—Brief Note—

Effect of Intraperitoneal Administration of Desialylated Erythropoietin on the Follicular Survival in Cryopreserved Canine Ovaries after Xenotransplantation

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Abstract: *The effect of intraperitoneal administration of desialylated erythropoietin (asialo EPO) on the follicular reserve of cryopreserved canine ovarian tissues was examined using non obese diabetic-severe combined immunodeficient (NOD-SCID) mice was examined. Vitrified-warmed canine ovarian tissues were placed under the dorsal skin of mice, and were then treated with saline or 400 or 4,000 U/kg/day of asialo EPO by intraperitoneal injection from one day before transplantation for 5 days. Transplanted tissues were removed and subjected to a histological examination 4 weeks after the transplantation. Without asialo EPO treatment, the mean number of primordial follicles in the cryopreserved ovarian tissues had decreased from 4.3/mm^2 to 0.2/mm^2 at 4 weeks after xenotransplantation. However, higher numbers of follicles were observed when recipient mice were treated with asialo EPO. The average number of primordial follicles per square millimetre of ovarian section was 0.2 in the saline group and 1.1 in the 400 U/kg/day of asialo EPO at 4 weeks after transplantation. These results indicate that the systemic administration of asialo EPO could be effectively used to enhance the survival of the follicles of transplanted cryopreserved canine ovaries.*

Key words: *Canine, Cryopreservation, Ovary, Transplantation*

Introduction

Progress in the diagnosis and treatment of cancer has created 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 such as embryo, oocyte or ovarian tissue cryopreservation are currently available for preserving the fertility of cancer patients. However, cryopreservation of ovarian tissue is the only option available for prepubertal girls and women in need of immediate chemotherapy [3–9]. The cryopreservation of ovarian tissues is also a potentially significant technology for the preservation of the genetic resources of working dogs as well as other laboratory and domestic animals [10, 11]. However, it has been demonstrated that a large proportion of follicles are lost during the initial ischemia which occurs after transplantation of murine [12, 13], ovine [14, 15], canine [11, 16] and human ovaries [17, 18]. Several attempts have been made to prevent or at least decrease the follicular loss of cryopreserved ovarian tissues after transplantation [19, 20]. Recently, it was reported that the survival of follicles in cryopreserved canine ovaries was significantly improved when the grafted tissues in recipient immuno-deficient mice were locally treated with desialylated erythropoietin (asialo EPO) [11]. It is well established that EPO functions not only as a hematopoietic factor but also as an inhibition of apoptosis and/or protects tissues in nerve cells, myocardial cells, renal proximal tubular epithelial cells, etc [21, 22]. Asialo EPO, digested by neuraminidase, has a higher specific activity than intact EPO [23]. In this study, the effect of constitutional administration (intraperitoneal injection) of asialo EPO on the follicular survival in cryopreserved canine ovaries after xenotransplantation was examined.

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Materials and Methods

Female non obese diabetic-severe combined immunodeficient (NOD-SCID) mice were purchased from a commercial supplier (Charles River Japan, Kanagawa, Japan). All animals were housed in polycarbonate cages, and maintained under a specific pathogen-free environment in light-controlled (lights-on from 07:00 to 19:00) and air-conditioned rooms (temperature: 24 ± 1 °C, humidity: 50 ± 10%). They had free access to standard laboratory chow (CA-1; CLEA Japan, Tokyo, Japan) and water *ad libitum*. Canine ovaries were obtained from healthy bitches undergoing routine ovariohysterectomy at local veterinary clinics in Obihiro. The ovaries from a 5-month-old mixed breed, a 5-month-old pug, 5-month-old and 6-month-old Yorkshire terriers and a 6-month-old toy poodle were transported to the laboratory within one hour of removal in a vacuum flask containing physiological sterile saline supplemented with 100 IU/ml penicillin (Callbiochem, La Jolla, CA , USA) at 37 °C, and were vitrified-warmed. The cryopreservation procedures were performed according to the method of Ishijima *et al.* [10]. Briefly, ovarian tissue was minced into 2.0–2.5 mm cubes, which were immersed in 1 M dimethyl sulfoxide (DMSO, SIGMA, St. Louis, MO, USA) at room temperature for 60 seconds, placed in a 1-ml cryotube (Nalge Nunc International KK, Tokyo, Japan) containing 5 μl of DMSO, and cooled on ice for 5 min. After addition of DAP 213 (2 M DMSO, 1 M acetamide, 3 M propylene glycol) solution [24] precooled on ice, the tube was cooled on ice for a further 5 min, then immersed in liquid nitrogen. For warming, the tube was removed from the liquid nitrogen, and the liquid nitrogen in the tube was discarded, the tube was then allowed to stand at room temperature for 60 sec. After the addition of 900 μl of 0.25 M sucrose (SIGMA, St. Louis, MO, USA) prewarmed to 37 °C, the suspension was quickly stirred by mild pipetting and washed with modified Dulbecco's phosphate buffered saline [25] five times. A portion of the excised ovaries was fixed with 10% formalin to prepare pre-transplant ovarian tissue samples.

NOD-SCID mice (6 to 9-week-old, n=11) were anesthetized by intraperitoneal administration of sodium pentobarbital (5 mg/ml, Nembutal, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), 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 from the ovarian bursa, and two pieces of vitrified-warmed canine ovarian tissues were introduced under the right and left sides of the dorsal skin (subcutaneous transplantation). 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 recovery had sufficiently occurred to allow movement. Mice were treated with 400 or 4,000 U/kg/day of desialylated erythropoietin (asialo EPO, Chugai Pharmaceuticals, Tokyo, Japan) by intraperitoneal injection from one day before the transplantation for 5 days. These doses were chosen as the minimum effective dose of EPO for tissues characterized by tight endothelial barriers is ~500 U/kg body weight administered intraperitoneally or intravenously and asialo EPO activates tissue protection without stimulating hematopoiesis [26]. Four 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 freezing and thawing, and subsequent xenotransplantation, follicles that visibly contained an ovum (oocyte) with a nucleus were counted according to the classification of Myers *et al.* [27]. Primordial follicles were defined as an oocyte surrounded by a partial or complete layer of squamous granulosa cells. Primary follicles showed a single layer of cuboidal granulosa cells. Secondary follicles were surrounded by more than one layer of cuboidal granulosa cells, with no visible antrum. Antral follicles possessed a clearly defined antral space. For ovarian tissue, about ten sections (5 μm in thickness) were sequentially prepared for a tissue specimen (a block). A total of 8-16 graft samples were examined for each experimental group. The distance between sections was 100 μm. The number of follicles in each section was counted, and the area of each section was calculated using ImageJ 1.41 software bundled with Java 1.6.0-10. The number of follicles in each stage in each section was expressed per square millimetre. The survival rates of follicles were calculated as the number of follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples × 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 in accordance with the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine.

Table 1. Follicular density of canine ovarian tissues before and after vitrification

Donor breed and age	Exp. Group	No. of follicles /mm^2 (Mean ± SD)				
		Primordial	Primary	Secondary	Antral	Total
Mixed	Fresh	13.5 ± 22.3^a	2.1 ± 2.0^a	0.3 ± 0.5^a	0^a	15.9 ± 24.0^a
5M	Cryo*	9.8 ± 17.2^a	2.5 ± 4.7^a	0.1 ± 0.5^a	0^a	12.4 ± 18.2^a
Pug	Fresh	1.1 ± 4.3^a	1.1 ± 1.7^a	0.6 ± 1.0^a	0^a	2.7 ± 5.5^a
5M	Cryo	1.0 ± 2.1^a	0.8 ± 1.3^a	0.3 ± 0.5^a	0^a	2.2 ± 3.4^a
Poodle	Fresh	4.1 ± 7.4^a	2.1 ± 1.9^a	1.4 ± 1.2^a	0^a	7.6 ± 8.7^a
6M	Cryo	4.4 ± 7.2^a	2.5 ± 2.0^a	1.1 ± 1.2^a	0.1 ± 0.1^a	8.0 ± 8.4^a
Yorkshire terrier	Fresh	19.3 ± 23.9^a	6.0 ± 6.4^a	0.2 ± 0.5^a	0^a	25.5 ± 28.2^a
6M	Cryo	17.5 ± 37.6^a	1.9 ± 2.3^b	0.2 ± 0.6^a	0^a	19.5 ± 38.4^a
Yorkshire terrier	Fresh	10.3 ± 17.3^a	4.4 ± 5.7^a	0.0 ± 1.6^a	0^a	14.7 ± 22.8^a
5M	Cryo	9.4 ± 14.6^a	3.9 ± 2.5^a	0.8 ± 0.9^a	0^a	14.1 ± 15.8^a

*: Crypreserved ovarian tissues. In total, four ovarian tissues (2.0–2.5 mm cubes) were examined in each experimental group for each donor.

Table 2. Effect of asialo EPO administration on the follicular survival in cryopreserved canine ovarian tissues at 4 weeks after xenotransplantation

Exp. Group	No. of tissues examined	No. of follicles /mm^2 (Mean ± SD)				
		Primordial	Primary	Secondary	Antral	Total
Fresh	8	6.8 ± 16.4^a	1.6 ± 1.9^a	0.4 ± 0.8^a	0	8.8 ± 17.8^a
cryopreserved	8	4.3 ± 13.1^a	1.5 ± 3.6^a	0.3 ± 0.5^a	0	6.0 ± 14.2^a
Saline	12	0.2 ± 0.6^b	0.1 ± 0.3^b	0$^{b, c}$	0	0.3 ± 0.2^b
400 U/kg/day	16	1.1 ± 3.5^b	0.3 ± 1.1^c	0.0 ± 0.2^b	0	1.4 ± 4.3^c
4,000 U/kg/day	16	1.0 ± 2.6^b	0.2 ± 0.6$^{b, c}$	0^c	0	1.2 ± 3.1^b

Different superscripts within a column indicate significantly different values (P<0.05).

Results

As shown in Table 1, the average number of primordial, primary, secondary and antral follicles and the ratio of each developmental stage of follicles in fresh ovarian sections from the five bitches were considerably varied. Namely, the mean numbers of primordial follicles per square millimetre in the fresh ovary ranged from 1.1 ± 4.3 to 19.3 ± 23.9 among donors. However, differences in the number of follicles in each stage between pre- and post-vitrification were limited in the five donor bitches. A significant difference was only seen in the mean number of primary follicles between fresh and cryopreserved ovarian tissues from the 6-month-old Yorkshire terrier. These results indicate that the follicular loss of the canine ovary was not drastically extended by the vitrification procedure itself. On the other hand, the detrimental effect of transplantation on the follicular survival was remarkable. As shown in Table 2, the mean number of primordial follicles in cryopreserved ovarian tissues reduced from 4.3 ± 13.1/mm^2 to 0.2 ± 0.6/mm^2 at 4 weeks after xenotransplantation. However, higher numbers of primordial and primary follicles were observed when recipient mice were treated with asialo EPO. The mean number of primordial follicles per square millimetre in ovarian sections was 0.2 ± 0.6 in the control (saline) group and 1.1 ± 3.5 in the 400 U/kg/day of asialo EPO at 4 weeks after transplantation. This number was 4.3 ± 13.1 before transplantation, which indicates primordial follicles in the asialo EPO (400 U/kg/day) had a higher survival rate (25.6%) than the control group (4.7%). Similarly, the average number of primary follicles per square millimetre in ovarian sections was 0.1 ± 0.3 in the saline group and 0.3 ± 1.1 in the 400 U/kg/day of asialo EPO group at 4 weeks after transplantation, in contrast to 1.5 ± 3.6 before transplantation, indicating primary follicles in the asialo EPO (400 U/kg/day) had a significantly higher survival rate (20.0%) than the control group (6.7%). The effect of the higher dose (4,000 U/kg/day) of asialo EPO was not significantly different from that of the low dose, 400 U/kg/day, of asialo EPO administration in this experiment (Table 2).

Discussion

Several reports including our present study (Table 1) have indicated that follicular loss in canine ovaries is not drastically altered by the cryopreservation procedure itself [10, 11, 16]. Previously, we showed that there was no difference in the morphology and in the average number of primordial and primary follicles between tissues vitrified and warmed in DAP213 and fresh ovarian canine tissues [10]. It is believed that the reason why primordial follicles are observably resistant to cryoinjury is because the oocytes they contain have a relatively inactive metabolism, and lack a 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 [10, 28–30]. The main reason behind the lack of success in transplantation following ovarian cryopreservation is that a large proportion of follicles are lost during the initial ischemia which occurs after transplantation. Most of the follicles which survive cryopreservation are lost due to ischemia during the time required for neovascularisation [31]. Several attempts have been made to prevent the follicular loss of cryopreserved ovarian tissues after transplantation. Treatment with a water soluble antioxidant (ascorbic acid) reduced apoptosis in the ovarian cortex *in vitro* [19]. However, local application of sphingosine-1-phosphate, an apoptosis inhibitor, did not prevent follicular loss after transplantation in sheep [32]. 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 [20]. Glutathione and uninastatin can improve the survival rate of follicles in human ovarian tissue after xenotransplantation [33]. More recently, we have shown that both local [11] and systemic administration (Table 2) of desialylated erythropoietin are effective for maintaining the survival of follicles in cryopreserved canine ovarian tissues after xenotransplantation. In both the local and systemic administration, asialo EPO was effective in maintaining the earlier stage follicles such as primordial and primary follicles. There are several reports on the extra-hematopoietic functions of EPO and its nonhematopoietic derivate, and EPO and asialo EPO may act to prevent apoptosis, stimulate protective molecules such as BDNF or inhibit proinflammatory cytokines [26, 34].

Despite these findings, a fully effective solution for the loss of follicles in transplanted tissues has not been found to date and further studies will be required to elucidate methods for enhancing the survival of transplanted cryopreserved ovarian follicles in canine as well as other mammalian species.

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