Beneficial effect of desialylated erythropoietin administration on the frozen-thawed canine ovarian xenotransplantation

Hiroshi Suzuki · Takako Ishijima · Saori Maruyama ·
Yoshiko Yanagimoto Ueta · Yasuyuki Abe ·
Hideki Saitoh

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Abstract

Purpose The main drawback of ovarian cryopreservation followed by transplantation is that a large proportion of follicles are lost after transplantation. Thus, effects of erythropoietin (EPO) and desialylated EPO administration on the frozen-thawed canine ovarian xenotransplantation were examined.

Methods The protective and survival-promoting effects of EPO and desialylated EPO on the follicles of frozen-thawed canine ovaries after transplantation were examined using NOD-SCID mice. Frozen-thawed dog ovarian tissue with 400 U/kg of EPO or asialo EPO was placed into the ovarian bursa.

Results At 4 weeks after the transplantation, the ovaries were removed and subjected to histological examination. The survival rate of early primary follicles was 15.2% in the EPO group and 157.6% in the asialo EPO group, in contrast to 10.1% in the untreated group.

Conclusions These results demonstrate that administration of asialo EPO could be effectively used to enhance the survival of the follicles of transplanted cryopreserved ovaries.

Keywords Cryopreservation · Dog · Erythropoietin · Fertility · Follicle · Ovary · Transplantation

Introduction

Advances in the diagnosis and treatment of childhood, adolescent and adult cancer have greatly increased the life expectancy of premenopausal women with cancer, but have also resulted in a growing population of adolescent and adult long-term survivors of malignancies [1] with infertility problems due to induced premature ovarian failure [2]. Several options are currently available to preserve fertility in cancer patients and provide the opportunity for mothering when they have overcome their disease: embryo cryopreservation, oocyte cryopreservation or ovarian tissue cryopreservation [2]. Among these, cryopreservation of ovarian tissue is the only option available for prepubertal girls and woman in need of immediate chemotherapy [3–8]. The main drawback of ovarian cryopreservation followed by transplantation is that a large proportion of follicles are lost during the initial ischemia which occurs after transplantation [9–16]. Therefore, reducing the damage due to the ischemic interval between transplantation and revascularization is essential to maintaining both follicular reserve and the function of the graft.

Erythropoietin (EPO) is an acidic glycoprotein hormone which promotes the differentiation and proliferation of erythroid progenitor cells, and is mainly produced by the kidney. EPO plays a central role in maintaining erythrocyte homeostasis *in vivo*, and it is clinically used for the treatment of anemia as well as pre- and post-operative management. Furthermore, it is well established that EPO functions not only as a hematopoietic factor but also to inhibit apoptosis and/or protect tissues in nerve cells,

Capsule Administration of asialo EPO enhanced the survival of the follicles of transplanted cryopreserved ovaries.

H. Suzuki (✉) · T. Ishijima · S. Maruyama ·
Y. Yanagimoto Ueta · Y. Abe
Research Unit for Functional Genomics,
National Research Center of Protozoan Diseases,
Obihiro University of Agriculture and Veterinary Medicine,
Inada, Obihiro 080-8555, Japan
e-mail: hisuzuki@obihiro.ac.jp

H. Saitoh
Chugai Pharmaceutical Co., Ltd.,
Nihonbashimuromachi, Tokyo 103-8324, Japan

myocardial cells, renal proximal tubular epithelial cells, etc. [17, 18]. These two functions of EPO are attributed to two different signal transduction pathways. When EPO acts on the EPO receptor homodimer, it induces hematopoiesis through the intracellular JAK2 signal transduction pathway. When EPO acts on the heterodimer of the EPO receptor and a common β receptor, however, it induces an anti-apoptotic effect through the intracellular ERK1/2 signal transduction pathway [19].

Since xenotransplantation of cryopreserved ovarian tissue can be used to evaluate the tissue developmental potential before an elective retransplantation [20, 21], the protective and survival-promoting effects of EPO and desialylated EPO (asialo EPO), digested by neuraminidase [22], on the follicles of frozen-thawed canine ovary after transplantation were examined by using non obese diabetic-severe combined immunodeficient (NOD-SCID) mice. Since asialo EPO has a higher specific activity compared with intact EPO [22], effect of asialo EPO was examined in addition to EPO.

Materials and methods

Female and male NOD-SCID mice were purchased from a commercial supplier (CLEA Japan, Tokyo, Japan), and were bred in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All animals were housed in polycarbonate cages, and maintained in a specific pathogen-free environment in light-controlled (lights-on from 07:00 to 19:00) and air-conditioned rooms (temperature: $24 \pm 1^\circ\text{C}$, humidity: $50 \pm 10\%$). They had access to standard laboratory chow (CE-2; CLEA Japan) and water *ad libitum*. The ovaries from a 4-month-old dog were frozen-thawed and transplanted into the ovarian bursa of 8-wk-old NOD-SCID mice. The freezing and thawing procedures and ovarian transplantation were performed according to the method of Ishijima *et al.* [21]. Ovarian tissue was minced into 1.0–1.5 mm cubes, which were immersed in 1M dimethyl sulfoxide (DMSO) at room temperature for 60 s and then placed in a 1-ml cryotube (Nalge Nunc International KK, Tokyo, Japan) containing 5 μl of DMSO, and the tube was cooled on ice for 5 min. After addition of DAP 213 (2M DMSO, 1M acetamide, 3M propylene glycol) solution [23] precooled on ice, the tube was cooled on ice for 5 min and immersed into liquid nitrogen. For thawing, the tube was removed from the liquid nitrogen, the liquid nitrogen in the tube was discarded and then the tube was allowed to stand at room temperature for 60 s. After the addition of 900 μl of 0.25M sucrose prewarmed to 37°C into the tube, the suspension was quickly stirred by mild pipetting and washed with PBI [24] five times. A portion of the extirpated ovaries

was fixed with 10% formalin to prepare pre-transplant ovarian tissue samples.

Adult NOD-SCID mice ($n=9$) were anesthetized by intraperitoneal administration of sodium pentobarbital (5 mg/ml, Nembutal, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and then the dorsal skin was incised to draw out the ovaries. An incision was made in the lateral side of each ovary to remove the mouse ovary in the ovarian bursa, leaving a part to ensure blood flow to a dog ovarian xenograft after transplantation, and a piece of frozen-thawed dog ovarian tissue was placed there (i.e. into the ovarian bursa). A hemostatic gelatin sponge (Spongel, S022Y01, Astellas, Japan) soaked with 400 U/kg of EPO (r-hu-EPO; EPOGIN S1500, Chugai Pharmaceuticals, Tokyo, Japan) or asialo EPO [22] was also placed into the ovarian bursa. As a control, Spongel soaked with an equivalent amount of physiological saline was placed into the ovarian bursa. The skin incision was closed with a clip (9-mm auto clip, 427631, Becton Dickinson). The operated mice were placed on a warm plate until sufficient recovery had occurred to allow movement. At 4 weeks after the operation, the transplanted ovaries were removed and fixed with 10% formalin and subjected to hematoxylin and eosin staining together with the pre-transplant ovarian tissue samples. To evaluate the effects of EPO and asialo EPO, follicles that visibly contained an ovum (oocyte) with a nucleus were counted according to the classification of Oktay *et al.* [25] as follows. Primordial follicles comprise follicles containing an oocyte partially or completely encapsulated by squamous pregranulosa cells; early primary follicles are follicles in which at least one of the pregranulosa cells had become columnar (enlarged); primary follicles are follicles in which all of the granulosa cells exhibit enlargement and a single layer of granulosa cells; transitional follicles comprise follicles containing an oocyte encapsulated by a 1–2 layer of columnar granulosa cells; preantral follicles are made up of follicles containing an oocyte encapsulated by more than 2 layers of granulosa cells with no antrum formation; antral follicles are follicles containing an oocyte encapsulated by more than 2 layers of granulosa cells with antrum formation. For pre-transplant ovarian tissues, ten tissue samples were randomly selected and the number of follicles in the ten tissue samples was counted. The number of follicles in a circle of 900 μm in diameter, i.e., a view field of 0.64 mm^2 , containing the highest number of follicles in each selected tissue sample was counted (for a total of 10 view fields). This number was recorded as the number of follicles before transplantation. For transplanted ovarian tissue, five sections (7 μm in thickness) were sequentially prepared for a tissue specimen (a block). A total of six graft samples were examined in each experimental group. The distance between sections was 40–50 μm . The number of follicles in a circle of 900 μm in

diameter, i.e., a view field of 0.64 mm^2 , containing the highest number of follicles in each section, was counted (in a total of 5 fields of view). The survival rates of follicles were calculated as number of follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples $\times 100$. Statistical analysis was performed by using Wilcoxon's signed rank test. P values less than 0.05 were considered to be significant.

The tissues and animals used in this study were treated under the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine.

Results

The average number of primordial, early primary, primary, transitional, preantral and antral follicles per 0.64 mm^2 in frozen-thawed ovarian sections was 14.8 ± 11.9 , 3.3 ± 2.1 , 4.1 ± 1.9 , 3.6 ± 1.7 , 0.8 ± 0.7 and 0, respectively. As shown in Table 1, the average number of primordial follicles per 0.64 mm^2 in ovarian sections was 0.3 ± 0.31 in the untreated control group, 0.4 ± 0.57 in the EPO group, and 3.9 ± 2.47 in the asialo EPO group at 4 weeks after transplantation, in contrast to 14.8 ± 11.9 before transplantation, which indicated the asialo EPO group in particular showed a significantly higher survival rate (26.6%) as compared with the untreated group (2.3%). The survival rate of early primary follicles was 15.2% in the EPO group and 157.6% in the asialo EPO group, in contrast to 10.1% in the untreated group. The proportion of early primary follicles to total follicles in untreated, EPO and asialo EPO group was 43, 42, and 54%, respectively. These results clearly indicate the primordial follicles have partially grown into early primary follicles in the asialo EPO group.

Moreover, it was found that the asialo EPO group had a tendency to higher survival rates of primary follicles and transitional follicles as compared with the untreated group.

These results demonstrate that administration of EPO, especially asialo EPO, can be effectively used for the enhancement of survival of transplanted organ tissues.

Discussion

It is believed that the reason the primordial follicle is observably resistant to cryoinjury is because the oocyte it contains has a relatively inactive metabolism, as well as the lack of meiotic spindle, zona-pellucida and cortical granules [2]. In fact, a high percentage of oocytes as well as granulosa cells survive the cryopreservation and thawing procedure [21, 26–28]. Our previous study has shown that there was no difference in morphology and in the average number of primordial and primary follicles between the vitrified-warmed by DAP213 and fresh ovarian tissues in dog [21]. Recovery rates of the grafts in cryopreserved ovarian tissues were equivalent and much better than those in fresh ovarian tissues when the tissues were transplanted to NOD-SCID mice and recovered at 4 weeks post-operation [21]. Also, it has been shown that proliferating cell nuclear antigen was detectable in many of the granulosa cells in the primary follicles of the grafts when canine ovarian tissues were cryopreserved by DAP213 and transferred into ovarian bursa of NOD-SCID mice [21]. However, a majority of primordial follicles in frozen-thawed canine ovarian tissues reportedly disappears after transplantation (Table 1). The main reason for the follicular loss after cryopreservation and xenografting seems to be the ischemic effect which takes place after transplantation rather than cryopreservation *per se* [16, 29]. Several attempts have been

Table 1 Effect of erythropoietin administration on the average number of follicles and the survival rate of follicles relative to the number of follicles in ovarian tissues before transplantation in frozen-thawed canine ovary at 4 weeks after transplantation

Exp. Group	Classification of follicle (% of survival)					
	Primordial	Early primary	Primary	Transitional	Preantral	Antral
Untreated	0.3 ± 0.31^a (2.3)	0.3 ± 0.42^a (10.1)	0.1 ± 0.23^a (3.3)	0 ^a (0)	0 (0)	0 -
EPO	0.4 ± 0.57^a (2.7)	0.5 ± 0.71^a (15.2)	0.3 ± 0.42^a (7.3)	0 ^a (0)	0 (0)	0 -
AsialoEPO	3.9 ± 2.47^b (26.6)	5.2 ± 3.62^b (157.6)	0.4 ± 0.53^a (9.8)	0.1 ± 0.12^a (1.9)	0 (0)	0 -

The results are shown as the mean $\pm$ SD. The different superscript letters within a column indicate significantly different values ($P < 0.05$). Total six graft samples were examined in each experimental group. Five sections ($7 \mu\text{m}$ in thickness) were sequentially prepared for a tissue specimen (a block). The distance between sections was $40\text{--}50 \mu\text{m}$. The number of follicles in a circle of $900 \mu\text{m}$ in diameter, i. e., a view field of 0.64 mm^2 , containing the highest number of follicles in each section, was counted (in a total of 5 fields of view). The survival rates (% of survival) of follicles were calculated as number of follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples $\times 100$

made to prevent or at least decrease the follicular loss of cryopreserved ovarian tissues after transplantation. However, an effective solution has not been found to date. It has been reported that functional vessels within the graft were detected by both magnetic resonance imaging and histological exam from day 7 onwards when rat ovaries were transplanted into the muscles of castrated nude mice [30]. Kim *et al.*, [31] showed that the ovarian tissue could tolerate ischaemia for at least 2 h at 0°C or at room temperature, and that a water soluble antioxidant (ascorbic acid) reduces apoptosis in ovarian cortex by up to 24 h in the case of incubation *in vitro*. It has been reported that treatment with vitamin E, a lipid soluble antioxidant, improved the survival of follicles in ovarian grafts by reducing ischemic injury [32]. Prolonged exogenous stimulation promoted primordial follicle maturation but also caused a loss of primordial follicles in xenotransplanted frozen-thawed ovaries [28, 33]. Our present study clearly shows that the administration of asialo EPO is effective for enhancing the survival of transplanted cryopreserved ovarian follicles (Table 1). In addition, the increasing number of early primary follicles in the frozen-thawed ovarian tissues treated with asialo EPO indicates a growth promoting effect of asialo EPO for primordial follicles. It is well known that EPO functions not only as a hematopoietic factor, but also inhibits apoptosis and/or protects several kinds of cells such as nerve [18] and myocardial cells [17]. However, a protective effect of EPO for ovarian or follicular cells has not been reportedly demonstrated both *in vitro* and *in vivo*. The reason why the protective effects of asialo EPO for follicular cells were much higher than those in EPO (Table 1) seems to be related with the finding that asialo EPO showed a four-times-higher specific activity *in vitro* compared with intact EPO [22]. Asialo EPO binds to its receptor faster than the intact form [22]. Although it is still unclear how asialo EPO initiates the differentiation and proliferation of the ovarian follicular cells, previous study indicates that female reproductive organs/tissues including ovarian follicles at various stages express EPO receptor, and that signal transduction of EPO contributes to the cyclic changes in the female reproductive organs [34]. In conclusion, the administration of asialo EPO appears to be effective for the prevention of follicular loss in frozen-thawed ovary after transplantation.

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