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ANIMAL EXPERIMENTATION

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

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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.

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

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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,

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±1°C, humidity: 50±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 x 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]	0.3±0.42 [a]	0.1±0.23 [a]	0 [a]	0	0
	(2.3)	(10.1)	(3.3)	(0)	(0)	-
EPO	0.4±0.57[a]	0.5±0.71 [a]	0.3±0.42 [a]	0 [a]	0	0
	(2.7)	(15.2)	(7.3)	(0)	(0)	-
AsialoEPO	3.9±2.47[b]	5.2±3.62[b]	0.4±0.53 [a]	0.1±0.12 [a]	0	0
	(26.6)	(157.6)	(9.8)	(1.9)	(0)	-

The results are shown as the mean±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 μm in thickness) were sequentially prepared for a tissue specimen (a block). 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 survival)of follicles were calculated as number of follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples x 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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NOTE *Bacteriology*

Enzyme-Linked Immunosorbent Assay for Screening of Canine Brucellosis Using Recombinant Cu-Zn Superoxide Dismutase

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ABSTRACT. *Brucella canis*, a facultative intracellular pathogen, is the causative agent of canine brucellosis. The diagnosis of canine brucellosis is based on bacteriological examination and serological methods including agglutination and gel diffusion tests. In this study, recombinant *B. canis* Cu-Zn superoxide dismutase (SOD) was used as an antigen for the enzyme-linked immunosorbent assay (ELISA). The recombinant SOD showed a specific reaction with serum infected with *B. canis* in Western blotting and ELISA. These results suggest that ELISA using recombinant SOD is useful in screening for canine brucellosis.
KEY WORDS: brucellosis, Cu-Zn superoxide dismutase, ELISA.

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Canine brucellosis is widely distributed around the world and is an important disease due to the economic losses it causes in animal production, and the risks to human health [7]. Reproductive disorders, such as abortions and premature births, are the clinical signals of this bacterial disease in pregnant animals. Diagnosis of the disease is based on bacteriological examination and serological tests [4]. Serological diagnosis is usually performed by the tube agglutination test, rapid slide agglutination test, and gel immunodiffusion test [4–6]. However, agglutination tests sometimes give false-positive reactions due to cross-reactions with other pathogens, and a general strategy for eliminating such cross-reactions is to use purified antigen with unique epitopes. We previously reported that a method in which crude hot saline extracted antigens are coated on to latex beads would be useful in the serological diagnosis of canine brucellosis [12]. Among the antigens extracted in this way, Cu-Zn superoxide dismutase (SOD) showed the strongest antigenic reaction [12]. In the present paper, we report a screening method for canine brucellosis using an enzyme-linked immunosorbent assay (ELISA) with recombinant SOD as antigen.

The gene encoding SOD was amplified from chromosomal DNA isolated from *B. canis* by means of PCR with 5'-GTGATGAAGTCCTTATTTATT-3' and 5'-TTATTCGATCACGCCGCAGGC-3' used as the pair of primers. The product was cloned into pCold TF vector (Takara Bio Inc., Shiga, Japan). The trigger factor (TF) and His-tagged SOD was expressed in the *E. coli* strain DH5α, and its purification was performed as described by the manufacturer (Novagen, Darmstadt, Germany). The antigen solution was separated using 10% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore, Billerica, MA, U.S.A.). The efficiency of transfer was determined using Coomassie brilliant blue R-250, and then the membranes were tested for reactivity with antibodies in canine sera. The tube agglutination test was performed as follows. Heat-inactivated *B. canis* QE-13 whole-cell antigens were obtained from Kitasato Laboratories. Equal volumes (0.5 m*l*) of the whole-cell antigens (optical density of 0.8 at 450 nm) and serum, which had been serially diluted 2-fold with PBS, were incubated at 50°C for 24 hr. Agglutination titers were determined from the final dilution of serum showing 50% agglutination. Samples showing a titer higher than 160 were considered to be positive [3, 10]. ELISA using recombinant SOD was performed as follows. To coat the recombinant SOD onto immunoplates for ELISA, 50 μl of it (50 μg/m*l*) was added to a 96-well Immuno plate (Nunc, Rochester, NY, U.S.A.) and left overnight at 4°C. Then, the wells were blocked using 0.5% bovine serum albumin (BSA) for 30 min. Sera diluted 1/200 were added to the wells. The wells were incubated at 37°C for 1 hr, were washed, and then horseradish peroxidase-labeled anti-dog IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added. The wells were incubated at 37°C for 1 hr, were washed, and a substrate, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA (SIGMA, St. Louis, MO, U.S.A.), was added. The absorbance was measured at 405 nm by an ELISA reader (model 450, Bio-Rad, Hercules, CA, U.S.A.).

To identify sera infected with B. canis, we performed the tube agglutination test on canine serum samples (*n*=224) randomly selected from dogs consecutively admitted to animal hospitals in Korea by hospital staff. In the test, antibodies to *B. canis* were detected in 30 of the 224 serum samples (Table 1). Recombinant SOD was subjected to Western blotting together with dog serum that had tested positive and negative in the tube agglutination test to test for antigenic

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Table 1. Serological analysis of canine sera

	TAT[a)]+ 30 (13.4%)		TAT- 194 (86.6%)	
	ELISA[b)]+	ELISA–	ELISA+	ELISA–
Sera (n=224)	30 (100%)	0 (0%)	2 (1.0%)	192 (99.0%)
WB[c)]+ in ELISA+	26 (86.7%)	–	0 (0%)	–

a) Tube agglutination test.
b) Samples with absorbance values of over 0.400 (OD_{405}).
c) Western blot analysis.

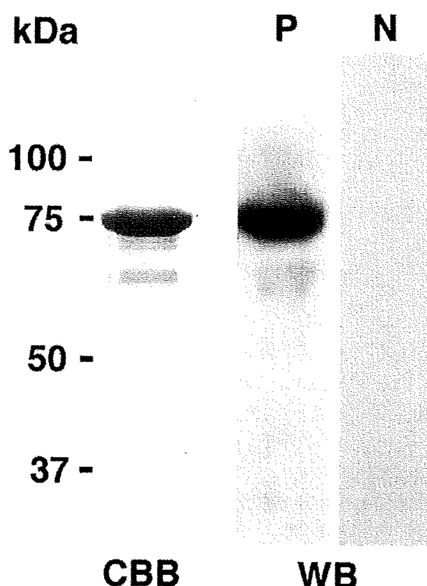

Fig. 1. Western blot analysis of recombinant SOD. TF fusion SOD was separated by SDS-PAGE under reduced condition and then transferred to nylon membranes. The membranes were stained with Coomassie brilliant blue (CBB) and used for analysis of *B. canis* infected (P) and uninfected (N) sera (WB).

reactivity. The recombinant SOD showed strong reactivity with positive sera, but not with negative sera (Fig. 1). These results indicated that the recombinant SOD reacted specifically with *Brucella* infected sera. We then determined whether ELISA using recombinant SOD can be applied to screening for canine brucellosis using the dog serum samples from Korea. All serum samples having absorbance values of over 0.410 or under 0.400 (OD_{405}) were either positive or negative in the tube agglutination test (Fig. 2). Six serum samples having an absorbance value between 0.410 and 0.401 (OD_{405}) produced a mixed result, and the tube agglutination test detected antibodies to *B. canis* in 4 of them (Fig. 2). Immunoreactive bands were detected for sera having an absorbance value of over 0.41 (OD_{405}) in Western blotting (Table 1). Thus, the absorbance measurements in ELISA with recombinant SOD and the titers in the tube agglutination test showed a similar tendency, suggesting that ELISA with recombinant SOD is useful in screening for canine brucellosis. However, for the serum samples having low absorbance values of around 0. 400 (OD_{405}), it was difficult to judge whether they were positive or negative for canine brucellosis.

The tube agglutination test using whole *B. canis* antigens has been used to diagnose brucellosis in dogs in Japan [10]. However, it has been noted that nonspecific reactions occur in a tube agglutination test using whole bacterial cell antigens as well as in the rapid slide agglutination test [2]. More recently, with the aim of developing a serological diagnosis method that is easier to perform, we coated latex beads with antigens extracted by hot saline for use in the agglutination test [12]. However, the crude antigens extracted in our method were not specific ones and therefore not useful in a highly sensitive serodiagnosis method such as ELISA [12]. Since SOD is a known antigenic protein of *B. abortus* [1], its potential value as a vaccine for brucellosis prevention and as a diagnostic reagent for the disease has been investigated [8, 9, 11], and the results in our study showed that recombinant SOD was useful for the detection of canine brucellosis. Further, since ELISA can be used for very small serum samples and handle many samples at a time, it would be suitable for the screening of valuable samples such as those from wildlife or small animals. Besides, conventional serological and bacteriological tests would be needed to diagnose for canine brucellosis.

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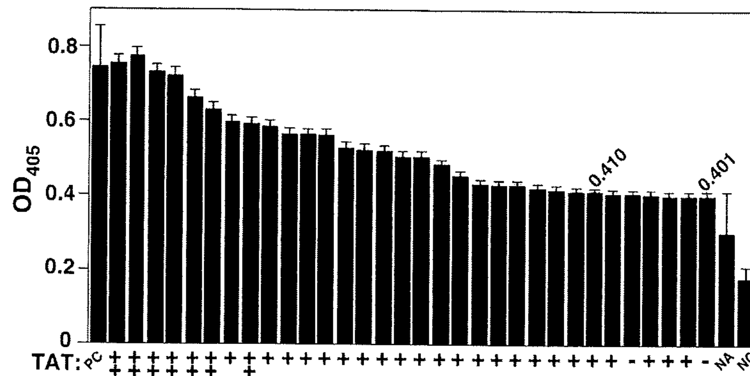

Fig. 2. ELISA absorbance values of dog sera using recombinant SOD. The bars indicate samples with absorbance values of over 0.400 (OD_{405}). Values are averages and standard deviations of triplicate wells from three identical experiments. PC, NC and NA indicate average absorbance value of ten positive control (infected) sera, ten negative control (uninfected) sera and the tube agglutination test negative sera (194 samples). Titers for the tube agglutination test (TAT) with *B. canis* antigen are indicated as—(<1:80), +(1:160), and ++(1:320), respectively.

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—Brief Note—

Follicular Loss of the Cryopreserved Canine Ovary after Xenotransplantation

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Abstract: *The effect of cryopreservation and subsequent xenotransplantation on the follicular reserve of the canine ovary by using non obese diabetic-severe combined immunodeficient (NOD-SCID) mice was examined. Vitrified-warmed canine ovarian tissues were placed into the ovarian bursa of mice, and then were removed and subjected to histological examination at 4 weeks after the transplantation. Over 30% of primordial follicles and 65% of early primary follicles survived after cryopreservation. However, regardless of breed or age, percentages of survived primordial follicles and early primary follicles after the transplantation ranged from 0–7% and from 0–15%, respectively. These results indicate that the majority of primordial follicles and early primary follicles in vitrified-warmed canine ovarian tissues disappear after xenotransplantation. Further studies will be required to be able to enhance the survival of transplanted cryopreserved ovarian follicles in canine.*

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

Introduction

Advances in the diagnosis and treatment of cancer have 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]. Although several options are currently available to preserve fertility in cancer patients, cryopreservation of ovarian tissue is the only option available for prepubertal girls and women in need of immediate chemotherapy [3–8]. On the other hand, the cryopreservation of ovarian tissues is a potentially significant technology for the preservation of the genetic resources of working dogs as well as other target animals [9]. However, it has been reported that a large proportion of follicles are lost during the initial ischemia which occurs after transplantation of mouse [10, 11], sheep [12, 13] and human ovaries [14, 15]. Thus, to corroborate the evidence for possible follicular loss after transplantation in canine ovarian tissues, we examined the effect of cryopreservation and subsequent xenotransplantation on the follicular reserve of the canine ovary by using non obese diabetic-severe combined immunodeficient (NOD-SCID) mice.

Materials and Methods

Female NOD-SCID mice were purchased from a commercial supplier (Charles River Japan, Kanagawa, 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 ± 1°C, humidity: 50 ± 10%). They had access to standard laboratory chow (CE-2; CLEA Japan, Tokyo, Japan) and water *ad libitum*. The ovaries from 5-month-old and 6 month-old mixed breeds, a 4-month-old miniature dachshund and a 11-month-old toy poodle were frozen-thawed and transplanted into the ovarian bursa of 8-wk-old NOD-SCID mice. The cryopreservation procedures and ovarian transplantation were performed according to the method of Ishijima *et al.* [9]. Briefly, ovarian tissue was minced into 1.0–1.5 mm cubes, which were immersed in 1 M dimethyl sulfoxide (DMSO) at room temperature for 60 sec 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 (2 M DMSO, 1 M acetamide, 3 M propylene glycol) solution [16] precooled on ice, the tube was cooled on ice for 5 min

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then immersed in liquid nitrogen. The grafts were stored in the liquid nitrogen for 2–6 months. For warming, 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 sec. After the addition of 900 μl of 0.25 M sucrose prewarmed to 37°C into the tube, the suspension was quickly stirred by mild pipetting and washed with PBI [17] five times. A portion of the excised ovaries was fixed with 10% formalin to prepare pre-transplant ovarian tissue samples.

NOD-SCID mice (n = 13) 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 in the ovarian bursa, leaving a part to ensure blood flow to the canine ovarian xenograft after transplantation, and a piece of frozen-thawed canine ovarian tissue was introduced into the ovarian bursa. A hemostatic gelatin sponge (Spongel, S022Y01, Astellas, Japan) was also placed in 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 freezing and thawing, and subsequent xenotransplantation, follicles that visibly contained an ovum (oocyte) with a nucleus were counted according to the classification of Oktay *et al.* [18] 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 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 for 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 the number of follicles in pre-transplant ovarian tissues / number of follicles in fresh ovarian tissue samples × 100, and the number of follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples × 100.

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

On autopsy, cryopreserved canine xenografts were distinguishable and were recovered in 13 of 13 mice (100%), and were identified in 20 of 26 (78%) of the transplanted sites. The average number of primordial, early primary, primary, transitional, preantral and antral follicles and the ratio of each developmental stage of follicles in fresh ovarian sections from the four bitches were varied. Namely, the mean numbers of primordial, early primary, primary, and transitional and preantral follicles per 0.64 square millimeter in the fresh ovary of the 6-month-old mixed breed were 38.4, 2.0, 0.2, and 0, respectively. While, for the 5-month-old mixed breed, the mean numbers of primordial, early primary, primary, transitional and preantral follicles per 0.64 square millimeter in fresh ovary of 6-month-old mixed breed were 8.2, 1.0, 2.4, 1.7 and 1.2 respectively. There were individual differences in the developmental stage of oocytes rather than the breed and/or age differences (Table 1). In addition, it seems that follicles are unevenly distributed in ovarian tissues, because the numbers of primordial and early primary follicles in the 5-month-old mixed breed and the 11-month-old toy poodle showed an increase in the number of follicles after cryopreservation. Namely, survival rates of primordial and early primary follicles after the

Table 1. Follicular loss of canine ovary after cryopreservation and subsequent xenotransplantation at 4 weeks after transplantation

Breed and age	Exp. Group	Mean number of follicles (/0.64 mm^2)					
		Pri mordial	Early primary	Primary	Transitional	Pre antral	Antral
Mixed	Fresh	38.4	2.0	0.2	0	0	0
6M	Cryo*	11.6	1.3	0.3	0	0.1	0
	Tp**	0.8	0.2	0.1	0.1	0.1	0.1
Mixed	Fresh	8.2	1.0	2.4	1.7	1.2	0
5M	Cryo	15.5	1.4	1.5	0.7	0.4	0
	Tp	0	0	0	0	0	0
Toy	Fresh	15.3	2.1	3.0	2.0	2.0	0.1
Poodle	Cryo	47.5	2.6	2.9	1.6	1.4	0
11M	Tp	1.5	0.1	0.1	0.1	0.7	0
Miniature	Fresh	14.8	3.3	4.1	3.6	0.8	0
Dachshund	Cryo	13.4	2.3	2.4	0.9	0.4	0
4M	Tp	0.3	0.3	0.1	0	0	0

*: Crypreserved ovarian tissues. **: Transplanted ovarian tissues.

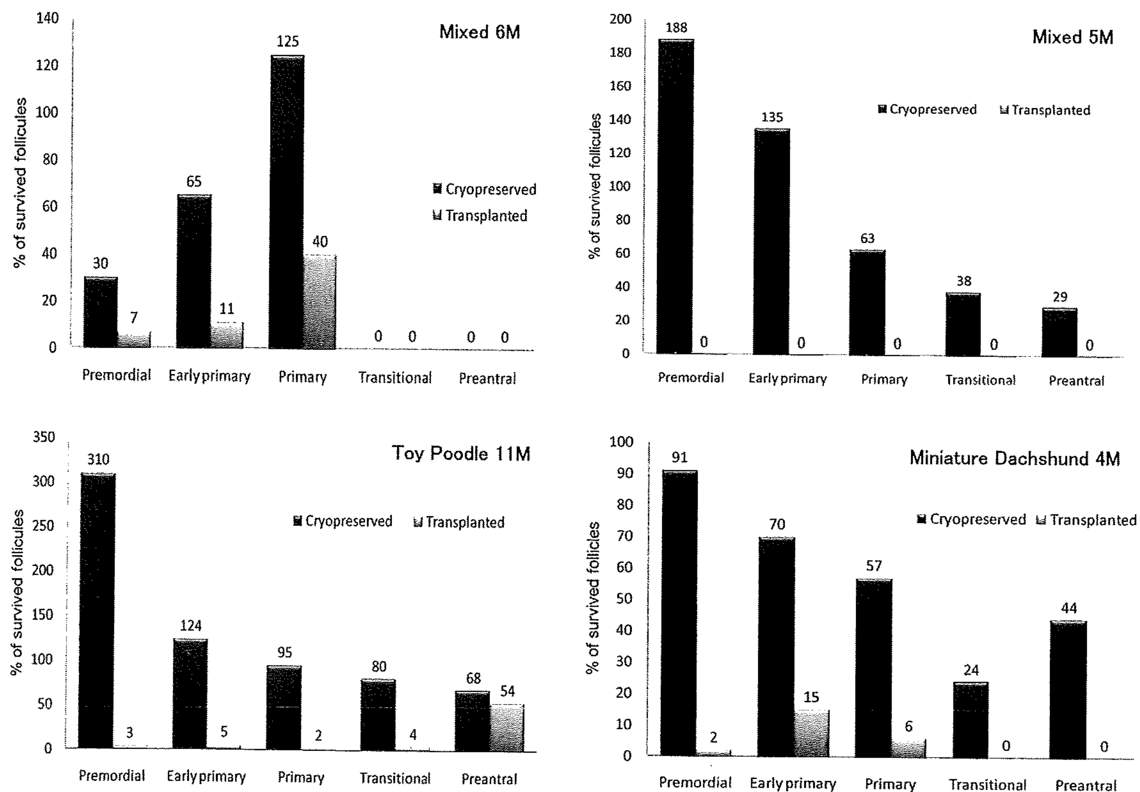

Fig. 1. Percentages of surviving follicles in cryopreserved and subsequently transplanted canine ovaries. The survival rates of follicles were calculated as number of follicles in cryopreserved ovarian tissues / number of follicles in fresh ovarian tissue samples × 100, and number of follicles in transplanted ovarian tissues / number of follicles in cryopreserved ovarian tissue samples × 100.

cryopreservation were 188% and 135% in the 5-month-old mixed breed, and 310% and 124% in the 11-month-old toy poodle, respectively (Fig. 1). However, as shown in Fig. 1, the survival rates of primordial and early primary follicles after the cryopreservation in the 6-month-old mixed breed were 30% and 65%,

respectively. These results indicate that the follicular loss of the canine ovary was not drastically extended by the cryopreservation procedure itself. On the other hand, the detrimental effect of transplantation on the follicular survival was remarkable, even though the follicles were unevenly distributed in the ovarian tissues. The percentages of surviving primordial follicles and early primary follicles ranged from 0–7% and from 0–15%, respectively (Fig. 1).

Discussion

Although the uneven distribution of follicles in the canine ovary make it difficult to interpret the results, it seems that a large proportion of follicles are lost after transplantation of ovaries in canine (Table 1 and Figs.1 and 2) as well as other mammalian species [10–15, 19]. In fact, a high percentage of oocytes as well as granulosa cells survived the cryopreservation and thawing procedure [9, 20–22]. Previously we showed that there was no difference in the morphology and in the average number of primordial and primary follicles between the vitrified-warmed by DAP213 and fresh ovarian canine tissues [9]. 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 a meiotic spindle, zona-pellucida and cortical granules [2]. Although 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 in DAP213 and transferred into the ovarian bursa of NOD-SCID mice [9], the majority of primordial follicles in vitrified-warmed canine ovarian tissues disappeared after transplantation (Table 1 and Fig. 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* [11, 23]. Several attempts have been made to prevent the follicular loss of cryopreserved ovarian tissues after transplantation. However, an effective solution has not been found to date. Kim *et al.* [24] showed that a water soluble antioxidant (ascorbic acid) reduces apoptosis in the ovarian cortex by up to 24 h *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 [25]. Further studies will be required to be able to enhance the survival of transplanted cryopreserved ovarian follicles in canine as well as other mammalian species.

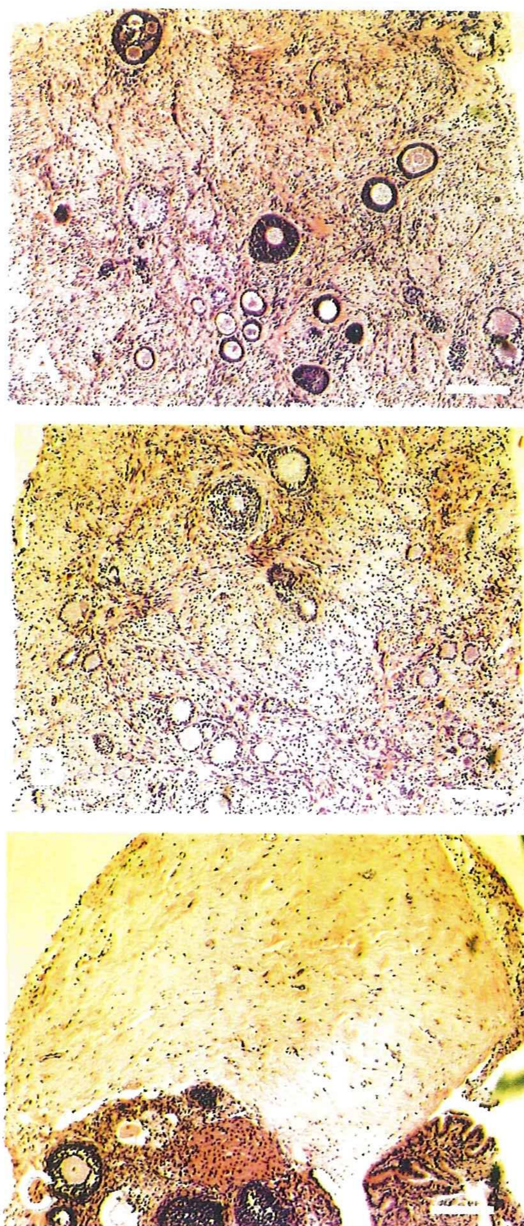

Fig. 2. Hematoxylin and eosin staining of ovarian tissues from a 5-month-old mixed breed. Canine ovaries were cryopreserved by vitrification. The fresh (A) and cryopreserved ovarian tissues (B) are morphologically equivalent. Panel C shows an ovarian tissue recovered from NOD-SCID mice 4 weeks after transplantation of the vitrified-warmed canine ovarian tissues into the bursa. Note the much deeper stain seen in the mouse ovarian tissue (lower) compared to the canine ovary (upper). Canine ovarian grafts (pale stain) successfully adhered to the mouse ovary. Many follicles are seen in canine fresh (A) and cryopreserved (B) ovarian tissues but not in transplanted tissue (C). White bar = 100 μm.

Acknowledgements

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RESEARCH NOTE

Individual fertility differences in the frozen-thawed spermatozoa among semen donors in the Labrador Retriever

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Abstract

Purpose We aimed to validate and determine the possible application of transcervical insemination of frozen semen for improved breeding in guide dogs for the blind in Japan.
Methods From February 2004 to March 2007, a total of 53 Labrador Retriever bitches, used for the breeding of guide dogs for the blind, were transcervically inseminated with frozen-thawed semen from 13 males by means of a cystoscope.
Results The overall whelping and pregnancy rate with the frozen semen was 42%. Pregnancy rates ranged widely from 0 to 100% depending on the semen donor male. Of 13 males, 6 males exhibited severely poor fertility (less than 20% pregnancy rate) and 3 males exhibited high fertility (over 70% pregnancy rate) on artificial insemination. However, the spermatozoa motility after thawing was not significantly different among these dogs. In addition, heterospermic insemination revealed the optimal timing for transcervical insemination with frozen-thawed semen to be by day 6 after the LH surge.
Conclusions Although transcervical insemination of frozen-thawed semen is effective for breeding of guide dogs for the blind, some modification of freeze-thawing procedures might be required to overcome individual fertility differences in the frozen-thawed spermatozoa among semen donor dogs,. In addition, the motility of spermatozoa after thawing might not be an appropriate indicator of the relative fertility of frozen-thawed spermatozoa in dogs.

Keywords Artificial insemination · Cryopreservation · Dog · Heterospermic insemination · Spermatozoa

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Introduction

Guide dogs make a remarkable contribution to the quality of life of the blind people, but many countries suffer from an acute shortage of guide dogs such that not all those who need them are able to be supplied. Approximately 1,000 guide dogs for the blind are presently at work in Japan. However, it is estimated that the demand for such guide dogs is approximately 7,800, including latent demand. Although about 120 trained dogs are provided to the society for the blind annually, the actual number of increase is only 20–30 dogs, since 90–100 of them retire due to ageing every year. This is far from an adequate supply of these dogs for the blind in Japan. In addition, only about 30% of the dogs that are trained in fact work out as guide dogs [1]. One of the important challenges from the point of view of animal and veterinary science, as well as social welfare, is to establish a stable and effective breeding and reproduction system for guide dogs for the blind. However, only about 150 breeding dogs, counting both males and females, are currently available in Japan. In addition, it is known that the Labrador Retriever, which is extensively used as a guide dog for the blind in Japan, is a carrier of certain severe, high frequency genetic defects, including some conditions that are specifically problematic for guide dogs such as hip dysplasia, elbow joint dysplasia, cataracts and retinopathy.

Thus, urgent countermeasures are required for a stable supply of guide dogs of high quality and in high quantity in Japan. Although the development and extension of assisted reproductive techniques in canines, such as artificial insemination, embryo transfer and in vitro fertilization has been remarkable, it is not as successful as in other mammalian species such as mice, pigs, cows and humans. However, successful artificial insemination with frozen canine semen has been well documented [2] since the first conception in 1969 [3]. Thus, in terms of the diffusion of the guide dogs for the blind, one of the solutions associated with these problems seems to be an application of artificial insemination by using frozen-thawed semen imported from developed countries such as the USA, UK and Australia. The transcervical insemination technique seems to be readily applicable to artificial insemination with frozen-thawed spermatozoa for large sized canines such as the Labrador Retriever, which is one of most popular breeds for the guiding dog [4]. Here we report successful delivery, with individual differences in fertility of frozen semen among the dogs, after transcervical artificial insemination with frozen-thawed spermatozoa of the Labrador Retriever.

Materials and methods

Animals and timing of artificial insemination

Both male and female Labrador Retrievers used as breeding dogs for guide dogs for the blind in the Hokkaido Guide Dog Association, Japan, were used in this study in the period from February 2004 to March 2007. Plasma progesterone concentrations of bitches were measured by enzyme linked fluorescent assay (SV-5010, SPOTCHEM VIDAS, Arkray, Kyoto, Japan) daily after the appearance of a blood-tinged vaginal discharge and vaginal swelling. The day when the plasma concentration of progesterone exceeded 2 ng/ml was estimated as the occurrence of the LH surge (Day 0) [5]. Bitches were inseminated with frozen-thawed semen 4–8 days after the estimated LH surge with some exceptions. A total of 13 semen donors with proven fertility in natural mating were used in this study. Unsuccessful pregnancy of the bitches after the experimental artificial insemination by frozen-thawed spermatozoa would considerably influence planning for the production and providing of the guide dogs in the guide dog association. Thus, in some cases, heterospermic insemination using both frozen-thawed and freshly ejaculated semen from different dogs was performed to avoid the failure of conception of the bitches. Paternity for the delivered pups was examined by using micro-satellite markers, as previously described [6].

Freezing and thawing of canine spermatozoa

The method of freezing and thawing of the spermatozoa was similar to that described by Christiansen and Schmidt [7] with diluent developed by Rota et al. [8]. Semen was collected by digital manipulation. The first fraction (seminal plasma) of the ejaculate was discarded, and 3–4 ml of the second (sperm-rich fraction) and third (seminal plasma) fractions mixture were pooled and then centrifuged at 700 g for 10 min. The sperm pellet was resuspended with recovered supernatant by gentle mixing at a concentration of 1×10^9 cells/ml. The sperm suspension was diluted with an egg yolk-Tris-citrate-glucose extender [8] at a concentration of 2×10^8 cells/ml at room temperature and then cooled to 4°C in a refrigerator for 4 h. The sample was further diluted with a second diluent, consisting of the first diluent supplemented with 16% (v/v) of glycerol [8], at a final concentration of 1×10^8 cells/ml. 0.5 ml of the diluted sperm suspension was loaded into a 0.5 ml plastic straw (Type 133, NFA101, Fujihira, Tokyo, Japan). Straws were placed in an atmosphere of liquid nitrogen vapor, i.e. placed horizontally 6 cm above the surface of the liquid nitrogen with a height of 8 cm in a styrene foam box (17.5 cm × 24.5 cm × 17.5 cm), and were kept there for 15 min, and then were plunged into the liquid nitrogen. For thawing, straws were put into a water bath at 38°C for 1 min. After examination of motility under light microscopy at a magnification of ×100, the frozen-thawed spermatozoa were subjected to transcervical insemination [4]. The motility of spermatozoa was classified into the following grades: +++, progressively motile at a high speed; ++, progressively motile at a moderate speed; +, progressively motile at a low speed; ±, motile without progression; −, immotile. The proportion of spermatozoa exhibiting a motility grade of +++ or ++ was used as the parameter for sperm motility.

Transcervical artificial insemination and diagnosis of pregnancy

Inseminations were performed on standing and non-sedated bitches. A catheter (8 Fr, Nippon Sherwood, Tokyo, Japan) equipped with a cystoscope for human use (Karl Storz, Germany) was inserted into the *corpus uteri* through the cervical canal [4]. And then 2×10^8 spermatozoa were inseminated through the catheter. Care was then taken to avoid backflow of the semen, so the catheter was withdrawn one minute after the insemination and the hind quarters of the bitch were kept elevated for 5 min. Conception was diagnosed by Doppler ultrasonography (VPU-011A, Toshiba, Tokyo, Japan).

The animals used in this study were treated and cared for under the Guiding Principles for the Care and Use of

Research Animals established by Obihiro University of Agriculture and Veterinary Medicine, Japan.

Results and discussion

As shown in Table 1, of the 53 bitches that were inseminated, 22 (42%) gave birth to a litter derived from frozen-thawed spermatozoa. All of the live pups exhibited normal appearance and growth. The motility of frozen-thawed spermatozoa from 13 dogs ranged from 55 to 75%. However, the delivery rates after insemination were considerably different between the dogs, ranging from 0 to 100%. Of 13 males, 6 males exhibited severely poor fertility (less than 20% pregnancy rate) and 3 males exhibited high fertility (over 70% pregnancy rate) on artificial insemination. Since the optimal time for insemination with frozen semen theoretically appeared to be around day 4 after the LH surge [9], and it has been suggested that artificial insemination with frozen semen is successful on days 4 and 6 [10], and days 5 and 7 [11, 12] after the LH surge in canine species, we thus performed transcervical insemination between days 4 and 8 after the estimated LH surge in the Labrador Retriever. However, as shown in Table 2, heterospermic insemination in the present study revealed the optimal timing for transcervical artificial insemination with frozen-thawed semen to be by day 6 after the estimated LH surge. When frozen-thawed spermatozoa were inseminated into the bitches by day 6 after the LH surge in heterospermic insemination, only the frozen-thawed spermatozoa contributed to conception in many of the cases (mating no. 1, 2, 3, 4, 6 and 10 in Table 2). When frozen-thawed spermatozoa from RYU were inseminated into bitches on day 4 or 5, however, those spermatozoa did not participate in the pregnancy (mating no. 11 and 12 in Table 2). Although 3 of 11 inseminations from RYU did not eventuate in pregnancy (Table 1), these failures of pregnancy included the above 2 cases (mating no. 11 and 12) and one abortion which was inseminated with frozen semen on LH 6 and 7. These results indicate that the 'hot spot' for the successful conception might be on day 6 after the LH surge. On the other hand, day 7 after the LH surge seems to be still fertile. Because when the frozen-thawed and freshly ejaculated semen were inseminated into the bitches on days 6 and 7, respectively, both frozen-thawed and freshly ejaculated semen successfully contributed to the conception, (mating no. 5, 7 and 13; Table 2). In mating no. 14, spermatozoa from NUR did not contribute to the conception in spite of insemination on day 6 after the estimated LH surge, but the cause of this fertilization or implantation failure appeared to be an individual difference rather than the timing of insemination. This is because the frozen-thawed spermatozoa from NUR never participated in pregnancy in any of the 7 trials in this study (Table 1).

Table 1 Results of transcervical artificial insemination with frozen-thawed spermatozoa in the Labrador Retriever guide dog

Semen donor	Motility of sperm after thawing (%)	No. of parturition/no. of bitches inseminated (%)	No. pups delivered	Litter size
ODY	65–75	4/5 (80)	13	3.3
RYU	70–75	8/11 (73)	28	3.7
BUZ	75	4/4 (100)	19	4.8
ERI	75	1/1 (100)	7	7.0
MAX	70	1/1 (100)	2	2.0
LEG	70	1/3 (33)	6	6.0
JAS	75	2/6 (33)	8	4.0
PEA	70	1/7 (14)	1	1.0
NUR	55–65	0/7 (0)	0	–
MAR	70	0/3 (0)	0	–
QUI	75	0/2 (0)	0	–
ATO	70	0/1 (0)	0	–
KEN	70	0/2 (0)	0	–
Total		22/53 (42)	84	3.8

Motility of spermatozoa was classified into the following grades: +++ progressively motile at a high speed, ++ progressively motile at a moderate speed, + progressively motile at a low speed; ± motile without progression, − immotile. The proportion of spermatozoa exhibiting a motility grade of +++ or ++ was used as the parameter for sperm motility

It has been reported that whelping rates after transcervical insemination with frozen-thawed spermatozoa using a stainless steel catheter (the Norwegian method) in canines ranged from 60 to 90% in several breeds which were inseminated on the second and third day after estimated ovulation [13]. However, the Norwegian method seems to require considerable skill for successful insemination through the cervix in large breeds such as the Labrador Retriever. The catheter could not be inserted through the cervix in approximately 3% of any sized bitches by the Norwegian method [13]. And less than 10% of the bitches of both large and giant breeds required sedation to enable abdominal fixation of the cervix [13]. In the present study, the catheter was successfully inserted into the uterus through the cervix without any sedation required in any of the trials. Thus, transcervical insemination using a stereoscope with a long sheath as is used for human cystoscopy might be an effective technique for artificial insemination of large sized bitches such as the Labrador Retriever. This technique enables spermatozoa to be deposited non-surgically into the uterus in standing, non-sedated bitches. Since the number of fertile spermatozoa and the longevity of the spermatozoa seem to be reduced, it would be more effective to make the deposit closer to the site of fertilization rather than intra-vaginanally. Since a fiber optic endoscope

Table 2 Results of heterospermic insemination with frozen-thawed and freshly ejaculated spermatozoa in the Labrador Retriever guide dog

Mating no.	Bitch	Dogs	Days after LH surge at insemination					No. of live pups
			4	5	6	7	8	
1	ERZ	BUZ		FT	FT			2
		MAR				NM		0
2	SHI	BUZ		FT	FT			3
		MAR			NM			0
3	PAG	BUZ		FT	FT			5
		RYU				EJ		0
4	BEG	BUZ		FT	FT			7
		FUN					NM	0
5	HAN	RYU			FT	FT		4
		PEA				EJ		4
6	BEL	RYU		FT	FT			6
		PEA				EJ		0
7	IND	RYU			FT	FT		5
		MAR				EJ		2
8	GUC	RYU		FT	FT	FT		5
		PEA				EJ		0
9	DRE	RYU		FT	FT	FT		1
		KEI				NM		0
10	BEL	RYU		FT	FT			1
		PEA				EJ		0
11	ION	RYU	FT					0
		PEA		NM				4
12	KER	RYU		FT				0
		TOR		NM		NM		7
13	HAN	RYU			FT	FT		4
		PEA				EJ		4
14	BEL	ODY	FT					0
		NUR			FT			0
		PEA			EJ		EJ	7
15	CHR	LEG	FT	FT	FT			6
		PAL			EJ	EJ		1

FT frozen-thawed spermatozoa, *EJ* freshly ejaculated spermatozoa, *NM* natural mating

and expensive equipment is required to achieve transcervical insemination, the spread of intrauterine insemination for the breeding of guide dogs, as well as other working dogs, may still face certain difficulties. However, in a view of the urgent demand of the increasing numbers of handicapped people, it is necessary to develop and diffuse new assisted reproductive techniques, including artificial insemination, to improve the effective use of genetic resources in dogs. Although the cause of the lower conceptus number and litter size in pregnancy is still unclear in our present study, it might be influenced by the quality of the frozen-thawed semen, the age and gravidity of the bitches used, and/or the optimal timing of insemination. Further studies would be required to develop a novel cryopreservation method which is available to male dogs proven fertile in natural matings.

The total of 15 homospermic and 38 heterospermic inseminations yielded 84 pups from frozen-thawed semen and 91 pups from freshly ejaculated semen, respectively. Heterospermic insemination in combination with cryopreserved spermatozoa and ejaculated semen appeared to be effective to avoid a shortage of production in a colony of guide dogs.

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トピックス

盲導犬の生殖工学

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はじめに

現在，わが国では約1,000頭の盲導犬が実働している．また，全国の盲導犬事業所から毎年130頭程度の盲導犬が，新規に視覚障害者に供給されている．しかしながら，盲導犬を希望する視覚障害者は約4,800人，潜在的希望者も加えるとその需要は約7,800人と推定されており，わが国で盲導犬事業が開始されてから50年を過ぎても慢性的な盲導犬不足が続いている．身体障害者補助犬の導入によって自立と社会参加を果たし得る障害者は数多く存在しており，その普及には，社会的受け入れ体制の整備とともに，良質な補助犬の育成体制の整備が不可欠であると考えられる．わが国においても，盲導犬，聴導犬，介助犬など，いわゆる補助犬の社会生活へのアクセス保障のみならず，補助犬の育成や使用者の責務までに踏み込んだ法整備が開始されたが，生物学，獣医学的な観点から補助犬の適切な活用および育成を支えようとするアプローチは十分ではない．本来，盲導犬を含む各種補助犬に要求される能力とその適合犬種の選定のための行動学，遺伝学的研究や補助犬の社会への浸透を確保するためのイヌ由来の人畜共通感染症に関する公衆衛生学的研究を含めた，新しい視点からのいわゆる「イヌ学」を総合的に推進する必要があると考えられるが，その基礎となる遺伝子資源の確保，保存，利用のための繁殖生理学，特に生殖工学的研究の立ち遅れがこれを阻んでいる．

盲導犬の普及を推進するためには，その訓練方法はもとより盲導犬の安定的，効率的繁殖が，もっとも重要な課題の１つであると思われるが，現在，全国の盲導犬訓練施設で使用されている繁殖犬はわずかに180頭余りで，ここから得られた産仔の盲導犬の合格率（適格犬数率）は30～40％と低い水準にある．この原因の１つとして挙げられるのは，盲導犬のきわめてユニークな育成システムである．すなわち，盲導犬の候補犬は雌雄ともに避妊・去勢を受けた後に訓練を開始するために，優秀な盲導犬であってもその遺伝子を繁殖によって次世代に伝える術を失うのである．これは，育種の概念に逆行した繁殖・育成システムであるとも言える．したがって，自然交配に終始することなく，人工授精，受精卵移植や卵巣移植などの人工繁殖技術の導入・開発によって，これまで廃棄の対象であった生殖細胞を活用し得る高度な効率化が，盲導犬普及率の改善のために必要であると思われる．

人工授精

イヌの凍結精子による人工授精は，1950年代から研究されており，現在，オーストラリア，アメリカ，イギリス，ニュージーランド，フィンランドなどの盲導犬協会・盲導犬事業所においては実用的な応用を果たしている．残念ながら，わが国においては，盲導犬事業への凍結精子を用いた人工授精の導入が遅れていたが，2002年に筆者らによって国内初の成功例が得られ，現在では実用化に至っている［１］．

イヌの自然交配においては，精液は腟内に射出されるが，凍結融解精子の運動性は新鮮射出精子と比較して劣るので，凍結融解精子を用いた人工授精においては，融解した精子を子宮体部に注入することが望ましい．補助犬に汎用されるラブラドールリトリバーのような大型犬では，解剖学的に腟が長いので子宮頸管を通過して子宮体部に精子を注入するには，適切なカテーテルを準備することが必要である．Norwegian カテーテルと呼ばれている金属製のカテーテルは，元来，キツネの人工授精のために開発されたものであるが，これは比較的小型の動物の腹部を触診しながらガイドして子宮頸管を通過するには適当であるが，大型犬への妥当性は乏しい．その点，1992年に Wilson が開発したヒト用の膀胱鏡を用いた経子宮頸管人工授精法は，カテーテルの先端をモニターで観察しながら実施するので，技術的にも容易で確実性が高い［２］．術者はカテーテルの先端が頸管を通過したことをモニターで確認してから，カテーテル内の凍結融解精子を子宮体部に注入することが可能である．ヒト膀胱鏡を用いた経子宮頸管人工授精法に問題点があるとすれば，それは用いる機材が比較的高価であることと思われる．国産の膀胱鏡ではシース（鞘）部の長さが不足しており，大型犬の人工授精に用いることは困難であり，ドイツのストルツ社製のヒト用膀胱鏡を輸入することが必要である．交配頻度がそれほど高くない比較的小規模の補助犬事業所に数百万円もする膀胱鏡を準備す