The beneficial effects of polyethylene glycol-superoxide dismutase on ovarian tissue culture and transplantation

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Abstract

Purpose Reducing the ischemic damage from free radicals that is inflicted on ovarian tissue is critical for successful ovarian tissue transplantation. Polyethylene glycol-superoxide dismutase (PEG-SOD) is mimetic of superoxide dismutase (SOD) and powerful free radical scavenger acts by reducing superoxide anions. The objective of study was to evaluate effects of PEG-SOD on mouse ovarian tissues in *in vitro* culture and in autotransplantation.

Methods Ovaries were collected and randomly divided into four groups that received different doses of PEG-SOD. To assess effects of PEG-SOD on *in vitro* cultures, four different doses of PEG-SOD were applied to *in vitro* culture media during *in vitro* culturing following ovarian tissue vitrification and warming. To evaluate effects of PEG-SOD on ovarian tissue transplantation, four different doses of PEG-SOD were applied for 2, 7, and 21 days to mice following vitrified-warmed mouse ovarian tissue autotransplantation.

Results The percentage of primordial follicles was maintained at the highest dose of PEG-SOD for 2 h *in vitro*, and there was a significant decrease in the percentage of apoptotic follicles at 2 h, but not at later time points. The highest dose of PEG-SOD

also maintained primordial, primary, and secondary follicles 2 days post-transplantation, but only primordial follicles were maintained up to 21 days after transplantation.

Conclusions PEG-SOD is protective mainly toward primordial follicles only for a short interval *in vitro*, presumably via antioxidant effects. PEG-SOD may be a promising additive for preserving ovarian tissue integrity, at least for primordial follicles, up to 21 days post-transplantation.

Keywords Ovarian tissue transplantation · Ovarian tissue cryopreservation · Polyethylene glycol-superoxide dismutase (PEG-SOD) · Fertility preservation

Introduction

Patients with cancer who require chemotherapy and/or radiotherapy can experience infertility due to premature ovarian failure. Cryopreservation of oocytes, embryos, and/or ovarian tissue is one option for the preservation of fertility in these patients [1, 2]. Although oocyte cryopreservation is the most reliable method for preserving fertility, there are several critical issues that are related to the application of this technique. For oocyte cryopreservation to be performed, the patients must reach puberty, and there must be sufficient time prior to cancer therapy to trigger oocyte maturation [1]. Alternatively, if a patient is pre-pubertal or requires immediate chemotherapy and/or radiotherapy, she can consider cryopreservation of the ovarian tissue [1–3]. Therefore, cryopreservation of ovarian tissue is a promising option for patients who might become infertile due to cancer treatment.

There are several critical considerations for the cryopreservation of ovarian tissue. Because ovarian tissue is more voluminous and structurally complex than oocytes, several problems must be circumvented. One potential problem is

Capsule In an animal model system, PEG-SOD is shown to be a promising free-radical scavenging additive for preserving ovarian tissue integrity destined for transplantation.

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ischemic injury following ovarian tissue transplantation [3]. Some pre-pubertal patients who are completely cured of cancer after the completion of chemotherapy and/or radiotherapy might want to become pregnant. Such pregnancies can be achieved using cryopreserved ovarian tissue transplantation to obtain oocytes or ovarian follicles. However, ovarian tissues are more likely to be in ischemic conditions following transplantation [4–6].

Treatment with antioxidants might minimize ischemic damage prior to angiogenesis and protect the ovarian tissue [7]. Polyethylene glycol-superoxide dismutase (PEG-SOD) is a superoxide dismutase (SOD) mimetic and a free radical scavenger that has been proposed to be an effective agent for reducing free radical-mediated injuries [8, 9]. However, although SODs are powerful antioxidants, it is difficult to take advantage their activities because these agents have extremely short half-lives [10, 11]. PEG-SOD is a modified version of the enzyme that has a longer half-life than pure SOD (>30 h) and is more strongly concentration-dependent [12]. Therefore, PEG-SOD is a more effective agent than SOD because PEG-SOD treatment doses can be lower [13, 14].

In the present study, the effect of PEG-SOD was evaluated by two methods, *in vitro* culture and transplantation. The objectives of this study were to evaluate the ability of PEG-SOD to protect ovarian tissue from oxidative stress during *in vitro* culture and to describe the role of PEG-SOD in protecting ovarian tissue from ischemic damage following transplantation.

Materials and methods

Animals

Female 5-week-old BDF-1 mice (F1 hybrid, C57BL/6 [♀] × DBA6 [♂]) were purchased from the Orient, Seongnam, South Korea. All animals were bred under temperature- and light-controlled conditions. The Institutional Animal Care and Use Committee of the Seoul National University Bundang Hospital approved the animal protocol. The mice were sacrificed using the cervical dislocation method. Figure 1 shows the experimental scheme for this study.

Vitrification and warming of the ovarian tissue

Ovaries were collected from the ovarian bursa and transferred directly into vitrification media using the two-step method described by Youm et al. [15]. First, the ovaries were equilibrated in 7.5 % ethylene glycol and 7.5 % dimethyl sulfoxide (DMSO) in 1× phosphate-buffered saline (PBS) supplemented with 20 % fetal bovine serum (FBS) for 10 min at room

temperature (RT). The ovaries were then transferred into vitrification media that consisted of 20 % ethylene glycol, 20 % DMSO, and 0.5 M sucrose in 1× PBS supplemented with 20 % FBS for 5 min at RT. Then, the ovaries were transferred to an electron microscopy grid, and the grid was plunged into liquid nitrogen. The vitrified ovarian tissues were stored in liquid nitrogen until warming. Finally, the vitrified ovaries were warmed in media containing four different concentrations of sucrose (1, 0.5, 0.25, and 0 M) in 1× PBS supplemented with 20 % FBS and cultured for 5 min at RT.

In vitro culture of ovarian tissue

The *in vitro* culture study consisted of 16 groups. The duration of the *in vitro* culture was varied across four groups (2, 4, 12, and 24 h), and within each of these groups. PEG-SOD (Sigma-Aldrich, MO, USA) the lyophilized powder which had over 1350 units/mg of protein and 1 mg/ml of solubility has been applied with a variety of 0, 10, 100, and 1000 U/ml PEG-SOD, respectively. Each group comprised 20 whole ovaries obtained from 10 mice. The ovaries were pooled and randomly assigned to each group by *in vitro* culture durations (2, 4, 12, and 24 h, respectively) and PEG-SOD concentrations (0, 10, 100, and 1000 U/ml, respectively). The culture medium consisted of α -MEM supplemented with 10 % FBS and 100 mIU of follicle-stimulating hormone (FSH) and was used to dissolve the PEG-SOD. The ovaries were incubated at 37 °C with 5 % CO₂ for 2, 4, 12, or 24 h. After incubation, the ovarian tissues were fixed in 4 % paraformaldehyde.

Ovarian tissue autotransplantation and polyethylene glycol-superoxide dismutase treatment

The mice were anesthetized via intraperitoneal injections of 9 ml/kg normal saline containing 0.6 ml/kg Zoletil (Virbac, France) and 0.4 ml/kg Rompun (Bayer, Germany). After 7 days of ovariectomy, the vitrified-warmed ovarian tissues were then autotransplanted under the kidney capsule. After autotransplantation, the mice were randomly divided into 12 groups in which the observation periods varied (2, 7, and 21 days, respectively), and within each of these groups, the dose of PEG-SOD was varied (0, 100, 1000, and 10,000 U/ml, respectively). Each group consisted of 10 mice. The treatments with PEG-SOD were performed via intraperitoneal injection once every 48 h. After the first week of treatment, the 21-day observation group was treated once every 4 days with PEG-SOD. The mice were sacrificed at the end of the observation period; the transplanted ovarian tissues were collected and fixed with 4 % paraformaldehyde. For the hormone assays, blood was also collected via cardiac puncture after the mice were sacrificed.

Fig. 1 Experimental scheme for this study

Histological analyses of the ovarian tissue morphologies

To evaluate the qualities of the ovarian follicles, the follicles were counted according to the classification method described below. Fixed ovarian tissues were embedded in paraffin blocks and sectioned (section thickness=4 μm). To prevent misreading, the paraffin slides were created with only one section per ovary. The follicles that contained oocytes were counted for the morphological analysis. The paraffin slides were stained with hematoxylin and eosin.

The ovarian follicle counts were performed according to the following criteria by Lundy et al. [16]:

- I. Primordial follicle: ovarian follicle that consisted of a flattened oocyte surrounded by a single layer of pregranulosa cells
- II. Primary follicle: ovarian follicle that consisted of oocytes surrounded by single layers of cuboidal granulosa cells (GCs)
- III. Secondary follicle: ovarian follicle that consisted of oocytes surrounded multiple layers of GCs
- IV. Antral follicle: ovarian follicle that has antra (follicular fluid)

The morphologies of the ovarian follicles were evaluated based on the following criteria [15, 17]:

- i. Grade 1 (G1): good, intact GCs and oocyte with normal morphologies
- ii. Grade 2 (G2): fair, GCs pulled away from edge of follicle but with intact oocyte
- iii. Grade 3 (G3): poor, displayed disruption and loss of GCs and theca cell, damaged or broken oocyte with poor morphologies

The ovarian follicles were counted using a light microscope at a magnification of $\times 100$ (Olympus, Tokyo, Japan).

Apoptosis assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kits (Roche, Penzberg, Germany) were used to detect ovarian follicle DNA fragmentation, and the assays were performed according to the manufacturer's protocol. Briefly, the ovarian tissues were permeated using proteinase K (Dako, Glostrup, Denmark) for 20 min at RT. The tissues were then washed with PBS and incubated with a mixture of the enzyme and labeling solutions for 1 h at 37 °C with 5 % CO_2 . After washing with PBS, the ovarian tissues were mounted in mounting media with DAPI (ImmunoBioScience, Mukilteo, WA, USA) and observed using a fluorescence microscope at $\times 100$ magnification (ZEISS, Jena, Germany).

Hormone level measurements

Enzyme-linked immunosorbent assay (ELISA) kits (Endocrine Technology, Newark, CA, USA) were used to evaluate the levels of FSH in the blood sera according to the manufacturer's protocol. The optical density was read at 450 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analyses

The statistical analyses were conducted using SPSS 12.0. The data were compared using chi-square tests, and differences were considered significant when $P < 0.05$ (Fig. 1).

Results

The effects of polyethylene glycol-superoxide dismutase treatment on vitrified and warmed ovarian tissue *in vitro*

Ovarian follicle integrity

Representative images of the histological analyses of the *in vitro* culture experiment is shown in Fig. 2. To evaluate the effects of PEG-SOD treatment on ovarian tissue integrity, ovarian follicle grading was performed. The intact (G1) primordial follicle ratio of the 2 h-1000 U/ml PEG-SOD treatment group was 76 %, which was the highest ratio among the 2-h *in vitro* culture groups (Table 1). The intact (G1) primordial follicle ratios of the groups of 4 h-10 U/ml PEG-SOD and 12 h-1000 U/ml PEG-SOD were 78 and 73 %, respectively (Table 1). These ratios were greater than 4 h-1000 U/ml group (62 %) and 12 h-100 U/ml group (58 %), respectively. After 24 h, no significant differences in intact (G1) primordial follicle ratio were observed between the four groups (Fig. 3b, Table 1). In case of primary follicles, 4 h-10 U/ml group (88 %), 12 h-100 U/ml group (91 %), and 24 h-10 U/ml group (80 %) showed the highest primary follicle ratio among the same *in vitro* culture duration groups. In 2 h of *in vitro* culture group, there were no significant differences in primary follicles. Regarding the intact (G1) secondary follicles after 24 h of *in vitro* culture, the ratio of the 1000 U/ml PEG-SOD treatment group (45 %) was significantly greater than those of the other 24-h culture groups (25, 22, and 25 % in 0, 10, and 100 U/ml groups, respectively) (Table 1). In terms of antral follicles, 2 h-0 U/ml group (51 %) and 12 h-0 U/ml group (33 %) had showed the significantly higher intact (G1) antral follicle ratio compared with 2 h-10 U/ml (40 %) group and

12 h-100 U/ml (19 %) group. Figure 4 shows the morphology of ovarian tissue after *in vitro* culture with or without PEG-SOD. Ovarian tissues after 12 and 24 h of *in vitro* culture showed disruption and loss of GCs, pyknotic nuclei, and abnormal oocyte while the ovarian tissues after 2 and 4 h of *in vitro* culture showed better oocyte and follicle integrity.

Ovarian follicle viability

To analyze the influence of PEG-SOD on ovarian follicle viability, we performed TUNEL assays on the ovarian tissues. After 2 h of *in vitro* culture, the apoptotic ovarian follicle ratios for the 100 and 1000 U/ml PEG-SOD treatment groups were 15 and 14 %, respectively (Table 1). These ratios were significantly lower than the ratios of 30 and 31 % that were observed in the 0 and 10 U/ml groups, respectively (Table 1). It was unable to count exact numbers of apoptotic follicles in the 12- and 24-h groups because the follicles were collapsed and broken just showing green apoptotic signals all over the ovarian tissue section.

Effects of polyethylene glycol-superoxide dismutase on the vitrified and warmed ovarian tissues after autotransplantation

Ovarian follicle integrity

Representative images of the histological analyses of the ovarian tissue from the transplantation experiment are shown in Fig. 5. When we examined the effects of PEG-SOD injection following ovarian tissue transplantation, the intact total ovarian follicle ratio in the 10,000 U/ml PEG-SOD treatment group was observed to be 56 %, which was significantly

Fig. 2 Representative images of the histological analyses of the *in vitro* culture experiment. The horizontal axis represents the PEG-SOD treatment dose. The vertical axis represents the *in vitro* culture duration. All of the representative images have same magnifications with the first image (2 h-0 U/ml) ($\times 100$)

Table 1 Proportions of follicles with good morphology (grade I) and apoptotic follicles according to supplementation of PEG-SOD in *in vitro* culture media after warming and *in vitro* culture for 2, 4, 12, and 24 h

<i>In vitro</i> Culture duration (hours_		PEG-SOD 0 U/ml	PEG-SOD 10 U/ml	PEG-SOD 100 U/ml	PEG-SOD 1000 U/ml
2	Number of ovaries	17	17	19	20
	Ratio of grade I follicles (%)				
	Primordial	95/160 (59 %) ^a	57/102 (56 %) ^a	141/233 (61 %) ^a	157/207 (76 %) ^b
	Primary	48/98 (49 %) ^a	24/54 (44 %) ^a	44/85 (52 %) ^a	52/92 (57 %) ^a
	Secondary	16/60 (27 %) ^a	36/75 (48 %) ^a	33/89 (37 %) ^a	30/82 (37 %) ^a
	Antral	39/77 (51 %) ^a	37/93 (40 %) ^b	40/97 (41 %) ^{a,b}	66/160 (41 %) ^{a,b}
	Total	198/395 (50 %) ^{a,b}	154/324 (48 %) ^a	256/502 (51 %) ^{a,b}	305/541 (56 %) ^b
4	Apoptotic follicles (%)	71/235 (30 %) ^a	69/222 (31 %) ^a	39/269 (15 %) ^b	48/334 (14 %) ^b
	Number of ovaries	17	17	19	20
	Ratio of grade I follicles (%)				
	Primordial	51/73 (70 %) ^{a,b}	86/111 (78 %) ^a	78/106 (74 %) ^{a,b}	61/99 (62 %) ^b
	Primary	26/36 (72 %) ^a	35/40 (88 %) ^b	42/56 (75 %) ^a	28/38 (74 %) ^a
	Secondary	22/49 (45 %) ^a	21/50 (42 %) ^a	31/54 (57 %) ^a	30/50 (60 %) ^a
	Antral	8/52 (15 %) ^a	14/34 (41 %) ^a	15/45 (33 %) ^a	10/40 (25 %) ^a
12	Total	107/210 (51 %) ^a	156/235 (66 %) ^b	166/261 (64 %) ^{a,b}	129/227 (57 %) ^{a,b}
	Apoptotic follicles (%)	131/312 (42 %) ^a	130/342 (38 %) ^a	135/431 (31 %) ^a	111/348 (32 %) ^a
	Number of ovaries	18	16	19	18
	Ratio of grade I follicles (%)				
	Primordial	86/126 (68 %) ^{a,b}	89/125 (71 %) ^{a,b}	72/124 (58 %) ^a	68/93 (73 %) ^b
	Primary	25/33 (76 %) ^a	32/46 (70 %) ^a	29/32 (91 %) ^b	27/55 (49 %) ^c
	Secondary	16/37 (43 %) ^a	20/46 (44 %) ^a	23/48 (48 %) ^a	18/46 (39 %) ^a
24	Antral	19/58 (33 %) ^a	9/43 (21 %) ^{a,b}	10/52 (19 %) ^b	13/62 (21 %) ^{a,b}
	Total	146/254 (58 %) ^a	150/260 (58 %) ^a	134/256 (52 %) ^a	173/294 (59 %) ^a
	Apoptotic follicles (%)	- (100 %)	- (100 %)	- (100 %)	- (100 %)
	Number of ovaries	18	16	20	17
	Ratio of grade I follicles (%)				
	Primordial	79/140 (56 %) ^a	55/105 (52 %) ^a	83/169 (49 %) ^a	71/116 (61 %) ^a
	Primary	18/43 (42 %) ^a	20/25 (80 %) ^b	24/43 (56 %) ^c	27/44 (61 %) ^c
	Secondary	16/63 (25 %) ^a	6/27 (22 %) ^a	14/56 (25 %) ^a	26/58 (45 %) ^b
	Antral	8/64 (13 %) ^a	8/46 (17 %) ^a	6/40 (15 %) ^a	12/61 (20 %) ^a
	Total	121/310 (39 %) ^a	89/203 (44 %) ^a	127/308 (41 %) ^a	136/279 (49 %) ^a
	Apoptotic follicles (%)	- (100 %)	- (100 %)	- (100 %)	- (100 %)

The different superscript letters represent significant differences between doses of PEG-SOD within one time interval

It was unable to count exact numbers of apoptotic follicles in the 12- and 24-h groups because the follicles were collapsed and broken just showing green apoptotic

Signals all over the ovarian tissue section

greater than 0 U/ml PEG-SOD treatment groups 2 days after transplantation (Fig. 3c, Table 2). Also, the intact (G1) ratios of primordial and primary follicles on 2 days after transplantation were significantly higher in the 10,000 U/ml group (primordial follicle 65 % and primary follicle 62 %) than the other groups (primordial follicle 23–45 % and primary follicle 19–45 %) in the same duration. For the intact (G1) secondary follicle ratio of the 2-day group, the 1000 U/ml (40 %) and 10,000 U/ml (51 %) groups showed significantly higher ratios than 0 U/ml (25 %) and 100 U/ml (16 %) groups. The

morphologically intact total ovarian follicle ratios were significantly greater in all the treatment groups (60, 57, and 64 % in 100, 1000, and 10,000 U/ml group, respectively) than in the control group (45 %) 7 days after transplantation (Fig. 2c, Table 2). In terms of intact (G1) antral follicle on 7 days after transplantation, 0 U/ml PEG-SOD treatment group (72 %) showed significantly increased intact (G1) ratio than 100 U/ml (65 %) and 10,000 U/ml (50 %) treatment groups. There were no significant differences in the intact total ovarian follicle ratios 21 days after transplantation (Fig. 3c, Table 2).

Fig. 3 **a** Intact (G1) total follicle ratios in the ovarian tissues after *in vitro* culture with polyethylene glycol-adsorbed-superoxide dismutase (PEG-SOD). **b** Intact (G1) primordial follicle ratios in the ovarian tissues after *in vitro* culture with PEG-SOD. **c** Intact (G1) total follicle ratios in the ovarian tissues after intraperitoneal treatment with PEG-SOD following ovarian tissue autotransplantation. **d** Intact (G1) primordial follicle ratios in the ovarian tissues after intraperitoneal treatment with PEG-SOD following ovarian tissue autotransplantation. The different superscript letters represent significant differences between doses of PEG-SOD within one time interval

However, 21 days after transplantation, the intact primordial follicle ratio of 10,000 U/ml PEG-SOD group was 72 %, which was greater than 0 U/ml PEG-SOD group (58 %) (Table 2, Fig. 3d). The intact (G1) antral follicle ratio on 21 days after transplantation was significantly higher in 1000 U/ml group (58 %) than 100 U/ml (44 %) and 10,000 U/ml groups (43 %). Furthermore, as shown in Fig. 5, follicles in 2 days after transplantation groups showed disruption of GCs and abnormal oocytes. In contrast to this, 7 and 21 days after transplantation groups had spherical shape of follicles, intact stromal cells, and normal oocytes.

Ovarian follicle viability

The ovarian follicle viabilities were evaluated after ovarian tissue transplantation and PEG-SOD injection based on the

Fig. 4 Serum follicle-stimulating hormone levels (FSH) in the blood of the ovarian tissues after autotransplantation into mice at 2, 7, and 21 days

apoptotic ovarian follicle ratios. As shown in Table 2, the apoptotic follicle ratios were higher in 2-day transplantation group than 7 and 21-day transplantation groups according to each dose of PEG-SOD. However, there was no significant difference when comparing within the groups of same transplantation durations.

Ovarian reserve after autotransplantation

The FSH levels were evaluated using ELISA to define the ovarian reserve after autotransplantation. The FSH levels decreased over time, but there were no significant differences between the groups (Fig. 3). On day 2, FSH levels were 11.7 ± 0.5 , 11.3 ± 1.2 , 12.2 ± 0.7 , and 11.9 ± 0.9 ng/ml in 0, 100, 1000, and 10,000 U/ml groups, respectively. On day 7, the FSH levels were 4.2 ± 0.9 , 4.3 ± 1.3 , 3.4 ± 0.9 , and 2.6 ± 0.9 ng/ml in 0, 100, 1000, and 10,000 U/ml groups, respectively. Also, on day 21, 2.1 ± 0.7 , 1.8 ± 0.7 , 1.4 ± 0.3 , and 1.8 ± 0.7 ng/ml of FSH levels were measured in 0, 100, 1000, and 10,000 U/ml groups, respectively.

Discussion

Ultimately, the success of ovarian tissue transplantation depends on the preservation of the ovarian follicles that are required to provide good quality, mature oocytes. This preservation can be achieved by reducing ischemic injury from superoxide anions and by preserving the morphology of the

Fig. 5 Representative images of the histological analyses of the ovarian tissue from the transplantation experiment. The horizontal axis represents the PEG-SOD treatment. The vertical axis represents the observation period after the ovarian tissue transplantation. All of the representative images have same magnifications with the first image (2 days-0 U/ml) ($\times 100$)

intact ovarian follicle. During cryopreservation and transplantation, ovarian follicles can be damaged by many external factors. For example, reactive oxygen species can induce apoptosis and other tissue injuries [18, 19]. The aim of our study was to analyze the ability of the antioxidant agent PEG-SOD

to protect *in vitro* cultured and transplanted ovarian tissue from oxidative stress and thereby reduce the likelihood of ischemic injury.

Polyethylene glycol-superoxide dismutase has been proposed to act as a protective agent that can reduce ischemic

Table 2 Proportions of follicles with good morphology (grade I) and apoptotic follicles according to treatment of PEG-SOD by intraperitoneal injection after transplantation for 2, 7 and 21 days

Observation period (days)		PEG-SOD 0 U/ml	PEG-SOD 100 U/ml	PEG-SOD 1000 U/ml	PEG-SOD 10,000 U/ml
2	Number of ovaries	19	18	18	19
	Ratio of grade I follicles				
	Primordial	9/40 (23 %) ^a	18/47 (38 %) ^b	24/53 (45 %) ^b	45/69 (65 %) ^c
	Primary	20/63 (32 %) ^a	11/59 (19 %) ^b	27/60 (45 %) ^c	43/69 (62 %) ^d
	Secondary	20/80 (25 %) ^a	12/75 (16 %) ^a	28/70 (40 %) ^b	41/80 (51 %) ^b
	Antral	32/86 (37 %) ^a	30/74 (41 %) ^a	31/85 (35 %) ^a	33/71 (47 %) ^a
	Total	81/269 (30 %) ^a	71/255 (28 %) ^a	110/268 (41 %) ^a	162/289 (56 %) ^b
7	Apoptotic follicles	34/170 (20 %) ^a	25/126 (20 %) ^a	33/177 (19 %) ^a	24/177 (21 %) ^a
	Number of ovaries	14	15	16	18
	Ratio of grade I follicles				
	Primordial	33/65 (51 %) ^a	18/27 (67 %) ^b	20/45 (45 %) ^{a,b}	14/51 (28 %) ^b
	Primary	35/52 (67 %) ^a	17/25 (68 %) ^a	29/51 (57 %) ^a	14/31 (45 %) ^a
	Secondary	72/114 (63 %) ^a	38/52 (73 %) ^{a,b}	42/75 (56 %) ^{a,b}	21/63 (33 %) ^b
	Antral	78/109 (72 %) ^a	39/60 (65 %) ^b	44/68 (65 %) ^{a,b}	30/60 (50 %) ^b
21	Total	85/188 (45 %) ^a	95/159 (60 %) ^b	173/304 (57 %) ^b	191/297 (64 %) ^b
	Apoptotic follicles	15/118 (13 %) ^a	13/107 (12 %) ^a	11/136 (8 %) ^a	10/118 (9 %) ^a
	Number of ovaries	18	14	16	19
	Ratio of grade I follicles				
	Primordial	55/95 (58 %) ^a	35/59 (59 %) ^{a,b}	42/65 (65 %) ^{a,b}	67/93 (72 %) ^b
	Primary	33/48 (69 %) ^{a,b}	13/21 (62 %) ^{a,b}	22/39 (56 %) ^a	27/38 (71 %) ^b
	Secondary	34/55 (62 %) ^a	23/51 (45 %) ^b	23/40 (58 %) ^{a,b}	48/72 (67 %) ^a
	Antral	28/61 (46 %) ^{a,b}	28/57 (44 %) ^a	29/50 (58 %) ^b	35/82 (43 %) ^a
	Total	150/259 (58 %) ^a	96/188 (51 %) ^a	116/194 (60 %) ^a	177/285 (62 %) ^a
	Apoptotic follicles	24/193 (12 %) ^a	16/150 (11 %) ^a	11/113 (10 %) ^a	18/165 (11 %) ^a

The different superscript letters represent significant differences between doses of PEG-SOD within one time interval

injury during the transplantation of organs such as the heart [20] and liver [21]. Therefore, PEG-SOD might be an effective free radical scavenger that can be used during ovarian tissue transplantation. Since the *in vitro* data represents ovarian tissue not exposed to a blood supply, the short-term protective effects of PEG-SOD on primordial follicles must then be most likely due to antioxidant properties. Also, the results of our study indicate that PEG-SOD played a role in preserving the morphologies of ovarian follicles, particularly during the first 2 h of *in vitro* culture (Fig. 3a). In the 2-h treatment group, the intact primordial ovarian follicle ratio was higher than those of the other groups at the PEG-SOD dose of 1000 U/ml (Fig. 3b). However, as the duration of the *in vitro* culture increased, the PEG-SOD-induced effects became weaker (Figs. 3a, b). We suggest that only early short-term treatment with PEG-SOD elicits a strong antioxidant effect on ovarian tissue that is cultured *in vitro*.

In contrast to the *in vitro* PEG-SOD treatment, *in vivo* PEG-SOD treatment can continuously preserve ovarian follicles. The highest dose (10,000 U/ml) maintained primordial, primary, and secondary follicles for 2 days relative to no PEG-SOD; maintained primordial and antral follicles for 7 days and maintained primordial follicles for up to 21 days, without effects on primary, secondary, and antral follicles. Thus, the major protective effect of PEG-SOD *in vivo* is seen in the cohort of primordial follicles. However, 21 days after transplantation, during a period of angiogenic stability, the results from all groups exhibited no significant differences (Table 2, Figs. 3c, d).

To enhance ovarian function, especially after ovarian tissue transplantation, it is important to prevent ischemic injury and to induce angiogenesis. This is because ovarian tissue transplantation is conducted without the initial anastomosis of blood vessels [22]. In the present study, the ovarian tissues were transplanted under the kidney capsule without anastomosis of the blood vessels during the early transplantation period (pre-angiogenesis). We speculate that PEG-SOD might play a role in the prevention of ischemic damage from oxidative stress until the completion of angiogenesis.

Oxidative stress can also induce apoptosis in ovarian follicles and inflict injuries that can result in a loss of viability [23]. Using *in vitro* culture experiments, we showed that, in the 2-h group, the apoptotic ovarian follicle ratios were significantly lower in the 100 and 1000 U/ml PEG-SOD groups than in the 10 U/ml PEG-SOD and control groups (Table 1). These results suggest that ovarian follicle damage from apoptosis might be prevented by treatment with high doses of PEG-SOD. Although there were no significant differences between the 4-h treatment groups, the results indicated that the apoptotic ovarian follicle ratio tended to decrease as the PEG-SOD dose increased across all dosages of PEG-SOD (Table 1). Consistent with the results from this study, Dolgachev V.

et al. also demonstrated that SOD can prevent apoptosis [9]. However, when the period of *in vitro* culture was extended over 2 h, the antiapoptotic effect exerted by PEG-SOD on the ovarian follicles was reduced. It means that when the *in vitro* culture time increased, the apoptosis ratios also increased accordingly, even in the PEG-SOD treatment group, so the PEG-SOD seems to be effective for certain periods during *in vitro* culture. In the experiments in which we injected PEG-SOD after transplantation, no significant differences in the apoptotic follicle ratios were observed across the different doses of PEG-SOD (Table 2).

The level of FSH is widely used as a biomarker of the ovarian reserve [24, 25]. As shown in Fig. 3, the FSH levels on day 2 were greatly higher than normal range (lower than 10 ng/ml [26]) due to the absence of negative feedback from ovary during the period between ovariectomy and ovarian tissue transplantation. However, as time went on, ovarian functions evaluated by measuring FSH level were recovered in the 7-day and 21-day groups. Although there were no significant differences in the levels of FSH between the groups, the FSH levels decreased over time, which indicated that the transplanted ovarian tissues functionally recovered after 7 days in case of mouse model. Restoration of the ovarian function post-transplantation was also indicated by the presence of antral follicles and corpora lutea in the ovarian tissue.

We did not check the estrous cycles of transplanted mice at that time of ovarian tissue collection because our observation was based on the days after transplantation rather than the estrous cycle. To diminish the errors that can be caused by different estrous cycles, we divided the mice randomly into the groups and increased the mice numbers for each group. Another limitation of this study is that there was no fresh control in both experiments. However, the dose-dependent effect of PEG-SOD on *in vitro* cultured and transplanted ovaries could be evaluated with comparison between non-treated control and PEG-SOD-treated groups.

In this study, we found that PEG-SOD preserved the quality of the ovarian follicles and prevented ischemic damage. However, the additional general functions of PEG-SOD remain unknown. Immunohistochemical experiments should be performed to analyze vascularization factors, such as CD31 and VEGF, and the molecular functions of other factors that are related to oxidative stress. Furthermore, the oocytes retrieved from the transplanted ovarian tissue treated with PEG-SOD should be assessed for their fertility potential including fertilization and live births.

In conclusion, we suggest that PEG-SOD can reduce the damage from oxidative stress during short-term *in vitro* culture and following ovarian tissue transplantation. However, additional detailed studies are required to elucidate the additional specific effects of PEG-SOD on ovarian tissue transplantation and *in vitro* culture.

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