium and vitrification solutions. The equilibration solution included 20% ethylene glycol (EG), and the vitrification solution included 40% EG, 18% Ficoll, and 0.3 M sucrose. All solutions were based on Dulbecco's phosphate

buffered saline (DPBS) containing 20% fetal bovine serum (FBS). Intact ovaries were first suspended in 1 mL of equilibration solution for 10 min, and then mixed with 0.5 mL of vitrification solution for 5 min. To investigate the effect of AFP, 0, 5, or 20mg/mL of AFP III was added into the vitrification solution and to investigate the effect of Nec-1, 0, 25, or 100 μM of Nec-1 was added into the vitrification solution. After warming, follicular morphology and apoptosis were assessed by histological analysis and the TUNEL assay. A part of ovaries vitrified in each group were warmed and autotransplanted. In the experiment assessing Nec-1, 0, 25, or 100 μM of Nec-1 was added to each warming solution. In addition, the same concentrations of Nec-1 were injected intraperitoneally to a final volume of 0.3 mL. After 2 weeks, follicular morphology and apoptosis of transplanted ovaries were assessed. Immunostaining with Ki-67 and vascular endothelial growth factor (VEGF) antibodies was performed for analyses of proliferative and angiogenic activity.

RESULTS: Morphological analysis after vitrification and warming showed a significantly higher intact follicle ratio in the AFP treated groups (control, 28.9%; 5 mg/mL AFP treated group, 42.3%; and 20 mg/mL AFP treated group, 44.7%). The rate of apoptotic follicles (TUNEL positive) was significantly lower in the AFP treated groups (control, 26.6%; 5 mg/mL AFP treated group, 18.7%; and 20 mg/mL treated group, 12.6%). After transplantation of the vitrified-warmed ovaries, morphological analysis showed a significantly higher intact follicle ratio in the 20 mg/mL AFP treated group compared with control and 5 mg/mL AFP treated groups. The rate of apoptotic follicles was

similar among the groups. Ki-67 positive follicle ratio increased significantly as the AFP dose increased (control, 3.3%; 5 mg/mL AFP treated group, 31.3%; and 20 mg/mL AFP treated group, 49.0%). Mean immunohistochemical intensity score of VEGF staining was significantly higher in both AFP treated groups in follicles and in the 20 mg/mL AFP treated group in the stromal cells. Morphological analysis after vitrification and warming showed a significantly higher intact follicle ratio in the Nec-1 treated groups (control, 45.1%; 25 μM Nec-1 treated group, 51.7%; and 100 μM Nec-1 treated group, 57.9%). The rate of apoptotic follicles was lower in the Nec-1 treated groups (control, 11.2%; 25 μM Nec-1 treated group, 8.5%; and 100 μM Nec-1, 7.2%). After transplantation of the vitrified-warmed ovaries, morphological analysis showed a significantly higher intact follicle ratio in the Nec-1 treated groups (control, 43.1%; 25 μM Nec-1 treated group, 60.6%; and 100 μM Nec-1 treated group, 70.7%). Nec-1 treated groups showed a lower rate of apoptotic follicles (control, 5.3%; 25 μM Nec-1 treated group, 2.5%; and 100 μM Nec-1 treated group, 2.0%). The Ki-67 positive follicle ratio was not different according to Nec-1 treatment. The mean intensity score of VEGF staining of the stromal cells was significantly higher in the 100 μM Nec-1 treated group.

CONCLUSIONS: The results of the present study suggest that supplementing AFP in the vitrification solution and Nec-1 during vitrification, warming, and transplantation has beneficial effects on the survival of ovarian tissue during cryopreservation and transplantation.

Keywords: fertility preservation, ovarian tissue cryopreservation, vitrification, antifreeze protein, necrostatin
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LIST OF ABBREVIATIONS

AFGP: antifreeze glycoprotein

AFP: Antifreeze protein

DPBS: Dulbecco's phosphate buffered saline

EG: ethylene glycol

FBS: fetal bovine serum

H&E: hematoxylin–eosin

Nec-1: necrostatin-1

RIP1: receptor-interacting protein kinase 1

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

VEGF: vascular endothelial growth factor

INTRODUCTION

Advances in the diagnosis and treatment of cancer have greatly increased life expectancy of premenopausal women. However, aggressive chemotherapy or radiotherapy for cancer treatment adversely affects gonadal function and subsequently leads to a loss of fertility and premature ovarian failure. Therefore, the preservation of ovarian function in female cancer survivors has become an important issue. Several options are available for preserving fertility in these women, such as cryopreservation of embryos, oocytes, or ovarian tissue (1, 2).

Cryopreservation of the embryo and oocytes is a clinically established procedure and has been used since many years for infertility treatment. Until recently, these procedures were considered as the first option for female fertility preservation. However, these procedures are preceded by controlled ovarian stimulation, a process requiring 2–3 weeks. For this, cancer treatment has to be delayed for several weeks.

Ovarian cryopreservation and transplantation is one of the most promising options for fertility preservation of female cancer survivors because it has several advantages compared with other options. First, since ovarian stimulation is not necessary for ovarian cryopreservation, it only minimally interferes with the treatment plan. In addition, a male partner is not required, and thus, this procedure is an option for single women and prepubertal girls.

Several reports have indicated that endocrine function and fertility is restored after transplantation of frozen-thawed ovarian tissue. Moreover, successful pregnancies have also been reported by several authors (3). Currently, more than 20 live births have been reported in humans after transplantation of cryopreserved ovarian tissue (4).

Despite these encouraging results, some concerns still limit the application of this procedure. Damage to ovarian tissue during cryopreservation thawing and transplantation is associated with 2 key factors: (1) cryodamage that occurs during cryopreservation thawing and (2) the ischemic injury that occurs during revascularization of the transplanted tissue from the surrounding vessels. One of the strategies for overcoming these obstacles is supplementing the patient with protective agents such as antioxidants (5-8), anti-apoptotic agents (9, 10), or angiogenic factors (11, 12) during cryopreservation and/or transplantation. However, not all the agents studied have shown beneficial effects on tissue survival, and there have been conflicting results among reports that have studied the same agent.

Antifreeze proteins (AFPs) are a class of polypeptides produced by animals such as antarctic fish; these proteins allow their survival in subzero environments (13). Several protective mechanisms such as a decrease in freezing temperature, inhibition of recrystallization during thawing, and protection of plasma membrane at low temperatures have been proposed (14). Recently, studies on the protective effects of AFP for cryopreservation of

animal cells and organs have been reported (15-18). However, no study has been performed on the use of AFP for ovarian tissue cryopreservation.

Necroptosis is a type of programmed necrosis that depends on the serine/threonine kinase activity of receptor-interacting protein kinase 1(RIP1)(19), and RIP1 represents the molecular target of cytoprotective agents such as necrostatins. Necroptosis participates in the pathogenesis of diseases, including ischemic injury, neurodegeneration, and viral infection (20). Necrostatin-1 (Nec-1) has been reported to efficiently suppress necroptotic cell death triggered by an array of stimuli in various cell types. Nec-1-inhibitable non-apoptotic death is an important contributor to ischemic injury in mouse models of cerebral ischemia and myocardial infarction (21, 22). Therefore, Nec-1 supplementation may help to reduce ischemic necrosis after ovarian transplantation.

The aims of this study were to investigate the effect of AFP and Nec-1 supplementation during ovarian tissue vitrification and transplantation procedures, and to development an improved technique of ovarian cryopreservation and transplantation for fertility preservation.

MATERIALS AND METHODS

Vitrification and warming of ovary

The experimental procedures performed were similar to those mentioned in a previous study (10) and are presented in Fig 1. The animals in this study were cared for and used in accordance with the institutional guidelines established by the Animal Care and Use Committee (IACUC) of Seoul National University of Bundang Hospital. ICR mice (Orient Co., Seoul, Korea) were maintained under 12-h light:12-h dark cycle at 23°C and fed *ad libitum*. After 1 week of adaptation, 4-week-old mice were killed by cervical dislocation, and both the ovaries were resected. Ovaries were vitrified using a two-step method involving exposure to equilibrium and vitrification solutions (23). Equilibration solution had 20% ethylene glycol (EG; Sigma Chemical Co., St. Louis, MO); vitrification solution was composed of 40% EG, 18% Ficoll (Sigma), and 0.3 M sucrose (Sigma). All solutions were based on Dulbecco's phosphate buffered saline (DPBS; Gibco BRL, Grand Island, NY) containing 20% fetal bovine serum (FBS; Gibco).

Intact ovaries were first suspended in 1 mL of equilibration solution for 10 min and then mixed with 0.5 mL of vitrification solution for 5 min. To investigate the effect of AFP, 0, 5, or 20 mg/mL of AFP type III (A/F Protein Inc, Waltham, MA, USA) was added to the vitrification solution, and to investigate the effect of Nec-1, 0, 25 or 100 μM of Nec-1 (Sigma) was added to the vitrification solution. After exposure to equilibrium and vitrification

solutions, ovaries were immediately transferred to 1.2-mL cryotubes (Nunc; Fisher Bioblock Scientific, Illkirch, France) and directly plunged into liquid nitrogen. After 2 weeks, the tissues were warmed by immersing the vials rapidly in a water bath at 37℃ and suspended serially in 0.5 M sucrose in DPBS containing 20% FBS for 5 min, 0.25 M for 5 min, and 0 M for 10 min.

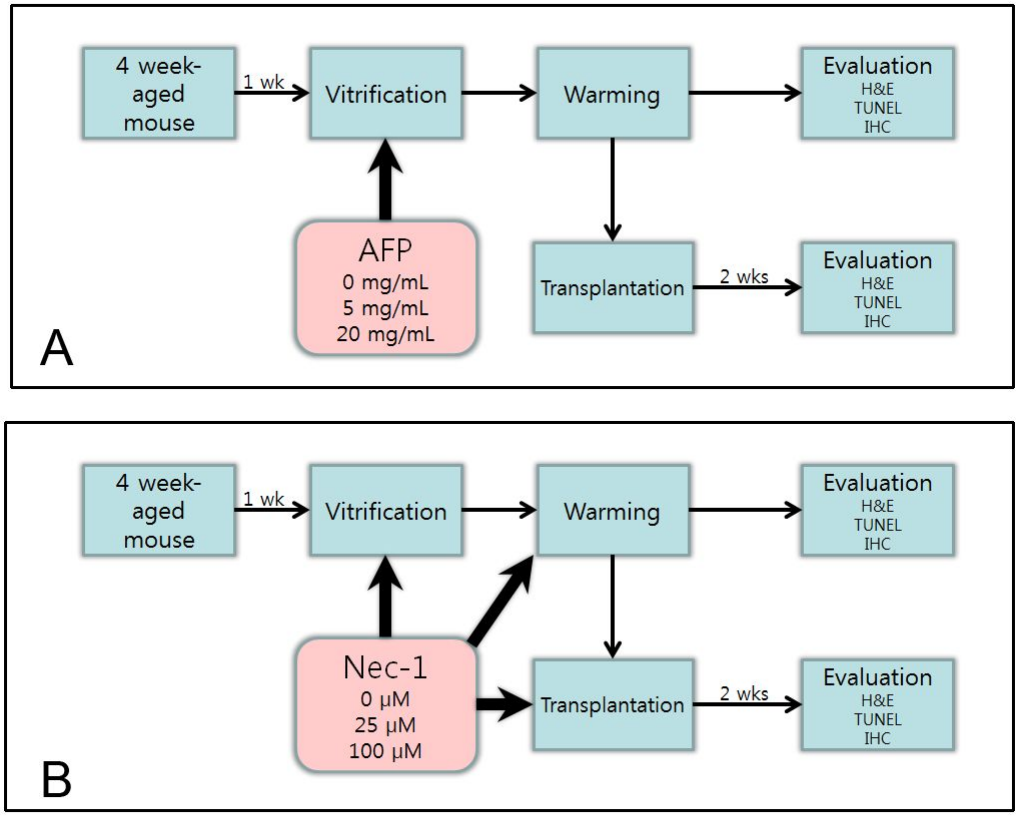

Fig 1. Schematic representation of the experimental designs. (A) Experiment for antifreeze protein. (B) Experiment for necrostatin-1. AFP: antifreeze protein, Nec-1: necrostatin-1, IHC: immunohistochemistry

Morphologic assessment of follicles

All the ovarian samples were fixed in 10% buffered formalin and then embedded in paraffin block. For routine histologic examination, paraffin embedded ovarian sections were cut (thickness, 5 μm). After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin (H&E). The numbers of each type of follicle were counted in the entire cut ovarian surface (400×). All follicles found in 1 section of each ovary were scored. Each type of follicle was categorized according to the following classification (24):

(1) Primordial: single layer of flattened pre-granulosa cells.

(2) Primary: single layer of granulosa cells; one or more being cuboidal cells.

(3) Secondary: two or more layers of cuboidal granulosa cells; antrum absent.

(4) Antral: multiple layers of cuboidal granulosa cells; antrum present.

The integrity of each follicle was evaluated using the following criteria (25):

(1) Primordial/primary follicle.

G1 – spherical with even distribution of granulosa cells.

G2 – granulosa cells pulled away from the edge of the follicle but oocyte still spherical.

G3 – pyknotic nuclei, misshapen oocyte or vacuolation.

(2) Secondary/antral follicle.

G1 – intact spherical follicle with evenly distributed granulosa and theca cells, small space, spherical oocyte.

G2 – intact theca cell, disruption of granulosa cells, spherical oocyte.

G3 – disruption and loss of granulosa and theca cells, pyknotic nuclei, missing oocyte.

Detection of apoptotic follicles by TUNEL assay

Paraffin-embedded ovarian sections were cut (thickness, 5 μm) and assessed for apoptosis using a commercial TUNEL assay kit (In Situ Cell Death Detection kit, Fluorescein, Roche Applied Science, Germany). After deparaffinization and rehydration, sections were rinsed in PBS (pH 7.2) and digested using proteinase K (20 μg/mL, 37°C, 30 min) in 10 mM Tris–HCl buffer. After rinsing in DPBS, the entire specimens were incubated with 50 μL of TUNEL reaction mixture at 37°C for 60 min in a humidified chamber in the dark, followed by rinsing with DPBS. Positive control slides were prepared by treating 1500 U/mL DNase I (Roche Applied Science) in 50 mM Tris–HCl (pH 7.5, including 1 mg/mL bovine serum albumin) for 10 min at room temperature to induce DNA strand breaks, prior to labeling procedures. Some ovarian tissue specimens were used as negative controls by substituting TdT with distilled water in the reaction mixture following the protocol.

The slides were then covered with DAPI (Vector Laboratories, Burlingame, CA) to counterstain DNA. TUNEL-stained and DAPI counterstained slides were examined under an inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Green fluorescence was visualized in TUNEL-

positive cells at an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm. DAPI reached excitation at approximately 360 nm and emitted at approximately 460 nm when bound to DNA, producing blue fluorescence in all nuclei. Only follicles with a visible nucleus were counted regardless of their types. Follicles were considered apoptotic if >30% of the follicular cells were TUNEL stained.

Ovarian autotransplantation

ICR mice were housed in air-filtered positive-pressure isolators with a 12 h light:12 h dark cycle at 23°C and fed *ad libitum*. After 1 week of adaptation, 4-week-aged mice were intraperitoneally anesthetized with ketamine (0.15 mg/g body weight) and xylazine (0.016 μg/g body weight). Both ovaries were removed via a dorsomedian incision. All surgical procedures were performed under aseptic conditions in a laminar flow hood. Excised ovaries were vitrified as described above. During vitrification, 0, 5, or 20 mg/mL of AFP type III was added to the vitrification solution for investigating AFP effect and 0, 25 or 100 μM of Nec-1 was added to the vitrification solution for investigating the effect of Nec-1. After 2 weeks, cryopreserved ovaries were warmed and immediately autografted into the dorsal subcutaneous space of the flank using the same anesthetic protocol. In the experiment assessing Nec-1, the same dose of Nec-1 that was added to the vitrification solution (0, 25 or 100 μM, respectively) was added to each warming solution. In addition, 30 min before transplantation, the same concentrations of Nec-1 were also

injected intraperitoneally to a final volume of 0.3 mL. Two weeks later, the transplanted ovarian tissues were retrieved, and their gross morphology was examined. Using only normal-appearing intact ovaries, the follicular normality and apoptosis were assessed as described above, and immunohistochemical analysis was performed.

Immunohistochemistry

Grafts were fixed in 4% paraformaldehyde and were embedded in paraffin. Subsequently, 4-μm serial cross-sections were mounted on glass slides. Sections were deparaffinized using absolute xylene (Yvsolab SA, Beerse, Belgium) and rehydrated in 2-propanol (Merck). A demasking step was performed by heating for 20 min with citrate buffer (DAKO, S2031) or Tri/EDTA (DAKO, S2367) in a microwave and then allowing it to cool at room temperature for 30 min. Endogenous peroxidase activity was blocked using the peroxidase-blocking solution (DAKO, S3006), before incubation with each antibody (anti-Ki-67 rabbit polyclonal antibody, Novus Biologicals, CO, USA; anti-VEGF mouse monoclonal antibody, Santa Cruz, CA, USA) for 30 min at room temperature. The slides were subsequently incubated with EnVision/HRP (DAKO, K4003) for 30 min at room temperature. Substrate-chromogen solution (DAKO, K3468) was used as a chromogen. The specimens were then counterstained with hematoxylin (DAKO, S3309) and mounted with a Mounting medium (DAKO, CS703).

The proliferation index was evaluated blindly as the percentage of follicles with Ki-67 positive granulosa cells for each class of follicle (Fig 2, 3).

Follicles with at least one stained granulosa cell were considered Ki-67 positive (26). The immunohistochemical staining of VEGF antibody was estimated blindly in each class of follicle and stromal compartment of the ovary (Fig 4, 5). Signal intensity was scored semiquantitatively on a 4-point scale from 0 to 3, where 0 = no staining, 1 = weak, 2 = moderate, and 3 = strong. The staining intensity was considered strong when the staining was similar to that of the positive control. Then, each score was multiplied by a distribution score of 1 to 4, where 1 = <5%, 2 = 5–25%, 3 = 25–50%, and 4 = >50% of the area stained. Thus, the final immunohistochemical intensity score was assigned a score from 0 to 12. At least 4 randomly chosen areas were evaluated for stromal compartment in each ovary.

Statistical analysis

The proportions of follicle stages and normality were calculated in each group. Statistical analysis was performed using analysis of variance (ANOVA), Student's *t*-test or Kruskal Wallis test for continuous variables, whereas chi-square or Fisher's exact tests were used for categorical variables, as appropriate. Results were considered statistically significant for p-values < 0.05. The statistical software package SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

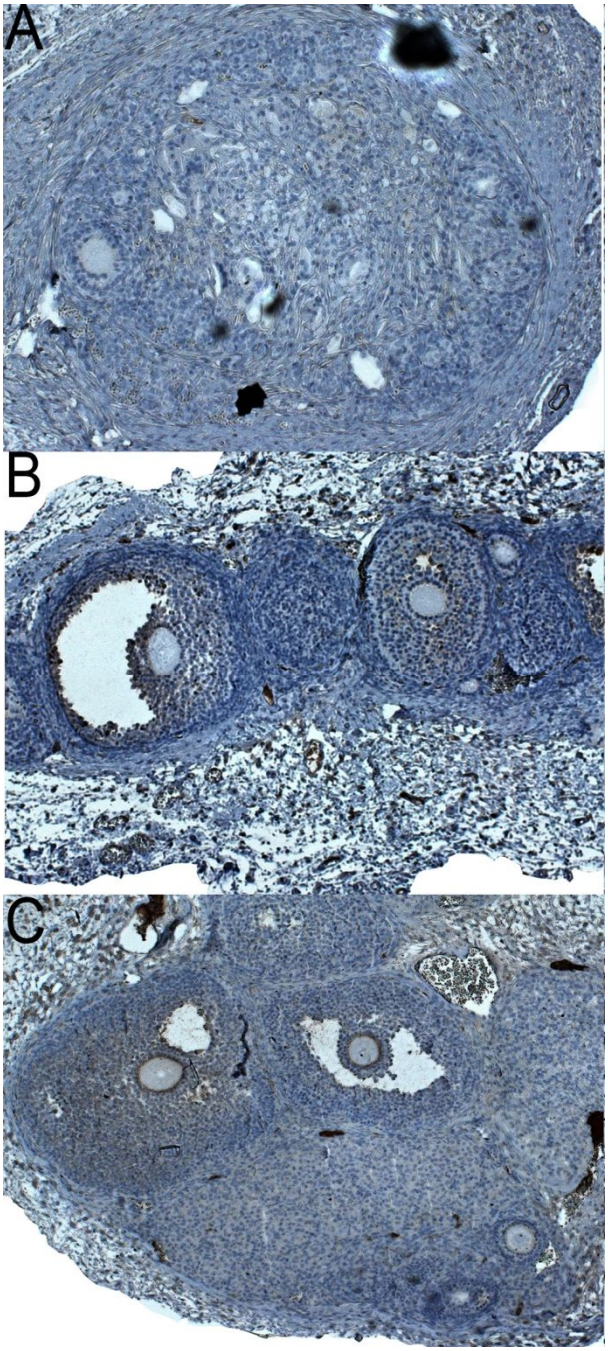

Fig 2. Images showing Ki-67 immunostaining of mouse ovarian follicles according to antifreeze protein (AFP) supplementation (x100). (A) Control. (B) AFP 5mg/mL supplementation during vitrification and warming. (C) AFP 20mg/mL supplementation during vitrification and warming.

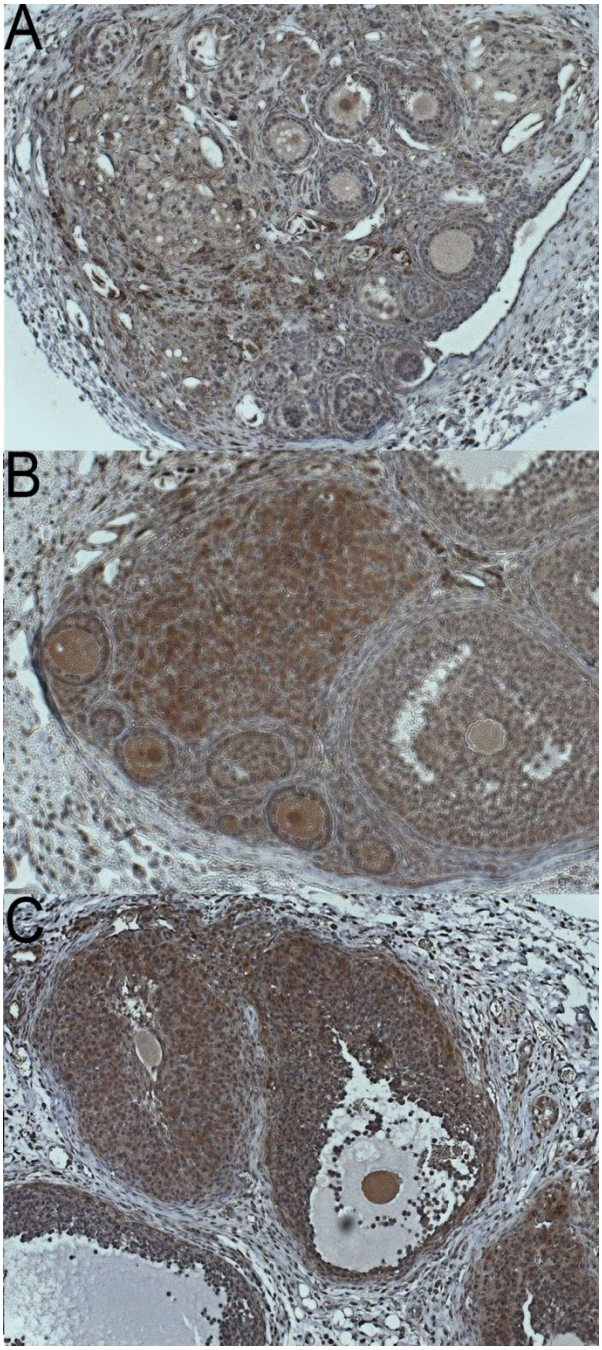

Fig 3. Images showing Ki-67 immunostaining of mouse ovarian follicles according to necrostatin-1 (Nec-1) supplementation (x100). (A) Control. (B) Nec-1 25μM supplementation during vitrification and warming. (C) Nec-1 100μM supplementation during vitrification and warming.

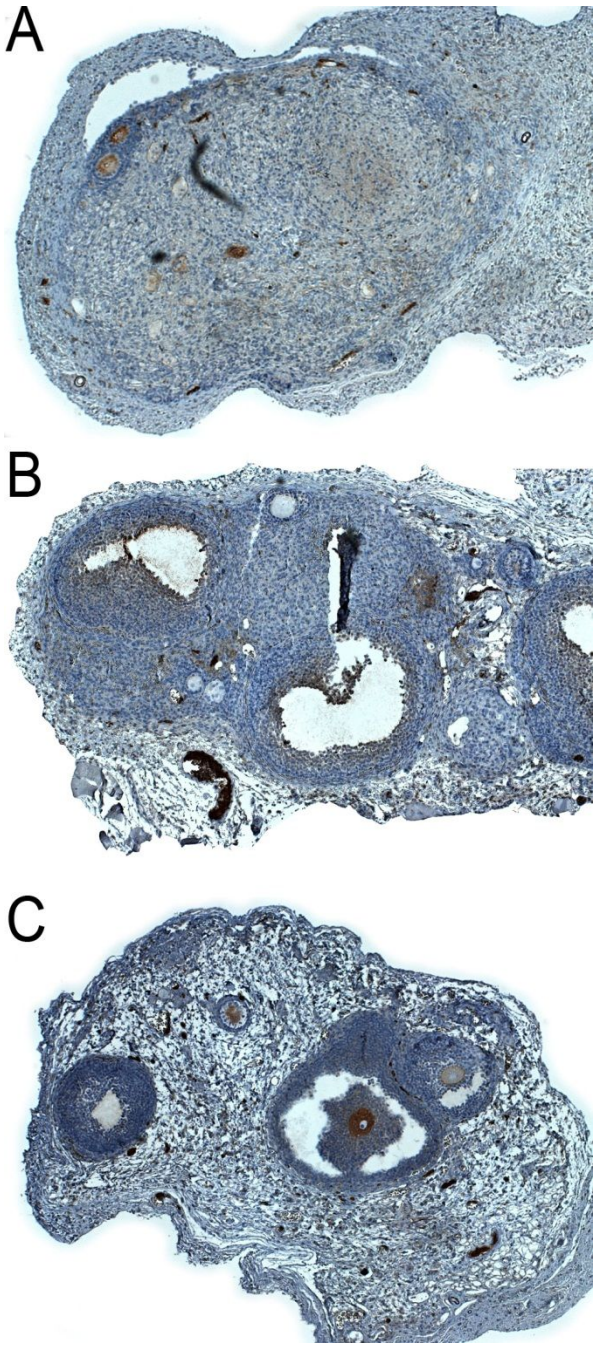

Fig 4. Images showing vascular endothelial growth factor (VEGF) immunostaining of mouse ovary according to antifreeze protein (AFP) supplementation (x100). (A) Control. (B) AFP 5mg/mL supplementation during vitrification and warming. (C) AFP 20mg/mL supplementation during vitrification and warming.

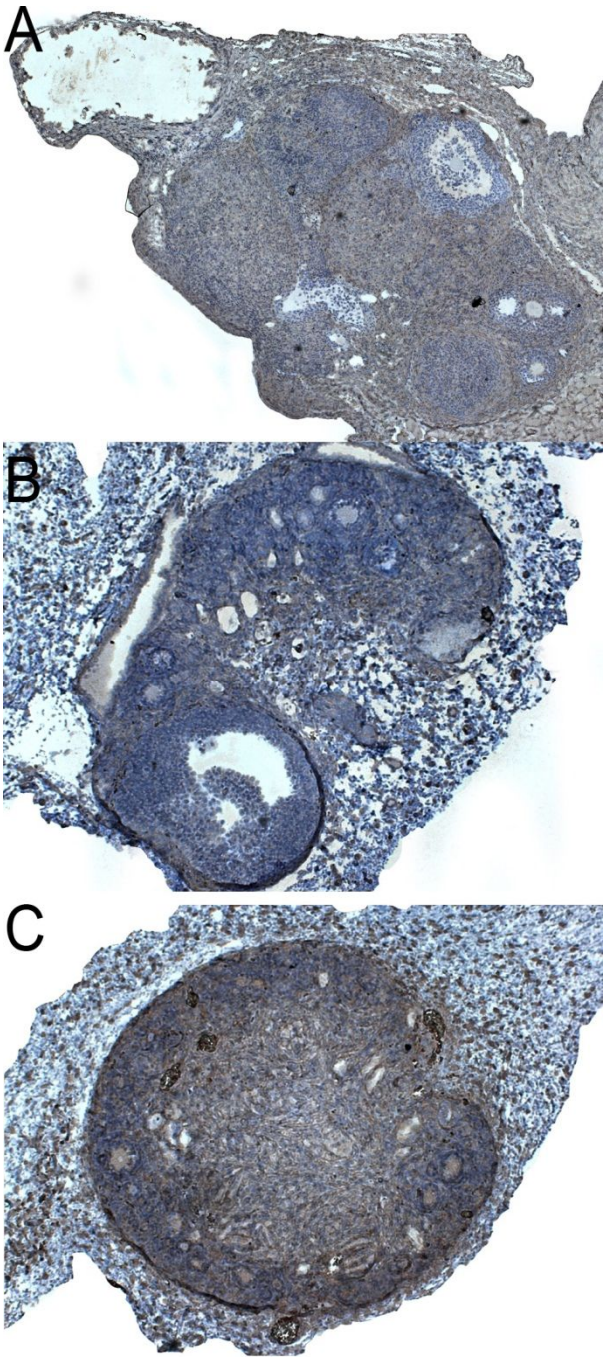

Fig 5. Images showing vascular endothelial growth factor (VEGF) immunostaining of mouse ovarian follicles according to necrostatin-1 (Nec-1) supplementation (x100). (A) Control. (B) Nec-1 25μM supplementation during vitrification and warming. (C) Nec-1 100μM supplementation during vitrification and warming.

RESULTS

Morphological analysis after vitrification and warming showed a significantly higher intact follicle (G1) ratio in the AFP treated groups (controls, 28.9%; 5 mg/mL AFP treated group, 42.3%; and 20 mg/mL AFP treated group, 44.7%) (Table 1). When follicle types were considered separately, AFP also showed protective effects. In all follicle types, the AFP treated groups showed a high proportion of intact (G1) follicles, although the values did not reach statistical significance. The rate of apoptotic follicles (TUNEL positive) was significantly lower in the AFP treated groups (control, 26.6%, 5 mg/mL AFP treated group, 18.7%; and AFP 20 mg/mL treated group, 12.6%), and the rate was decreased more with higher dose of AFP supplementation (Fig 6).

After transplantation of the vitrified-warmed ovaries, the percentage of grossly normal ovary seemed to increase as the AFP dose increased, but the difference was not statistically significant (control, 70.3%; 5 mg/mL AFP treated group, 78.9%; and 20 mg/mL AFP treated group, 88.0%). Morphological analysis of grossly normal ovaries showed a significantly higher intact follicle (G1) ratio in the 20 mg/mL AFP treated group compared with the control and the 5 mg/mL AFP treated groups (Table 2). The rate of apoptotic follicles was similar among the groups (control, 8.3%; 5 mg/mL AFP treated group, 7.8%; and 20 mg/mL AFP treated group, 7.5%) (Fig 7).

The proportion of Ki-67 positive follicles significantly increased as the AFP dose increased (control, 3.3%; 5 mg/mL AFP treated group, 31.3%; and 20 mg/mL AFP treated group, 49.0%). Except for primordial follicles, the 20 mg/mL AFP treated group showed a significantly higher Ki-67 positive follicle ratio for all follicle types compared to the control group (Table 3). The mean immunohistochemical intensity score of VEGF staining of total follicles was significantly higher in the AFP treated groups than the control group. For stromal cells, only 20 mg/mL AFP treated group showed significantly higher mean immunohistochemical intensity score of VEGF staining (Table 4).

In the experiment assessing Nec-1, morphological analysis after vitrification and warming showed a significantly higher intact follicle ratio in the Nec-1 treated groups as the dose increased (control, 45.0%; 25μM Nec-1 treated group, 51.7%; and 100μM Nec-1 treated group, 58.4%). The rate of apoptotic follicles appeared to decrease in the Nec-1 treated groups (control, 11.2%; 25μM Nec-1 treated group, 8.5%; and 100 μM Nec-1 treated group, 7.2%), although statistical significance was not reached (Table 5, Fig 8).

After transplantation of the vitrified-warmed ovaries, morphological analysis showed a significantly higher intact follicle ratio in the Nec-1 treated groups (control, 43.1%; 25μM Nec-1 treated group, 60.6%; and 100μM Nec-1 treated group, 70.7%) as the Nec-1 dose increased. When follicle types were considered separately, in all follicle types, except the antral follicles, the Nec-1 treated groups showed a significantly higher proportion of intact (G1)

follicles. The Nec-1 treated groups showed a lower apoptotic follicle rate (control, 5.3%; 25μM Nec-1 treated group, 2.5%; and 100μM Nec-1 treated group, 2.0%), but these differences did not reach statistical significance (Table 6, Fig 9).

The proportion of Ki-67 positive follicles was not different between the control and Nec-1 treated groups (Table 7). The mean immunohistochemical intensity score of VEGF staining of total follicles was significantly higher in the control group compared to the Nec-1 treated groups. However, the 100μM Nec-1 treated group showed a significantly higher mean immunohistochemical intensity score of VEGF staining in stromal cells (Table 8).

Table 1. Proportions of follicles with good morphology (grade I) and apoptotic follicles according to supplementation of antifreeze protein III in vitrification solution after warming.

	Control	AFP 5mg/mL	AFP 20mg/mL	*p*
Number of ovaries	12	14	12	
Grade I follicles				
Primordial	18/49 (36.7%)[a]	39/90 (43.3%)[a]	31/53 (58.5%)[b]	0.071
Primary	18/58 (31/0%)[a]	26/42 (61.9%)[b]	32/75 (42.7%)[a]	0.009
Secondary	32/119 (26.9%)[a]	43/112 (38.4%)[a]	38/109 (34.9%)[a]	0.163
Antral	12/51 (23.5%)[a]	16/49 (32.7%)[a]	25/45 (55.6%)[b]	0.004
Total	80/277 (28.9%)[a]	124/293 (42.3%)[b]	126/282 (44.7%)[b]	< 0.001
Apoptotic follicles	89/334 (26.6%)[a]	64/342 (18.7%)[b]	41/326 (12.6%)[c]	< 0.001

AFP: antifreeze protein

a, b, c: different characters indicate significant difference by post-hoc analysis.

Table 2. Proportions of follicles with good morphology (grade I) and apoptotic follicles according to supplementation of antifreeze protein III in vitrification solution after autotransplantation of vitrified-warmed ovary.

	Control	AFP 5mg/mL	AFP 20mg/mL	*p*
Number of ovaries	37	38	25	
Grossly Intact ovaries	26/37 (70.3%)[a]	30/38 (78.9%)[a]	22/25 (88.0%)[a]	0.251
Grade I follicles				
Primordial	37/94 (39.4%)[a]	55/99 (55.6%)[b]	69/93 (74.2%)[c]	< 0.001
Primary	30/64 (46.9%)[a]	31/78 (39.7%)[a]	22/38 (57.9%)[a]	0.182
Secondary	33/52 (63.5%)[a]	31/49 (63.3%)[a]	50/63 (79.4%)[a]	0.096
Antral	3/6 (50.0%)[a]	8/8 (100.0%)[a,b]	17/18 (94.4%)[b]	0.008
Total	103/216 (47.7%)[a]	125/234 (53.4%)[a]	158/212 (74.5%)[b]	< 0.001
Apoptotic follicles	11/132 (8.3%)[a]	9/115 (7.8%)[a]	8/107 (7.5%)[a]	0.970

AFP: antifreeze protein

a, b, c: different characters indicate significant difference by post-hoc analysis.

Table 3. Proportions of Ki-67 positive follicles according to supplementation of antifreeze protein III in vitrification solution after autotransplantation of vitrified-warmed ovary.

	Control	AFP 5mg/mL	AFP 20mg/mL	*P*
Number of ovaries	8	18	18	
Ki67 positive follicles				
Primordial	0/26 (0%)[a]	0/18 (0%)[a]	4/50 (8.0%)[a]	0.147
Primary	0/20 0(%)[a]	4/42 (9.5%)[a]	14/40 (35.0%)[b]	0.001
Secondary	2/12 (16.7%)[a]	12/54 (22.2%)[a]	36/58 (62.1%)[b]	< 0.001
Antral	0/2 (0%)[a]	36/52 (69.2%)[b]	46/56 (82.1%)[b]	0.015
Total	2/60 (3.3%)[a]	52/166 (31.3%)[b]	100/204 (49.0%)[c]	< 0.001

AFP: antifreeze protein

a, b, c: different characters indicate significant difference by post-hoc analysis.

Table 4. Mean intensity score of vascular endothelial growth factor (VEGF) expression in follicles and stromal cells according to supplementation of antifreeze protein III in vitrification solution after autotransplantation of vitrified-warmed ovary.

	Control	AFP 5mg/mL	AFP 20mg/mL	*p*
Number of ovaries	8	18	18	
VEGF intensity score				
Primordial	0.08±0.06[a]	0.73±0.16[b]	0.40±0.09[b]	0.001
Primary	0.14±0.14[a]	0.65±0.1[a]	0.83±0.14[a]	0.104
Secondary	0.67±0.33[a]	1.69±0.30[a]	2.14±0.26[a]	0.231
Antral		2.83±0.47	3.28±0.40	0.471
Total follicles	0.15±0.06[a]	1.44±0.17[b]	1.58±0.15[b]	<0.001
Stromal cells	2.30±0.44[a]	2.21±0.22[a]	3.97±0.29[b]	< 0.001

AFP: antifreeze protein

All data are mean±S.E.

a, b: different characters indicate significant difference by post-hoc analysis.

Table 5. Proportions of follicles with good morphology (grade I) and apoptotic follicles according to supplementation of necrostatin-1 in vitrification solution after warming.

	Control[a]	Nec-1 25μM [b]	Nec-1 100μM [c]	*p*
Number of ovaries	35	30	29	
Grade I follicles				
Primordial	77/124 (62.1%)[a]	59/97 (60.8%)[a]	114/166 (68.7%)[a]	0.341
Primary	108/222 (48.6%)[a]	115/205 (56.1%)[a,b]	107/164 (65.2%)[b]	0.005
Secondary	138/388 (35.6%)[a]	157/354 (44.4%)[b]	128/278 (46.0%)[b]	0.010
Antral	60/117 (51.3%)[a]	69/117 (59.0%)[a]	62/96 (64.6%)[a]	0.141
Total	383/851 (45.0%)[a]	400/773 (51.7%)[b]	411/704 (58.4%)[c]	< 0.001
Apoptotic follicles	25/223 (11.2%)[a]	27/317 (8.5%)[a]	22/304 (7.2%)[a]	0.275

Nec-1: necrostatin-1

a, b, c: different characters indicate significant difference by post-hoc analysis.

Table 6. Proportions of follicles with good morphology (grade I) and apoptotic follicles according to supplementation of necrostatin-1 in vitrification and warming solution and injection to recipient after autotransplantation of vitrified-warmed ovary.

	Control	Nec-1 25μM	Nec-1 100μM	*p*
Number of ovaries	46	50	54	
Grade I follicles				
Primordial	55/119 (46.2%)[a]	107/163 (65.6%)[b]	103/140 (73.6%)[b]	< 0.001
Primary	34/87 (39.1%)[a]	64/117 (54.7%)[b]	74/106 (69.8%)[c]	< 0.001
Secondary	40/99 (40.4%)[a]	76/130 (58.5%)[b]	69/107 (64.5%)[b]	0.001
Antral	11/20 (55.0%)[a]	4/4 (100.0%)[a]	17/19 (89.5%)[b]	0.022
Total	140/325 (43.1%)[a]	251/414 (60.6%)[b]	263/372 (70.7%)[c]	< 0.001
Apoptotic follicles	5/95 (5.3%)[a]	3/121 (2.5%)[a]	2/101 (2.0%)[a]	0.374

Nec-1: necrostatin-1

a, b, c: different characters indicate significant difference by post-hoc analysis.

Table 7. Proportions of Ki-67 positive follicles according to supplementation of necrostatin-1 in vitrification solution after autotransplantation of vitrified-warmed ovary.

	Control	Nec-1 25μM	Nec-1 100μM	*p*
Number of ovaries	10	10	11	
Ki67 positive follicles				
Primordial	5/22 (22.7%)[a]	22/76 (28.9%)[a]	33/67 (49.3%)[b]	0.015
Primary	12/19 (63.2%)[a,b]	11/20 (55.0%)[a]	15/17 (88.2%)[b]	0.084
Secondary	16/16 (100%)[a]	16/22 (72.7%)[b]	8/11 (72.7%)[a,b]	0.069
Antral	4/4 (100%)[a]	11/11 (100%)[a]	4/6 (66.7%)[a]	0.063
Total	37/61 (60.7%)[a]	60/129 (46.5%)[a]	60/101 (59.4%)[a]	0.075

Nec-1: necrostatin-1

a, b, c: different characters indicate significant difference by post-hoc analysis.

Table 8. Mean intensity score of vascular endothelial growth factor (VEGF) expression in follicles and stromal cells according to supplementation of necrostatin-1 in vitrification solution after autotransplantation of vitrified-warmed ovary.

	Control	Nec-1 25μM	Nec-1 100μM	*p*
Number of ovaries	10	10	11	
VEGF intensity score				
Primordial	5.00±0.84[a]	1.46±0.22[b]	1.38±0.26[b]	0.012
Primary	3.47±0.79[a]	1.56±0.29[a]	2.11±0.30[a]	0.480
Secondary	6.16±0.77[a]	1.55±0.28[b]	3.89±0.70[a,b]	0.001
Antral	1.67±0.67[a]	1.67±0.21[a]	9.00±0.00[a]	0.081
Total follicles	4.75±0.46[a]	1.54±0.13[b]	2.21±0.36[b]	<0.001
Stromal cells	5.95±0.39[a]	4.94±0.38[a]	10.61±0.33[b]	<0.001

Nec-1: necrostatin-1

All data are mean±S.E.

a, b: different characters indicate significant difference by post-hoc analysis.

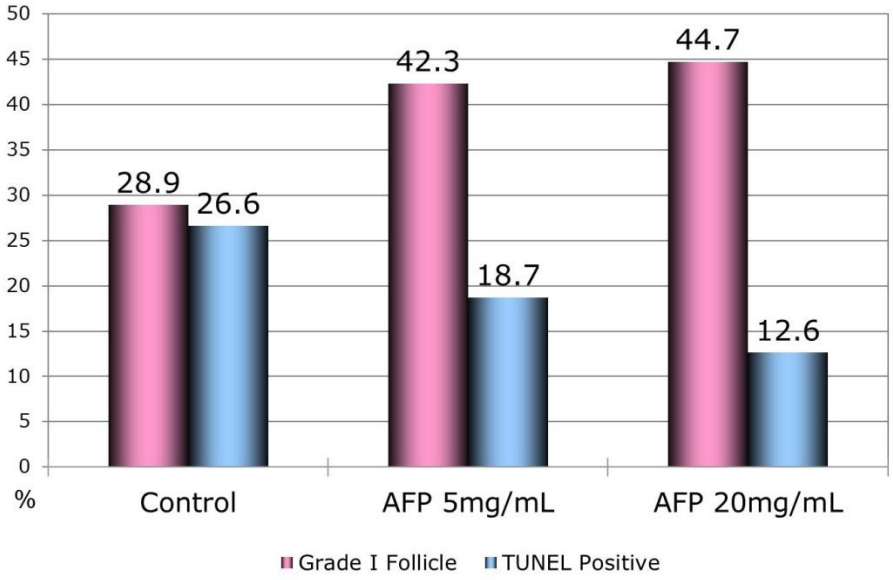

Figure 6. Morphologically intact (grade I) follicle and apoptotic follicle ratios according to supplementation of antifreeze protein (AFP) in vitrification solution after warming.

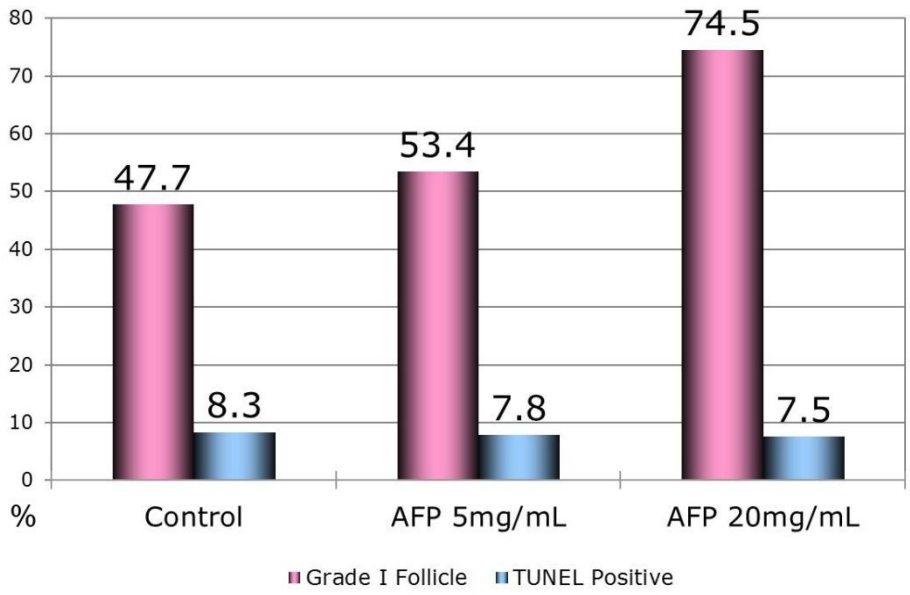

Figure 7. Morphologically intact (grade I) follicle and apoptotic follicle ratios according to supplementation of antifreeze protein (AFP) in vitrification solution after autotransplantation of vitrified-warmed ovary.

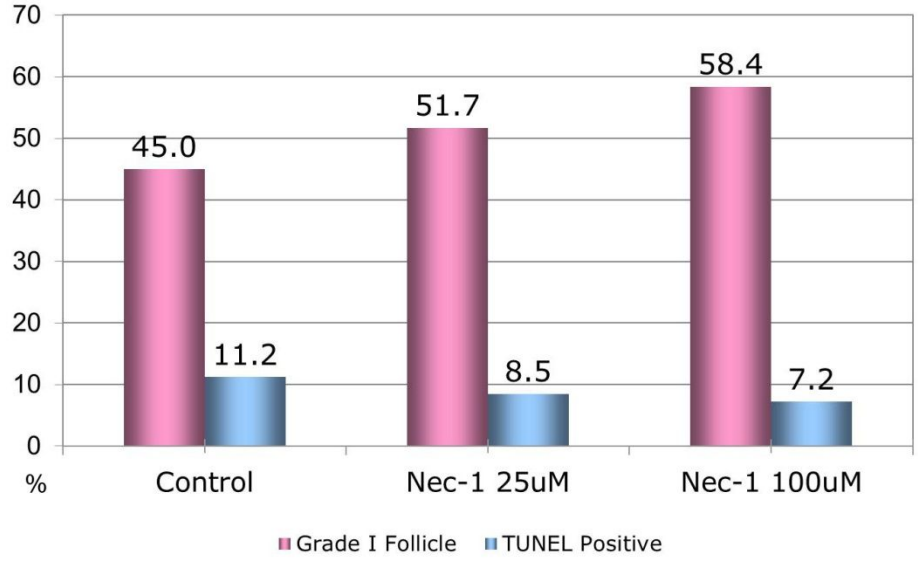

Figure 8. Morphologically intact (grade I) follicle and apoptotic follicle ratios according to supplementation of necrostatin-1 (Nec-1) in vitrification solution after warming.

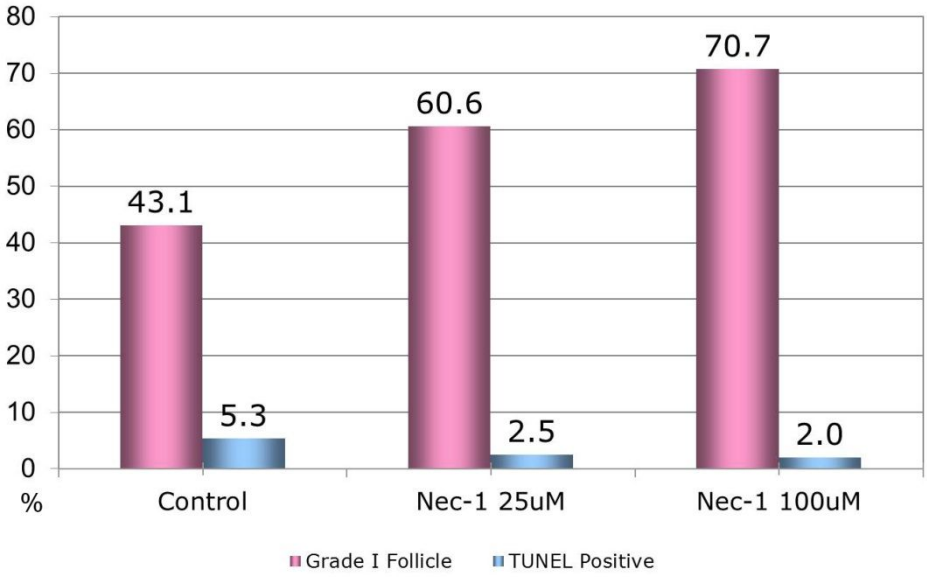

Figure 9. Morphologically intact (grade I) follicle and apoptotic follicle ratios according to supplementation of necrostatin-1 (Nec-1) in vitrification solution after autotransplantation of vitrified-warmed ovary.

DISCUSSION

The present study comprised 2 experiments: one was designed to investigate the effect of AFP on ovarian tissue cryopreservation, and the other investigated the effect of Nec-1 supplementation. Regarding AFP effects, the AFP treated group showed a significantly higher intact follicle ratio. In addition, the rate of apoptotic follicles was significantly lower in the AFP treated groups compared to the controls. These positive effects of AFP supplementation were also evident after transplantation, but statistically significant difference was only noted in the higher dose (20 mg/mL) AFP group. The proliferation of follicles significantly increased with AFP supplementation in a dose-dependent manner. Finally, VEGF expression increased in the follicles and the ovarian stromal compartment. These results demonstrate that AFP supplementation during vitrification improves the survival of ovarian tissue during cryopreservation and after transplantation.

AFPs are a class of polypeptides produced by animals such as antarctic fish; these proteins allow their survival in subzero environments (13). Five groups of these proteins exist, one of which is glycosylated (antifreeze glycoproteins, AFGPs). Structural characterization and properties of AFPs and AFGPs and the molecular mechanisms involved in inhibiting ice growth are still not completely clear. Suggested cryoprotective mechanisms include (1) a decrease in freezing temperature or thermal hysteresis and the inhibition

of the normal growth habit of ice (27-29), (2) inhibition of recrystallization during warming (30, 31), and (3) protection of the plasma membrane (32).

Previous studies have demonstrated that AFPs are effective cryoprotectants for oocytes, embryos, and spermatozoa. The vitrification of immature oocytes and two-cell-stage embryos of mice or pigs treated with AFGPs at 40 mg/mL showed improvements in morphological integrity, suggesting that these proteins can inhibit ice formation and stabilize the plasma membrane (32). O'Neil et al. found significantly enhanced rates of fertilization after mature mouse oocyte vitrification with 6 mol/L Me_2SO (dimethyl sulfoxide) plus 1 mg/mL AFGP (33). Supplementation with AFPs has also shown beneficial effects in bovine, ram, and chimpanzee spermatozoa (16, 34, 35). However, controversial results have also been reported. In these studies, AFPs failed to show any specific benefits, either in relation to the glass-forming tendency of highly supercooled cryoprotectants or in relation to the survival of various cells, which include vitrified bovine blastocysts, two-step-cryopreserved oyster oocytes, and equine embryos (14). More recent studies with mature and immature mouse oocytes have demonstrated that supplementation of AFP type III in the vitrification medium protects oocytes from chilling injury (15, 36).

There have been few studies which investigated the effect of AFP on ovarian tissue cryopreservation, but the results of many other studies on cryopreservation of other reproductive cells and embryos were in line with the present study. The mechanism of cryoprotective effect during vitrification in

the present study is not clear. However, protective mechanism suggested by previous studies may be applicable to ovarian tissue cryopreservation. Jo et al suggested that one protective mechanism in mouse oocytes involved preservation of structural and functional integrity associated with the maintenance and recovery of spindle reassembly during vitrification and warming (15). Bagis et al reported an effect of AFP in mouse ovarian transplantation using AFP-III transgenic mouse ovaries. The authors demonstrated that the litter size of mice that were transplanted vitrified ovaries from AFP type III transgenic mice was not different from the control group, whereas the litter size of mice that were transplanted vitrified ovaries from non-transgenic mouse was significantly decreased. These results suggest that AFP type III has beneficial effect for ovarian vitrification and transplantation. A potential protective mechanism may involve the interaction of AFP with function and structure of the cell membrane (37). In the present study, AFP type III was added only in the vitrification solution, and therefore, the beneficial effect of AFP did not directly affect post-transplantation ischemic damage. However, the protective effect during vitrification and warming was enough to improve outcomes after transplantation. It is obvious that AFP has protective effects during ovarian vitrification and warming. Further study is necessary to elucidate the exact mechanism underlying this protective effect.

In the present study, the AFP treated groups had significantly higher Ki-67 positive follicle ratio and higher expression of VEGF in both follicles and

ovarian stroma compared to the controls. The proportion of Ki-67 positive follicles is a more sensitive indicator of growth than morphology alone (26). Thus, increased Ki-67 positive follicle ratio in the AFP treated groups confirms the beneficial effects of AFP on the survival of transplanted ovarian tissue. VEGF is a potent angiogenic factor and a major modulator of vascular growth and remodeling (38, 39). In the female reproductive tract, VEGF plays a crucial role in follicular growth and corpus luteum development and function (40, 41). It was demonstrated that angiogenesis-associated VEGF expression increased in rat ovarian grafts after transplantation (42). In another study, mice treated with VEGF and granulocyte colony-stimulating factor (G-CSF) maintained a significantly greater number of primordial follicles in transplanted mouse ovaries (43). Therefore, increased expression of VEGF in the AFP treated group could represent a mechanism underlying better survival after transplantation. However, whether AFP supplementation directly enhanced VEGF expression could not be determined from the present study.

Several studies reporting a detrimental effect of AFP showed that high AFP concentrations were associated with a destructive effect on cells and tissues. In those studies, at relatively low concentration of AFPs enhanced the survival rate of red blood cells, whereas at high concentrations these proteins reduced survival rates (30,31). A recent study involving immature mouse oocytes showed that high doses of AFP have a harmful effect on oocyte survival (36). In the present study, a high dose of AFP (20 mg/mL) demonstrated better results compared to the lower dose (5 mg/mL). Further study is necessary to

confirm whether AFP at higher doses is harmful, and to determine a more optimal dose of AFP for ovarian tissue cryopreservation.

In a review on the effect and mechanisms of AFPs, Wang explained that AFPs have both protective and destructive actions depending on many relevant factors such as composition and concentration of cryoprotectant, type and concentration of AFPs, cooling and warming rate, and cell surface features (14). In the present study, the composition of equilibrium and vitrification solutions, exposure time to cryoprotectants, and the vitrification method are well-established protocols after long-term clinical use in embryo vitrification and confirmed by our previous study for optimization of vitrification method using human ovarian tissue (23). Thus, relevant factors influencing AFP effect have already been optimized to show protective rather than destructive effects.

Regarding Nec-1 effects, in the present study, morphological analysis after vitrification and warming showed a significantly higher intact follicle ratio in Nec-1 treated groups as the Nec-1 dose increased, and the rate of apoptotic follicles decreased in the Nec-1 treated groups (though the decrease was not statistically significant). These results persisted after transplantation of the vitrified-warmed ovaries. These results demonstrate that Nec-1 supplementation during vitrification improves survival of ovarian tissue during cryopreservation and after transplantation. However, in contrast to AFP supplementation, Nec-1 treatment did not increase the proportion of Ki-67 positive follicles and VEGF expression decreased in the follicles after Nec-

1 treatment, although significantly higher VEGF expression was observed in the stromal cells..

Necroptosis, a type of programmed necrosis initiated by the activation of tumor necrosis factor alpha and/or Fas, is distinct from caspase-dependent apoptotic cell death. Necrostatin-1 is a cell-permeable specific inhibitor of necroptosis that functions via a mechanism distinct from that of apoptosis and that of nonspecific oxidative stress-induced necrosis. Thus, the inhibition of necroptosis offers a new therapeutic target for ischemic damage in addition to inhibition of apoptosis and reactive oxygen species activity. In the previous study, Nec-1 reduced ischemic tissue damage in a mouse middle cerebral artery occlusion stroke model, indicating that necroptosis may be an important mode of ischemic cell death in vivo (21).

Necroptosis depends on the serine/threonine kinase activity of RIP1 (19) that represents the molecular target of cytoprotective agents, i.e., necrostatins. Necroptosis participates in the pathogenesis of diseases, including ischemic injury, neurodegeneration, and viral infection (20). Nec-1 efficiently suppresses necroptotic cell death triggered by an array of stimuli in various cell types. Nec-1-inhibitable non-apoptotic death is an important contributor to ischemic injury in mouse models of cerebral ischemia and myocardial infarction (21, 22). An ischemic period exists after ovarian transplantation because this procedure involves implantation of ovarian tissue without vascular anastomosis. The time for reperfusion after transplantation of ovarian tissue was reported to be 2–5 days (44, 45). According to previous studies,

Nec-1 supplementation can reduce ischemic damage during this period. In the present study, follicular damage was reduced with Nec-1 supplementation during vitrification and transplantation. During transplantation, Nec-1 was added to the warming solution and injected into the recipient 30 min before transplantation. Follicle morphology was improved more after transplantation than that after warming, although this is not a direct comparison. Considering the results of the present as well as previous studies, the protective effects of Nec-1 may occur more during transplantation than during vitrification. Therefore, it can be postulated that Nec-1 supplementation can help to reduce both cryodamage during vitrification and ischemic necrosis after ovarian transplantation.

Several mechanisms underlying the protective action of Nec-1 have been suggested. Nec-1 is a specific inhibitor of RIP1 and a series of genes that may act downstream and/or serve as regulators of RIP1 (46, 47). In a neonatal hypoxia-ischemia model, Northington et al demonstrated that necrostatins inhibit RIP1-RIP3 interaction, decrease oxidative injury, and suppress upstream proinflammatory signaling and multiple cytokines (48). Lim et al showed that the cardioprotective effect of Nec-1 functions via cyclophilin-D, preventing the mitochondrial permeability transition pore opening, which occurs in myocardial reperfusion-mediated cell death. Nec-1 reduces the acute loss of plasma membrane integrity at an early time point after ischemia (49). In a traumatic brain injury model, Nec-1 reduced brain neutrophil influx and microglial activation, suggesting a novel anti-inflammatory effect (50). In

another study, Nec-1 inhibited the induction of LC-3-II, a marker of autophagy (21). Further study is necessary to elucidate the mechanism of protective effect of Nec-1 in ovarian tissue transplantation.

Damage to the ovarian tissue during cryopreservation thawing and transplantation is associated with 2 key factors: one is cryodamage that occurs during the cryopreservation-thawing procedure, and the other is the ischemic injury that occurs during revascularization of the transplanted tissue from the surrounding vessels. The present study suggests that AFP prevents cryodamage during vitrification and warming of ovarian tissue, and that Nec-1 has a protective effect against ischemic damage after transplantation. Xu et al reported a synergistic protective effect of humanin, anti-apoptotic agent, and Nec-1 on hypoxia and ischemia/reperfusion injury in a mouse cerebral ischemia model (51). Consistent with the previous study, the combined use of AFP and Nec-1 is likely to show a synergistic effect, though further study is necessary to confirm this.

One of the limitations of the present study is the different control results in the AFP and Nec-1 experiments. For example, after vitrification and warming, the morphologically intact follicle ratio of the control group was 28.9% and 45.0% in AFP and Nec-1 experiments, respectively. This discordance of results between the 2 experiments may originate from the difference in experimental conditions. The 2 sets of experiments were performed at different times and under different laboratory conditions. Thus, direct comparison between the 2 experiments may not be appropriate for evaluating

the effects of AFP and Nec-1. In order to address this limitation, another experiment directly comparing AFP and Nec-1 should be performed.

In conclusion, AFP and Nec-1 may represent promising supplementary agents for reducing cryodamage during vitrification and ischemic damage transplantation of ovarian tissue. Further studies combining the use of these 2 agents with human ovarian tissue and evaluating toxicity as well as studies on the mechanism underlying this protective effect are necessary for the application of this technique in clinical practice.

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국문 초록

서론: 난소동결보존과 이식은 여성 암 생존자에서 가임력보존의 가장 이상적인 방법으로 기대된다. 그러나 동결보존된 난소의 생존율이 낮아 아직 임상에서 널리 사용되기에는 부족한 점이 많다. 난소 동결보존과 이식 시 난소 손상은 주로 동결 시 발생하는 동결손상과 이식 시 발생하는 허혈성 손상에 기인한다. 동결방지제와 항괴사제는 이러한 동결손상과 허혈성괴사를 감소시킬 것으로 기대된다. 항동결단백 (antifreeze protein, AFP)은 극지생물의 체내에서 생성되는 폴리펩티드의 한 종류로 이들이 극지에서 살아가는 것을 가능하게 하는 물질이다. 최근 들어 항동결단백이 동물세포와 기관의 동결보존 시 보호효과를 보인다는 연구가 보고되었으나 항동결단백의 난소동결에서의 효과에 대해서는 연구가 이루어진 바가 없다. Necrostatin-1 (Nec-1)은 receptor-interacting protein 1 kinase (RIP1)의 억제제로 이를 통한 necroptosis 를 억제하는 역할을 하는 것으로 알려진 물질이다. 본 연구의 목적은 항동결단백과 Nec-1 의 첨가가 난소조직 동결보존에 미치는 효과를 탐색하는 것이다.

방법: 4 주령 ICR 마우스를 사용하여 유리화 동결 실험을 진행하였으며 유리화 동결 과정은 평형용액과 유리화동결용액에 노출시키는 2 단계 방법으로 진행되었다. 평형용액은 20% ethylene glycol (EG), 유

리화동결용액은 40% EG, 18%Ficoll, 0.3M sucrose 로 구성되었으며 두 용액 모두 20% fetal bovine serum (FBS)을 포함한 Dulbecco's phosphate buffered saline (DPBS)을 기본용액으로 하여 제작되었다. 적출된 난소를 1mL 의 평형용액에 10 분간 처리한 후 0.5mL 의 유리화동결용액에 5 분간 처리하였다. 항동결단백 첨가의 효과를 탐색하기 위해서는 각각 0, 5, 20mg/mL 의 항동결단백을 유리화동결용액에 첨가하였으며, Nec-1 첨가의 효과를 탐색하기 위해서는 각각 0, 25, 100 μM 의 Nec-1 을 유리화동결용액에 첨가하였다. 동결과 해동 후 난포형태와 세포자연사 여부를 조직학적 검사와 TUNEL assay 를 이용하여 평가하였다. 유리화동결된 난소 중 일부는 해동하여 자가이식을 시행하였다. Nec-1 효과를 보기 위한 실험에서는 각각 0, 25, 100 μM 의 Nec-1 을 해동용액에 첨가함과 아울러 각각 0, 25, 100 μM 의 Nec-1 용액 0.3mL 를 이식 30 분 전에 복강내 주사하였다. 이식 2 주 후 이식된 난소의 난포형태 분석과 세포자연사 여부 평가를 시행하였다. Ki-67 과 혈관내피성장인자 (vascular endothelial growth factor, VEGF)항체의 면역조직화학 염색을 시행하여 이식된 난소의 증식과 혈관생성을 평가하였다.

결과: 유리화동결과 해동 후 형태학적 분석 결과 항동결단백 처리군에서 유의하게 높은 정상난포 비율을 보였다 (대조군: 28.9%, 항동결단백 5mg/mL 투여군: 42.3%, 항동결단백 20mg/mL 투여군: 44.7%). 세포자연사가 진행된 난포 (TUNEL 양성 난포)의 비율은 항동결단백

처리군에서 유의하게 낮았다 (대조군: 26.6%, 항동결단백 5mg/mL 투여군: 18.7%, 항동결단백 20mg/mL 투여군: 12.6%). 해동-이식 후 형태학적 분석 결과 항동결단백 20mg/mL 처리군에서 유의하게 높은 정상난포 비율을 보였다. 세포자연사가 진행된 난포의 비율은 각 군에서 유의한 차이가 없었다. Ki-67 양성 난포의 비율은 항동결단백의 투여용량에 따라 유의하게 증가하는 양상을 보였다 (대조군: 3.3%, 항동결단백 5mg/mL 투여군: 31.3%, 항동결단백 20mg/mL 투여군: 49.0%). VEGF 의 immunohistochemical intensity score 는 난포에서는 항동결단백 투여군 모두에서, 기질세포에서는 항동결단백 20mg/mL 투여군에서 유의하게 높았다. Nec-1 투여후 유리화동결과 해동 시 형태학적 분석 결과 Nec-1 처리군에서 유의하게 높은 정상난포 비율을 보였다 (대조군: 45.1%, Nec-1 25 μM 투여군: 51.7%, Nec-1 100 μM 투여군: 57.9%). 세포자연사가 진행된 난포의 비율은 Nec-1 처리군에서 낮은 양상을 보였다 (대조군: 11.2%, Nec-1 25 μM 투여군: 8.5%, Nec-1 100 μM 투여군: 7.2%). 해동-이식 후 형태학적 분석 결과 Nec-1 처리군에서 유의하게 높은 정상난포 비율을 보였다 (대조군: 43.1%, Nec-1 25 μM 투여군: 60.6%, Nec-1 100 μM 투여군: 70.7%). 세포자연사가 진행된 난포의 비율은 Nec-1 처리군에서 낮은 양상을 보였다 (대조군: 5.3%, Nec-1 25 μM 투여군: 2.5%, Nec-1 100 μM 투여군: 2.0%). Ki-67 양성 난포비율은 각 군에서 차이를 보이지 않았고, 기질세포에서의

VEGF 의 immunohistochemical intensity score 는 Nec-1 100 μM 투여군에서 유의하게 높은 결과를 보였다.

결론: 본 연구의 결과 난소조직의 유리화 동결 시 항동결단백의 첨가와 유리화동결, 해동 및 이식 시 Nec-1 의 첨가가 난소조직의 동결보존과 이식 시 유익한 효과를 나타낼 수 있다는 점을 확인할 수 있다.

주요어 : 가임력보존, 난소조직 동결보존, 유리화동결, 항동결단백질, necrostatin

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