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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'-TTATTC-GATCACGCCGCAGGC-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.

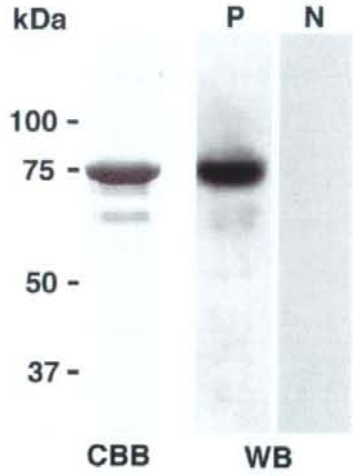

Fig. 1. Western blot analysis of recombinant SOD. TF fusion SOD was separated by SDS-PAGE under reduced condition and then transferred to nylon membranes. The membranes were stained with Coomassie brilliant blue (CBB) and used for analysis of *B. canis* infected (P) and uninfected (N) sera (WB).

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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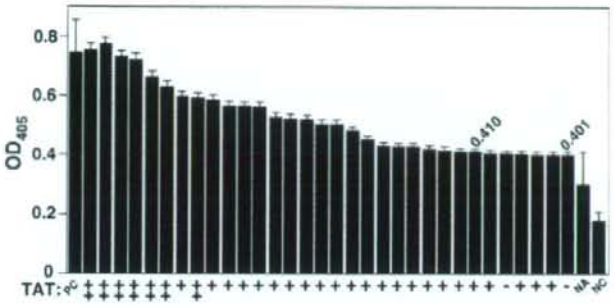

Fig. 2. ELISA absorbance values of dog sera using recombinant SOD. The bars indicate samples with absorbance values of over 0.400 (OD_{405}). Values are averages and standard deviations of triplicate wells from three identical experiments. PC, NC and NA indicate average absorbance value of ten positive 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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Running head: Follicular loss after the ovarian transfer

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.

Keywords: 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 seconds 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 minutes. 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 minutes 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 seconds. 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 x 100, and the number of

follicles in transplanted ovarian tissues / number of follicles in pre-transplant ovarian tissue samples x 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 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.

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