Table 1. Effect of ZIP on the fertility of transgenic mouse freeze-thawed spermatozoa and subsequent embryo transfer

Line	Strain	Treatment	No. of fertilized / No. of inseminated oocytes (%)	No. of implantation sites / No. of embryos transferred (%)	No. of live fetuses / No. of embryos transferred (%)
A	C57BL/6J	ZIP	222/300 (74)*	123/205 (60)	70/205 (34)
		Non	5/20 (25)	3/5 (60)	2/5 (40)
B	C57BL/6J	ZIP	277/626 (44)*	172/277 (62)	104/277 (38)
		Non	15/85 (18)	7/15 (47)	4/15 (27)
C	C57BL/6J	ZIP	158/367 (43)	75/157 (48)*	31/157 (20)
		Non	15/30 (50)	0/15 (0)	0/15 (0)
D	C57BL/6J	ZIP	203/292 (70)*	120/202 (59)	58/202 (29)
		Non	1/34 (3)	Not done	Not done
E	C57BL/6J	ZIP	184/245 (75)*	93/184 (51)*	51/184 (28)
		Non	19/69 (28)	4/19 (21)	3/19 (16)
F	C57BL/6J	ZIP	184/440 (42)*	135/151 (89)	74/151 (49)
		Non	0/36 (0)	Not done	Not done
G	FVB	ZIP	131/279 (47)*	65/131 (50)	42/131 (32)
		Non	11/61 (18)	4/11 (36)	3/11 (27)

*$P<0.05$, compared to the control (non-treated).

cally by the chi-square test and Tukey's test for nonparametric multiple comparisons. In all statistical tests, the difference was considered significant when P was <0.05.

The fertilization rates were higher in ZIP/IVF (42–75%) than in conventional IVF in 6 of 7 lines; lines A–F had a C57BL/6J genetic background and line G had a FVB genetic background (Table 1). However, the embryos produced by conventional IVF from line C, the line that did not show a higher fertilization rate using ZIP/IVF, failed to produce any live born pups after embryo transfer. In line C there might be abnormally fertilized oocytes including, for example, parthenogenetic oocytes. However, the abnormal fertilization could not be confirmed because an embryo was defined as fertilized at the 2-cell stage 24 h after insemination in this study. Overall, 20–49 and 27–40% of the 2-cell embryos developed to term following ZIP/IVF and conventional IVF, respectively. Lines C, D, and F produced live fetuses following ZIP/IVF but not from conventional IVF.

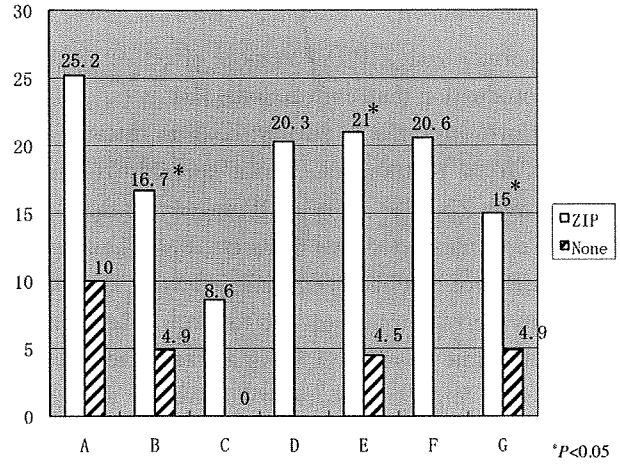

Fig. 1. The overall efficiency of mouse production by means of ZIP/IVF and conventional IVF. Efficiency rates were 8.6–25.2% using ZIP/IVF. In lines B, E, and G, the overall efficiency was 16.7, 21.0, and 15.0%, respectively, with ZIP/IVF, higher than values for conventional IVF (4.9, 4.5, and 4.9%, respectively). The overall efficiency of lines D and F using conventional IVF could not be calculated because of the small number or lack of oocytes fertilized and thus not transferred.

Figure 1 shows the overall efficiency of mouse production after ZIP/IVF and conventional IVF using frozen-thawed spermatozoa. In 3 lines (B, E from C57BL/6J and G from FVB) of transgenic mice, the overall efficiency of ZIP/IVF was significantly greater than conventional IVF ($P<0.05$). The FVB/N mouse strain has a low sperm concentration but high fertility, which is maintained through the freezing process [13]. In this study, the overall efficiency using cryopreserved FVB transgenic spermatozoa was improved by using ZIP/IVF. The number of live born pups from 100 ZIP oocytes determined the overall efficiency. Figure 1 clearly indicates that, with cryopreserved C57BL/6J and FVB trans-

Table 2. Effect of ZIP on the fertility of C57BL/6J transgenic mouse freeze-thawed spermatozoa and subsequent embryo transfer

Line	No. of fertilized / No. of inseminated oocytes (%)	No. of implantation sites / No. of embryos transferred (%)	No. of live fetuses / No. of embryos transferred (%)
H	81/148 (55)	32/81 (40)	20/81 (25)
I	77/138 (56)	31/77 (40)	14/77 (18)
J	38/133 (29)	5/38 (13)	3/38 (8)
K	95/200 (48)	34/95 (36)	20/95 (21)
L	85/200 (43)	40/85 (47)	16/85 (19)
M	174/517 (34)	80/174 (46)	53/174 (30)
N	102/460 (22)	55/102 (54)	27/102 (26)
O	256/750 (34)	140/256 (55)	77/256 (30)
P	183/434 (42)	135/168 (80)	74/168 (44)
Q	293/395 (74)	108/210 (51)	51/210 (24)
R	187/281 (67)	78/187 (42)	38/187 (20)
S	78/132 (59)	21/40 (53)	8/40 (20)
T	46/126 (39)	9/32 (28)	6/32 (19)
U	144/560 (26)	82/144 (57)	45/144 (31)
Total	1839/4474 (41)	850/1689 (50)	452/1689 (27)

genic spermatozoa, ZIP/IVF is more effective for ART mouse production than conventional IVF.

As shown in Table 2, the fertilization rates of ZIP oocytes from 14 transgenic mice spermatozoa lines (H–U) were 22–74%. When ZIP zygotes at the 2-cell stage were transferred into oviducts, 8–44% of transferred embryos that had been fertilized using spermatozoa from the 14 lines developed to term. The average fertilization rate was 41% (1,839/4,474) and the percentage of live fetuses from embryo transfer was 27% (452/1,689). The fertilization rates varied among lines as a result of variations in spermatozoa. Fertility dose not correlate with sperm concentration or with total motility and progressive fraction counts. Large variations were especially seen among the C57BL/6J mice because of the variations of spermatozoa among the mice [13].

Cryopreservation of mouse spermatozoa has become a commonly used tool for preserving transgenic mice in many laboratories. The ZIP method, which we previously developed and reported [5], has been suggested to be a useful technique for the exploitation of stored mouse spermatozoa. In addition to ZIP [5], PZD [9], and laser-assisted zona drilling [3] techniques have also been reported; both have advantages and disadvantages. With PZD, the technique is difficult because zona pellucida need to be dissected manually by needle and the PZD zygotes must be cultured *in vitro* up to the morula or blastocyst stage prior to embryo transfer, because blastomeres often escape from the slit in the zona pellucida during the early stages of embryonic development and attach to the epithelial cells of the oviduct [5]. However, PZD requires only a microscope and needle so its cost is very low. In laser-assisted zona drilling, the laser equipment used for the drilling is very expensive and, in some cases, a sucrose solution (0.5 M) for shrinking the oocytes is needed to avoid laser-induced cytoplasmic damage; however, 500 oocytes can be treated in 1 h without any damage [2]. ZIP, in contrast, is easy to perform with little or no damage and new expensive equipment is unnecessary because many reproductive laboratories already have piezo micro-manipulators. The piezo electric actuator is mainly used for injection of certain materials into oocytes or embryos, and manipulating 100 oocytes for ZIP requires approximately 1 h [4]. Both ZIP oocytes and laser-assisted zona drilling oocytes can be transferred into oviducts [2, 3, 4]. Recently, there have been reports of new methods for IVF using frozen-thawed mouse spermatozoa involving the addition of solutions, for example, adding methyl-beta-cyclodextrin to the preincubation medium [14] or adding monothioglycerol to the cryoprotective medium [11]. With these methods, the collection of motile thawed spermatozoa from preincubation medium is necessary and previously cryopreserved spermatozoa cannot be

used. However, the ZIP method is stable and easy. Moreover, ZIP allows the use of the vast number of previously cryopreserved spermatozoa at the many laboratories and gene resource banks.

In conclusion, all 21 lines of the transgenic mice freeze-thawed spermatozoa produced live fetuses using ZIP/IVF. The average fertilization rates and percentages of live fetuses are shown in Table 2. Five breeding pairs were produced from only 90 ZIP oocytes using spermatozoa cryopreserved for the maintenance of transgenic mouse colonies. Thus, it is easy to provide breeding pairs of mice from gene resource stocks using this method, indicating that ZIP/IVF is a useful tool for ART. ZIP/IVF offers significant advantages in the production of genetically modified mouse strains and the maintenance of transgenic mouse colonies.

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Immunogenicity and growth inhibitory efficacy of the prime–boost immunization regime with DNA followed by recombinant vaccinia virus carrying the P29 gene of *Babesia gibsoni* in dogs

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ABSTRACT

In recent studies, heterologous prime–boost approaches, employing plasmid DNA and viral vector pathogen-delivering sequences, have been considered an effective protection strategy for intracellular parasite infections. Here, we evaluated the efficacy of such a strategy against the canine *Babesia gibsoni* infection. The DNA (pCAGGS-P29) and recombinant vaccinia virus (vvP29) both encoding the P29 of *B. gibsoni* were used in this study. The dogs were immunized 3 times with priming DNA and boosted once with recombinant virus. The dogs immunized with P29 developed a significant level of IgG2 antibody against P29. The response was strongly boosted by the inoculation of vvP29. The peripheral IFN-gamma responses of the dogs immunized with P29 were significantly higher than those of controls after the parasite inoculation. Moreover, the P29 immunized group showed a significantly low level of parasitemia. In conclusion, this study supports the efficacy of a prime–boost strategy for dogs against canine *B. gibsoni* infection.

1. Introduction

Babesia gibsoni is an intra-erythrocytic, tick-transmitted protozoan that can cause clinical babesiosis in dogs. Infection is endemic in Asia, Africa, Europe, the Middle East and North America. Canine *B. gibsoni* infection is characterized by remittent fever, thrombocytopenia, sever anemia, and sometimes death (Boozer and Macintire, 2003; MacWilliams, 1987). In natural or experimental *B. gibsoni* infection, 60% of dogs are recovered from the acute stage and then shift to the chronic stage. In chronically infected dogs, the *B. gibsoni* parasite can be maintained for several years, and the dogs became a reservoir of the parasites for the next generation. Furthermore, it is known that a chronically infected bitch transmits the parasite in the uterus, which causes a fatal infection in her pups (Fukumoto et al., 2005a). For the reasons, the disease is frequently present in dogs and has recently become a serious clinical problem.

For the control and alleviation of *B. gibsoni* infection in dogs, vaccination is generally considered to be the most effective means. A traditional vaccine development study targeted the induction of the humoral immune response by the immunization of inactivated pathogens (Brown and Palmer, 1999). However, in the case of protection against intracellular parasites, the induction of the both humoral and cell-mediated immune response is considered to be important (Brown and Palmer, 1999; Tsuji and Zavala, 2003). Thus, the optimal immunization methods for inducing such type of response are required for the study. In recent years, it was reported that the heterologous prime–boost immunization regime with priming DNA followed by recombinant virus both expressing the same antigen has been shown to effectively trigger an immune response against several infectious intracellular pathogens (Amara et al., 2001; Gilbert et al., 2002; Hanke et al., 1998).

In our previous study, we identified a P29 gene expressed on *B. gibsoni* merozoites (Fukumoto et al., 2003). The P29 was considered to play roles at the parasite invasion of the host erythrocyte by the maintenance of physical strength. We also showed that the P29 was recognized as the immunodominant antigen of the dog infected with *B. gibsoni*. In this study, we determined the immunogenicity and growth inhibitory effect of heterologous immunization with priming DNA–boosting recombinant vaccinia virus, both carrying the P29 gene of *B. gibsoni*.

2. Materials and methods

2.1. Parasite

The NRCPD strain of *B. gibsoni* parasite (Fukumoto et al., 2000; Ishimine et al., 1978) was used in this study. The *B. gibsoni*-infected erythrocytes for challenges were collected from a dog experimentally infected with *B. gibsoni*.

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2.2. Construction of plasmid expressing the P29 gene

The entire P29 gene (Fukumoto et al., 2003) was inserted into the EcoRI restriction enzyme site under the control of the CAG promoter of the mammalian expression vector pCAGGS (Niwa et al., 1991; Tokui et al., 1997), designated pCAGGS-P29. pCAGGS-P29 was amplified in a DH5a strain of *Escherichia coli*, and the purification was performed using the QIAGEN Plasmid Mega Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The expression of the P29 was analyzed using rabbit kidney 13 (RK13) cells in vitro prior to an in vivo trial with dogs. RK13 cells were transfected with pCAGGS-P29 using a lipofectine reagent (Gibco BRL, Rockville, MD) by the standard method. The expression of the P29 was analyzed by the immunofluorescence antibody test (IFAT using an anti-P29 monoclonal antibody 2 days after transfection.

2.3. Construction of the recombinant vaccinia virus expressing the P29 gene

The entire P29 gene was inserted into the SalI site of the vaccinia virus transfer vector pAK8 (Yasuda et al., 1990). RK13 cells infected with the parent vaccinia virus LC16mO (mO) (Yasuda et al., 1990) strain were transfected with the pAK8-P29 using a lipofectine reagent (Gibco). Thymidine kinase-negative (TK−) viruses were isolated by a plaque assay of 143TK− cells in the presence of 5-bromo-2′-deoxyuridine at a concentration of 100 μg/ml (Yasuda et al., 1990). The plaque assay was done 3 times to clone the recombinant virus. The recombinant vaccinia virus expressing P29 (vvP29) was propagated in RK13 cells in Eagle's minimum essential medium supplemented with 7.5% fetal bovine serum (FBS). To analyze the expression of P29 in vitro, RK13 cells were inoculated with 5 plaque-forming units (PFU) of vvP29 or mO per cell. Two days after inoculation, the cells were harvested and then subjected to IFAT or Western blotting as described above.

2.4. Immunization of dogs

Purebred female specific pathogen-free beagle dogs (14–15 months) were used in this study. All dogs were purchased from Chugai medical animal institute (Nagano, Japan). All dogs were physically examined by the veterinarian of the Obihiro University of agriculture and veterinary medicine and received the routine vaccination including canine parvovirus, canine adenovirus (types 1 and 2) and distemper. Nine dogs were randomly divided into three groups ($n = 3$). The P29 immunized group received pCAGGS-P29 and vvP29. The control immunized group received pCAGGS empty plasmid and the parent vaccinia virus mO strain. The remaining group received no immunization treatment. The immunization regime used DNA priming three times and a vaccinia virus boosting once because this immunization schedule showed most effective result for the induction of a strong immune response against malaria infection than other vaccination regimes in humans (Dunachie and Hill, 2003). For the plasmid DNA immunization, dogs were injected intramusculary (IM) in the quadriceps muscle with a 1-ml syringe and a 21G needle. Each single dose consisted of 200 μg of DNA dissolved in 1 ml of PBS containing 25%(w/v) sucrose. The dogs were immunized three times at two-week intervals. Two weeks after the final DNA immunization, the dogs were boosted with 5×10^8 PFU of the recombinant vaccinia virus vvP29 or mO intravenously (IV). After the DNA or recombinant vaccinia virus immunization, dogs were examined every day for 14 days by a veterinarian and no side effect were observed.

2.5. Determination of antibody responses against P29 by enzyme-linked immunosorbent assay (ELISA)

The antibody responses of the immunized dogs were measured using the ELISA with GST-P29 as described in our previous paper (Fukumoto et al., 2003). The total IgG, IgG1, and IgG2 subclass antibody responses against P29 were analyzed. All serum samples were used in 1:200 dilutions for ELISA. All HRP-conjugated secondary antibodies were purchased from Bethyl laboratory (Montgomery, TX).

2.6. Determination of the total IgE response by ELISA

Total IgE of the immunized dogs were measured using a capture ELISA (Dog IgE ELISA Quantitation kit, Bethyl laboratories) to analyze the allergic reaction caused by the immunization of DNA and recombinant vaccinia virus. The ELISA was performed following the manufacture's instructions. The sera collected at pre-immunization and 2 weeks after the each immunization (day −56, −42, −28 and 0 of Fig. 4) were used for analysis. All serum samples were used in 1:200 dilutions. The concentration of total IgE was calculated from the standards (ranges: 10,000–7.8 ng/ml).

2.7. Parasite growth inhibition assay in dogs

Two weeks after the booster immunization, dogs were i.v. infected with 2×10^8 of *B. gibsoni*-infected RBCs collected from a dog experimentally infected with the *B. gibsoni* parasite (NRCPD strain). Parasitemia in peripheral blood, packed cell volume (PCV), RBC number, and hemoglobin concentration were monitored at one-day intervals.

2.8. Peripheral IFN-gamma response

The serum samples collected at days 0 and 8 after parasite inoculation were used for the assay. The samples were kept at −80 °C until use. IFN-gamma was measured using a capture ELISA (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

2.9. Statistical analysis

The parasitemia and antibody responses in the immunized dogs were statistically analyzed by the Student's *t*-test.

2.10. Animal experiment

All animal experiments in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

3. Results

3.1. Expression of the P29 in vitro by pCAGGS-P29

To investigate whether the pCAGGS-P29 plasmid expressed P29, we transfected the plasmid into RK13 cells and analyzed it by Western blotting and IFAT. In the Western blotting, the anti-P29 MAb specifically recognized a 29 kDa band of RK13 cells transfected with pCAGGS-P29 and not with empty plasmid pCAGGS. The molecular weight of P29 expressed by pCAGGS-P29 was similar to that of the native P29 from *B. gibsoni*. In the IFAT, the MAb specifically reacted to RK13 cells transfected with pCAGGS-P29 (Fig. 1).

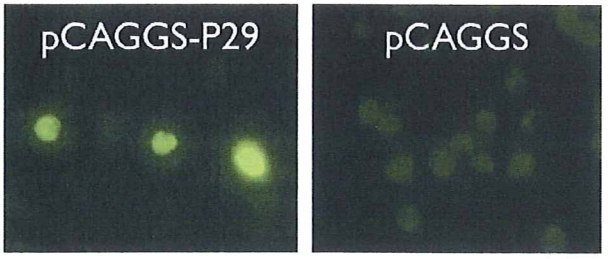

Fig. 1. Expression of the P29 in RK13 cells transfected with pCAGGS-P29. Expression of the P29 was analyzed by IFAT using anti-P29 monoclonal antibody. pCAGGS-P29, cells transfected with pCAGGS-P29; pCAGGS, cells transfected with control plasmid pCAGGS.

3.2. Expression of the P29 in vitro by the vvP29

RK13 cells were infected at 5 PFU/cell with a vvP29 or with a parent vaccinia virus mO. After incubation for 2 days, the cells were harvested and analyzed by Western blotting or IFAT using anti-P29 MAb. In Western blotting, a specific 29 kDa band was detected in the cells infected with vvP29 and not in mO (Fig. 2A). In IFAT, the anti-P29 antibody reacted specifically to the cells infected with vvP29 (Fig. 2B).

3.3. Antibody responses

To determine the profile of the immune response of the dogs immunized with the heterologous regime, the IgG response and its subclass were analyzed (Fig. 3). The total IgG responses against P29 of the P29 immunized dogs were under detectable level by each DNA immunization. However, two weeks after the booster immunization with vvP29, the antibody response was significantly increased (Fig. 3A, day −14 vs. day 0). The IgG subclass against P29 was also analyzed. The IgG1 antibody response maintained a low level (Fig. 3B). The IgG1 response of the dogs immunized with P29 did not show significance compared to that of the control groups. In contrast, the IgG2 antibody was significantly increased after the booster immunization with vvP29 (Fig. 3C, day −14 vs. day 0). When we compared IgG1 and IgG2, IgG2 was detected as major subclass all through the experimental period (Fig. 3B and C). The IgG2 response of the P29 group showed a significantly higher level (days 0–8, 16, 28, and 36) when compared to those of the control groups (Fig. 3C).

3.4. Allergic reaction of the immunized dogs

To determine the allergic reaction of the immunized dogs, serum total IgE concentration of immunized period (day −56 to 0 of fig. 3) was analyzed. All serum samples showed the concentration of less than 7.8 ng/ml (under detectable level) of serum total IgE responses (figure not shown).

3.5. Peripheral IFN-gamma response

The peripheral IFN-gamma response of the dogs after the challenge infection was analyzed by the capture ELISA. As shown in Table 1, at day 0 post-infection, all dog groups showed an undetectable level of IFN-gamma production. At 8 days post-infection, only the dog group immunized with P29 showed a significant level of the IFN-gamma response not detected in the control groups.

3.6. Inhibition of the parasite growth in the dogs

To determine the protective effect of the immunization with P29 by the heterologous prime–boost regime, the dogs were inoculated with *B. gibsoni* parasite on day 0 and parasitemia was monitored. The parasitemia was significantly inhibited (day 20) in the

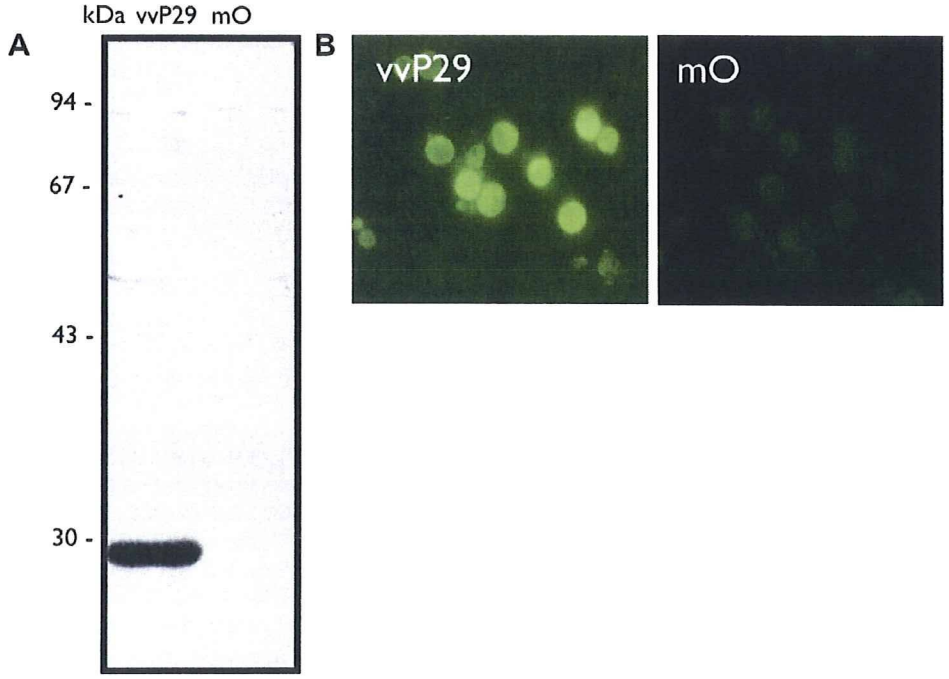

Fig. 2. Expression of the P29 in RK13 cells infected with vvP29. (A) Western blot analysis of the P29 expressed in RK13 cells. The expression of the P29 was detected using an anti-P29 monoclonal antibody. vvP29, lysates of recombinant vaccinia virus vvP29-infected cells; lane 2, control lysates of vaccinia virus mO-infected cells. (B) IFAT analysis of the P29 expressed in RK13 cells. The cells were stained with anti-P29 monoclonal antibody. vvP29, cells infected with the vvP29; mO, cells infected with the control parent virus mO.

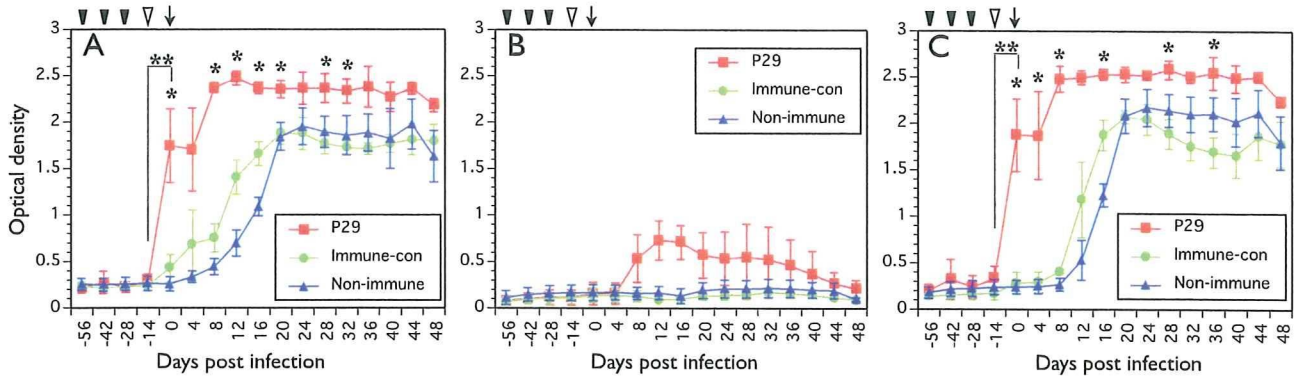

Fig. 3. Determination of the antibody response against P29 of dogs immunized with P29. Antibody response against each immunization was monitored at two weeks after each immunization. (A) Total IgG, (B) IgG1, and (C) IgG2. P29, sera collected from dogs immunized with pCAGGS-P29 and vvP29; immune-con, sera collected from dogs immunized with control plasmid pCAGGS and mO; non-immune, sera collected from non-immunized control dogs. The day at challenge infection of the parasites was designated as day 0 (solid arrow). The dogs were immunized with DNA at day −56, −42 and −28 (solid arrowhead), and immunized with vaccinia virus at day −14 (white arrow head). The asterisks (*) on the error bar show the significant difference ($P < 0.05$) between a dog group immunized with P29 and the control groups. The double asterisks (**) show the significant difference between day −14 and day 0. The results are shown as the mean values, and the error bars represent the standard deviations.

Table 1
Peripheral IFN-gamma responses of the immunized dogs after the *B. gibsoni* parasites inoculation.

Dog group ($n = 3$)	IFN-gamma response (pg/ml)	
	Day 0	Day 8
P29	UD[a]	64.64 ± 17.43[b]
Immune-con	UD	UD
Non-immune	UD	UD

[a] UD, under detectable level.
[b] Results was shown in mean ± standard deviation.

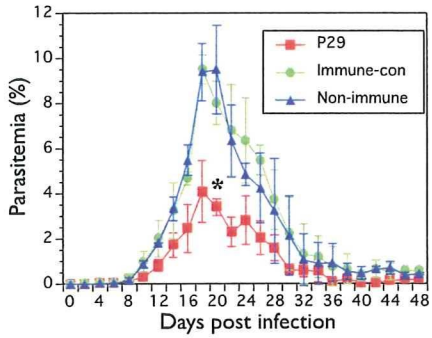

Fig. 4. Parasitemia of dogs after challenge infection with *B. gibsoni*-infected RBCs. P29, dogs immunized with pCAGGS-P29 and vvP29; immune-con, dogs immunized with control plasmid pCAGGS and mO; non-immune, non-immunized control dogs. The results are shown as the mean values, and the error bars represent the standard deviations. The asterisks show the significant difference ($P < 0.05$) between a dog group immunized with P29 and the control groups.

dog group immunized with P29 when compared to both of the control groups. There was no significant difference in the two control groups. At the peak of parasitemia, the ratio of the inhibitory effect was 54.5% compared to that of the immunized control group and 56.0% compared to that of the non-immunized group. There was no significant difference in clinical symptoms manifested as severe anemia (Fig. 5).

4. Discussion

This study demonstrates the immunogenicity and efficacy of a heterologous prime–boost immunization with priming DNA followed by recombinant vaccinia virus and the potential use of P29 as an immunogen against *B. gibsoni* infection.

For protection against animal babesiosis, the induction of an immune response, such as opsonizing IgG2 antibody and macrophages activated by the IFN-gamma produced by CD4 T cells, is considered to be important (Brown and Palmer, 1999). Individual immunization of the DNA (Bout et al., 2002; Kumar et al., 2002) or recombinant virus is known to induce immune responses of this type (Bender et al., 1996; Bennink et al., 1984). However, the inductivity of an immune response by individual immunization with DNA or a recombinant virus is limited and could induce moderate responses in mammals (Roy et al., 2000; Schneider et al., 1999, 2001; Swain et al., 2000), including dogs (Ramiro et al., 2003). To overcome these problems, in recent studies, it was shown that the combination of a heterologous prime–boost immunization with DNA followed by a recombinant vaccinia virus could induce a strong immune response in mammals (Amara et al., 2001; Ramiro et al., 2003). In a previous study, we demonstrated this immunization strategy using priming DNA followed by a recombinant vaccinia virus, both of which express a cell surface antigen P50 of *B. gibsoni*, and induced a high IgG2/IgG1 ratio of immune response in dogs (Fukumoto et al., 2007). On the other hand, the IgG1 and IgG2 antibody subclass induced by the immunization of recombinant P50 antigen expressed in insect cells with an adjuvant did not show any significant difference (Fukumoto et al., 2005b). Therefore, this prime–boost immunization regime also seems to be an effective strategy in dogs, but information regarding dogs is quite limited (Carson et al., 2009; Fukumoto et al., 2007; Ramiro et al., 2003; Ramos et al., 2008). To further demonstrate the efficacy of the heterologous immunization regime for dogs, we constructed the DNA and a recombinant vaccinia virus, both of which express the P29 gene of *B. gibsoni*, and we demonstrated their immunogenicity and growth inhibitory effects on *B. gibsoni* parasites.

To determine the immunogenicity of the heterologous regime of P29 with a limited number of dogs, we selected only one immunization regime, i.e., three times with priming DNA and a one-time boost with a recombinant vaccinia virus, both of which express P29, because the efficacy of this kind of regime had been shown in previous human study (McConkey et al., 2003), although dog groups immunized with DNA or vaccinia virus only was not determined. We analyzed the IgG response and the peripheral IFN-gamma as the marker for an immune response. The specific IgG against P29 was not detected in dogs (day −56 to −14) by the several

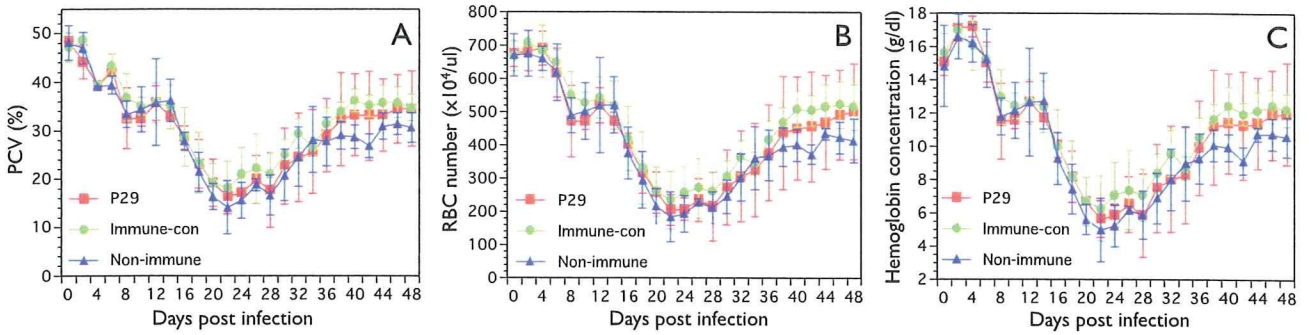

Fig. 5. Hematological parameters of the dogs after challenge infection with *B. gibsoni*-infected RBCs. (A) Packed cell volume (PCV), (B) RBC number, and (C) hemoglobin concentration. P29, dogs immunized with pCAGGS-P29 and vvP29; immune-con, dogs immunized with control plasmid pCAGGS and mO; non-immune, non-immunized control dogs. The results are shown as the mean values, and the error bars represent the standard deviations. Any significant difference was not observed between a dog group immunized with P29 and the control groups.

immunizations with DNA expressing P29. However, the specific IgG increased significantly (day −14 vs. day 0) with the booster immunization with the recombinant vaccinia virus vvP29. After the challenge infection of the *B. gibsoni* parasites, the IgG response against P29 in the dog group immunized with P29 significantly increased (day 8), and the IgG response maintained a significantly higher level throughout the experimental period than that in the control groups (days 8–48). To identify the immune response, we analyzed the IgG subclass against P29. The IgG2 subclass was detected as the major antibody subclass. Regarding the peripheral IFN-gamma response after the challenge infection with *B. gibsoni*, only the dog group immunized with P29 showed a detectable IFN-gamma response. These results suggested that the boosting effect of this type of immunization regime might be useful for the induction of IFN-gamma-producing $CD4^+$ T cell immune response for dogs.

To confirm the growth inhibitory effect of the heterologous prime–boost immunization with P29, a *B. gibsoni* parasite was inoculated 2 weeks after the vvP29 booster immunization. The peripheral parasitemia was monitored at one-day intervals. The parasite growth in the dog group immunized with P29 was significantly inhibited when compared to that of the control groups. The growth inhibitory rate of this parasite was quite similar to that in our previous study using the P50 gene as a target, and it did not show any significant difference between the P29 and P50 immunization experiments. In our previous study, P29 was suggested as an intracellular component (Fukumoto et al., 2003), and P50 was expressed on the parasite cell surface as a type-I transmembrane protein (Fukumoto et al., 2001). It was not clear how P29 interacted with the host and had a similarly protective action with P50; these results indicate that the activation of $CD4^+$ T cells and macrophage might be related to the inhibition of the parasite growth of P29, which could be a candidate antigen for further study of the control of canine *B. gibsoni* infection. However, individual antigen usage of P29 or P50 showed a limited growth inhibitory effect against the parasite and did not protect animals from clinical symptoms manifested as severe anemia.

These results indicated that further study of the detailed pathogenesis of this disease and the search for a more effective antigenic gene would be of value. In addition, the combined usage of multiple gene immunization in a prime–boost regime would be necessary for the development of an effective vaccine controlling canine *B. gibsoni* infection.

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REPRODUCTION
RESEARCH

Fertilizability, developmental competence, and chromosomal integrity of oocytes microinjected with pre-treated spermatozoa in mice

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Abstract

The aim of the present study was to investigate the safety of sperm pre-treatment during the ICSI procedure using a mouse model. Mouse spermatozoa were treated with methyl-β-cyclodextrin, lysolecithin, Triton X-100, and dithiothreitol (DTT), and injected into mouse oocytes. The injected oocytes were monitored for chromosomal integrity and pre- and post-implantation development. The chromosomal integrity of the injected oocytes was impaired by *in vitro* incubation and chemical antagonism. Particularly in the 60-min DTT group, severe chromosome damage increased. Despite the chromosomal damage, the resultant embryos frequently developed to the blastocyst stage. However, the embryos in the 60-min DTT group had significantly higher chromosomal damage and decreased developmental competence to live fetuses. These results indicate that excessive sperm pre-treatment such as DTT for 60 min generates severe chromosome damage in injected oocytes, and that the damage decreases developmental competence to live fetuses but not to blastocysts.

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Introduction

Spermatozoa undergo functional changes upon fertilization. Ejaculated spermatozoa are not yet potentially fertilizable. Their fertilizing capacity is acquired in the female genital tract in a process called sperm capacitation. When spermatozoa reach the fertilization site, the acrosome reaction, the exocytotic release of acrosomal enzymes, takes place due to penetration of the oocyte. Thus, only spermatozoa that have undergone these functional changes can be incorporated into the oocytes. As reviewed in Boerke *et al.* (2008), bicarbonate, Ca^{2+}, and albumin can induce sperm capacitation. Furthermore, capacitated spermatozoa are characterized by the redistribution of sperm membrane proteins, glycolipids, and cholesterol. These redistributions trigger spermatozoan hyperactivation, binding to zona, and the zona-induced acrosome reaction. Especially in realignment of sperm membrane proteins, fusion between the plasma membrane and outer acrosomal membrane and subsequent exocytosis of acrosome enzymes are regulated.

ICSI is characterized by direct/microsurgical introduction (injection) of spermatozoa into the oocytes (Yanagimachi 2005). The injection procedure allows the spermatozoa to fertilize independent of their motility. However, since injected spermatozoa are selected by an operator using outward criteria, such as progressive motility and morphological normality, it is likely that non-capacitated and acrosome-intact spermatozoa are injected into the oocytes and participate in fertilization events.

It is still unknown whether or not the use of spermatozoa with an unphysiological status (e.g. non-capacitated and intact acrosome) influences embryonic development. The hydrolytic enzymes released from sperm acrosomes induced the morphological deformation of injected mouse oocytes (Morozumi & Yanagimachi 2005). The compelling demembranization of spermatozoa and *in vitro* induction of the acrosome reaction by chemicals enhanced embryonic development *in vitro* (Morozumi *et al.* 2006). Tateno & Kamiguchi (2007) proposed that capacitated and acrosome-reacted spermatozoa might lower the incidence of chromosomal aberrations in ICSI embryos. The capacitation and acrosome reaction could be mimicked *in vitro* by cholesterol efflux with methyl-β-cyclodextrin (MBCD; Choi & Toyoda 1998, Takeo *et al.* 2008) and destabilizing membrane with lysolecithin (LL; Morozumi & Yanagimachi 2005, Morozumi *et al.* 2006) or Triton X-100 (TX; Perry *et al.* 1999) respectively. Therefore,

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it may be better to use spermatozoa with an *in vitro*-induced capacitation and acrosome reaction for ICSI (Roldan 2006). On the other hand, in ICSI of domestic animals, sperm pre-treatment such as dithiothreitol (DTT), which leads to a reduction of the protamine disulfide bond in sperm nuclei, was frequently applied due to low fertilizability of domestic animal spermatozoa in *in vitro*-matured oocytes (Rho *et al.* 1998, Galli *et al.* 2003, Ock *et al.* 2003, Watanabe & Fukui 2006, Watanabe *et al.* 2009).

Since the influence of sperm pre-treatment on embryonic and fetal normality is not fully understood, we investigated the chromosome integrity of zygotes and embryos derived from spermatozoa pre-treated with MBCD, LL, TX, and DTT. The safety of these sperm pre-treatments on *in vitro*-produced embryos for pre- and post-implantation production was investigated.

Results

Changes of mouse spermatozoa pre-treated with various chemicals

The effects of sperm pre-treatments were visualized by fluorescent dye (Fig. 1). Sperm pre-treatment with MBCD decreased the fluorescence intensity of the filipin-labeling sperm membrane cholesterol by 30.3% (Fig. 1A and B). The chemicals LL and TX removed almost all (99.5 and 99.2% respectively) of the acrosome membrane (Fig. 1D), whereas 92.5% of spermatozoa without pre-treatment had an intact acrosome (Fig. 1C). Furthermore, DTT reduced the disulfide bond in the sperm head in a time-dependent manner.

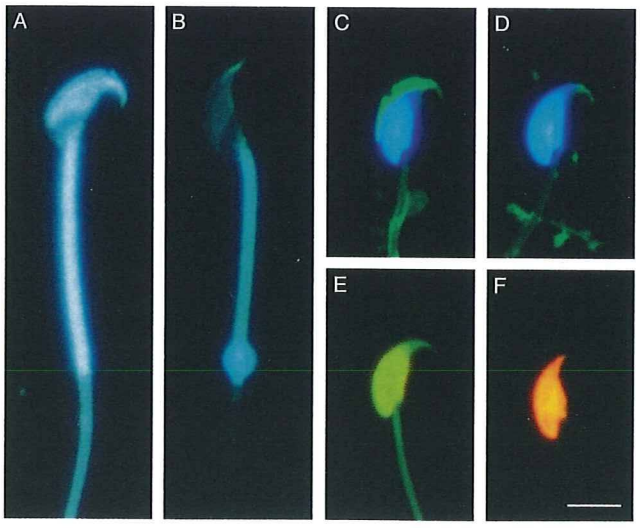

Figure 1 Visualization of the effects of sperm pre-treatments. Localization of sperm membrane cholesterol before (A) and after (B) MBCD treatment was evaluated by filipin. Acrosome before (C) and after (D) LL/TX treatments was stained with FITC-PNA. (E and F) Status of disulfide bond of DTT-treated spermatozoa was detected with acridine orange: (E) disulfide bond intact and (F) disulfide bond-reduced sperm head. Bar=5 μm.

The spermatozoa with a disulfide bond-reducing head (Fig. 1F) increased to 22.6, 83.7, and 94.9% for 10, 30, and 60 min treatments respectively. On the other hand, the control spermatozoa rarely had a disulfide bond-reducing head (0.3%).

Chromosomal integrity of mouse oocytes microinjected with in vitro *incubated and pre-treated spermatozoa*

The results of the chromosomal analysis of ICSI oocytes with spermatozoa incubated *in vitro* and pre-treated with the four chemicals are summarized in Table 1. As shown in Table 1, the cultivation of epididymal spermatozoa in Hepes-buffered Toyoda–Yokoyama–Hoshi medium (H-TYH) for 60 and 90 min increased ($P<0.05$) zygotic chromosomal breaks to 30.0 and 34.4% respectively. All pre-treatments of sperm induced zygotic chromosomal breaks of 30.8–83.0%, whereas in the control, which did not receive either incubation or pre-treatment, the percentage of breaks was 13.0% ($P<0.05$). When spermatozoa were treated with DTT for more than 30 min, chromosomal abnormalities remarkably ($P<0.001$) increased. Particularly, DTT treatment for 30 min and over generated severe aberrants (0.19–0.34). Within a subgroup in DTT treatment, the highest chromosome aberration rate was 83.0% in the group treated for 60 min. The culture of spermatozoa for 30 min following DTT treatment for 30 min (total 60 min) induced no further chromosomal damage.

In vitro *development of mouse eggs fertilized with spermatozoa pre-treated with various chemicals*

The majority of oocytes injected with pre-treated spermatozoa fertilized (86.5–97.2%) and developed (96.6–98.6%) to the two-cell stage (Table 2). Furthermore, even though spermatozoa were pre-treated with these chemicals, the injected oocytes frequently developed to the blastocyst stage (70.0–87.5%) with active mitosis. The developmental competence in the oocytes injected with these chemicals was comparable to that of the control. On the other hand, as shown in Table 3, the chromosomal aberration in spermatozoa pre-treated with DTT for 60 min continued up to the blastocyst stage, significantly different ($P<0.01$) to the control.

In vivo *development of mouse embryos derived from spermatozoa pre-treated with various chemicals*

The results of embryonic development during the post-implantation are shown in Table 4. When the embryos derived from spermatozoa pre-treated with DTT for 60 min were transferred to the surrogate females, 42.7 and 23.7% of the transferred embryos implanted and developed to live fetuses respectively. These values were

Table 1 Chromosomal analysis of mouse zygotes derived from spermatozoa with *in vitro* incubation and pre-treatments.

Treatment	Duration	Number of zygotes analyzed	Total number of (%) zygotes with chromosome aberrations[†,‡]	Types of chromosome aberrations[*]		Aberrations per zygote
				Minor	Severe	
H-TYH	0 min (control)	46	6 (13.0)	1	0	0.22
	30 min	35	8 (22.9)^{F}	1	0	0.40
	60 min	50	15 (30.0)^{b,C,E}	0.93	0.07	0.94
	90 min	61	21 (34.4)^{b}	1	0	0.83
MBCD	90 min	49	20 (40.8)^{b}	1	0	0.82
LL	1 min	52	16 (30.8)^{c}	1	0	0.46
TX	1 min	57	23 (40.4)^{b}	1	0	0.61
DTT	10 min	30	11 (36.7)^{c,A}	1	0	0.30
	30 min	60	27 (45.0)^{a,B,F}	0.81	0.19	1.40
	60 min	53	44 (83.0)^{a,A,B,C,D}	0.66	0.34	5.36
DTT+culture	30 min each	50	29 (58.0)^{a,D,E}	0.86	0.14	1.70

H-TYH, Hepes-buffered Toyoda–Yokoyama–Hoshi medium; MBCD, methyl-β-cyclodextrin; LL, lysolecithin; TX, Triton X-100; DTT, dithiothreitol.
[†]Statistical significance compared to control: $^{a}P<0.001$; $^{b}P<0.01$; $^{c}P<0.05$. [‡]Statistical significance, comparing the same letters: $^{A,B,C}P<0.001$; $^{D,E}P<0.01$; $^{F}P<0.05$.
[*]The proportions of each aberration type in the total number of zygotes with chromosome aberrations.

lower than those of the control group (60.9 and 39.8%: $P<0.01$). On the other hand, the rates of implants and live fetuses in ICSI embryos derived from spermatozoa treated with LL (76.8 and 60.7%) or DTT for 30 min (75.4 and 60.0%) were higher ($P<0.05$ and $P<0.01$) than the control group. Moreover, sperm pre-treatment did not affect the proportion of live fetuses per implant. All live fetuses were normal in morphology regardless of whether or not sperm pre-treatment had been performed. Furthermore, all analyzed fetuses had 40 intact chromosomes (Fig. 2).

Discussion

It was believed that sperm pre-treatment was effective for *in vitro* embryo production, and the beneficence depended on the exposure dose and duration of the chemical used. Although the defective effect of MBCD treatment was attributed to the prolonged incubation *in vitro* rather than to chemical antagonism (Table 1), it was clearly demonstrated in the present study that as a whole, all sperm pre-treatments were harmful to the spermatozoa; chemical antagonism or *in vitro* incubation and both during sperm pre-treatment generated chromosomal breaks. In particular, excessive treatment such as DTT for 60 min produced chromosomally damaged embryos, although embryonic development up to the blastocyst stage was not impaired. Two-cell embryos derived from spermatozoa treated with DTT for 60 min induced a lower development of live fetuses, suggesting that the percentage of blastocyst formation was not a direct indicator of developmental competence of embryos to offspring.

In the present study, the chromosomal aberration of ICSI oocytes derived from spermatozoa without any incubation and pre-treatment (control) was relatively higher (13.0%) compared with some previous studies (5.7–8.3%; Szczygiel & Ward 2002, Watanabe 2004, Tateno & Kamiguchi 2007). In this respect, sperm samples in the present study might have contained

Table 2 *In vitro* development of mouse embryos derived from spermatozoa pre-treated with various chemicals.

Treatment	Duration	Number of oocytes injected	Number of (%) surviving oocytes	Number of (%) oocytes fertilized	Number of (%)[a] embryos developed to the two-cell stage	Number of (%)[a] embryos developed to the blastocyst stage	Number of cells/ blastocyst	Mitotic index[b]
Control	–	72	59 (81.9)	55 (93.2)	54 (98.2)	43 (78.2)	58.1	0.11
MBCD	90 min	84	71 (84.5)	69 (97.2)	68 (98.6)	57 (82.6)	58.5	0.16
LL	1 min	85	74 (87.1)	64 (86.5)	63 (98.4)	56 (87.5)	56.6	0.12
TX	1 min	87	71 (81.6)	65 (91.5)	63 (96.9)	51 (78.5)	50.4	0.13
DTT	30 min	73	61 (83.6)	59 (96.7)	57 (96.6)	46 (78.0)	47.3	0.14
	60 min	69	54 (78.3)	50 (92.6)	49 (98.0)	35 (70.0)	57.3	0.11

MBCD, methyl-β-cyclodextrin; LL, lysolecithin; TX, Triton X-100; DTT, dithiothreitol.
[a]Value was calculated from number of oocytes fertilized. [b]Mitotic index was calculated as the ratio between the number of mitotic cells and the total number of cells.

Table 3 Chromosomal analysis of mouse blastocysts derived from spermatozoa pre-treated with various chemicals.

Treatment	Duration	Number of blastocysts analyzed	Total number of (%) embryos with chromosome aberrations†	Types of chromosomal aberrations*		
				Ploidy	Ploidy+ structural	Structural
Control	–	36	10 (27.8)	0.40	0	0.60
MBCD	90 min	41	11 (26.8)	0.36	0.09	0.54
LL	1 min	34	7 (20.6)	0.14	0	0.86
TX	1 min	40	13 (32.5)	0.15	0.31	0.54
DTT	30 min	30	15 (50.0)	0.33	0.07	0.60
	60 min	18	12 (66.7)[a]	0.25	0.17	0.58

MBCD, methyl-β-cyclodextrin; LL, lysolecithin; TX, Triton X-100; DTT, dithiothreitol. †Statistical significance compared to control: [a]$P<0.01$. *The proportions of each aberration type in the total number of embryos with chromosome aberrations.

a number of membrane-damaged spermatozoa, since it is reported that the factor released from the spermatozoa induced DNA nicks (Perez-Crespo *et al.* 2008). Nevertheless, ICSI oocytes with incubated or pre-treated spermatozoa have much higher rates of chromosome damage than that of the control group.

The targets of each treatment in the present study were different; cholesterol on sperm plasma membrane for MBCD, sperm plasma membrane *per se* for LL and TX, and the disulfide bond in sperm nuclei for DTT. Therefore, it was inferred that DTT was the most severe treatment compared with the others. The defective effect on the chromosomal integrity of spermatozoa along with the disulfide bond-reducing ability occurred in a time-dependent manner. The under-protaminated spermatozoa are less normal (Bianchi *et al.* 1993, Manicardi *et al.* 1995, Sakkas *et al.* 1996). Thus, DNAs of DTT-treated spermatozoa, lacking a disulfide bond, are breakable. Since DTT destroys the sperm plasma membrane depending on the duration of treatment (Ock *et al.* 2003), excessive treatment of DTT (such as 60 min) would be harmful to sperm chromosomes. In turn, there is supporting evidence that prolonged DTT treatment (50–60 min; Yong *et al.* 2005, Watanabe & Fukui 2006) and a combination of TX and DTT treatments (Nakai *et al.* 2006) degrade the fertilizability and developmental competence of porcine oocytes. Additionally, DTT treatment with a lower dose (2 mM) and for a shorter period (15 min) does not generate chromosomal nicks (Szczygiel & Ward 2002). The outcomes in Table 1 also show that prolonged exposure (60 min) to DTT increased chromosomal breaks, while DTT+culture for 30 min each induced no further damage compared with the 30-min DTT group.

Medium components (Hepes, ion balance, and others) affect sperm chromosome integrity (Tateno *et al.* 2000, Watanabe 2004, Tateno & Kamiguchi 2007). Changes in the sperm membrane induced by pre-treatment might be one of the reasons for the induction of chromosomal lesions due to direct exposure to medium components. As described above, most chromosomal lesions by MBCD treatment are caused by *in vitro* incubation for 90 min. These findings suggest that a shorter treatment is needed for chromosomal integrity in spermatozoa. However, it should be noted that LL and TX treatments were performed for only 1 min and increased chromosomal breaks. It was noteworthy that the spermatozoa were damaged within only a few minutes (~5 min) between individual demembranization of the spermatozoon and injection into oocytes. Since chromosomal damage of membrane-disrupted spermatozoa by freeze–drying was decreased by modifying the pH value of storage media (Kaneko *et al.* 2003*b*), and by pre-treating with diamide (Kaneko *et al.* 2003*a*) and EGTA (Kusakabe *et al.* 2008), the optimization of conditions during sperm pre-treatment might reduce chromosomal damage.

Table 4 *In vivo* development of embryos derived from spermatozoa pre-treated with various chemicals, and chromosomal analysis of resultant fetuses.

Treatment	Duration	Number of embryos transferred	Number of recipients	Number of (%) implants*,†	Number of (%) live fetuses*,† (range, %)	Live fetuses/ implant	Number of fetuses analyzed	Chromosomal integrity (%)
Control	–	128	9	78 (60.9)	51 (39.8) (13–67)	65.4	27	27 (100)
MBCD	90 min	70	5	46 (65.7)	37 (52.9) (27–81)	80.4	18	18 (100)
LL	1 min	56	4	43 (76.8)[b]	34 (60.7)[a] (44–75)	79.1	17	17 (100)
TX	1 min	56	4	40 (71.4)	31 (55.4) (25–81)	77.5	18	18 (100)
DTT	30 min	65	5	49 (75.4)[b,A]	39 (60.0)[a,A] (0–80)	79.6	16	16 (100)
	60 min	131	10	56 (42.7)[a,A]	31 (23.7)[a,A] (0–58)	55.4	19	19 (100)

MBCD, methyl-β-cyclodextrin; LL, lysolecithin; TX, Triton X-100; DTT, dithiothreitol. *Statistical significance compared to control: [a]$P<0.01$; [b]$P<0.05$. †Statistical significance, comparing the same letters: [A]$P<0.001$.

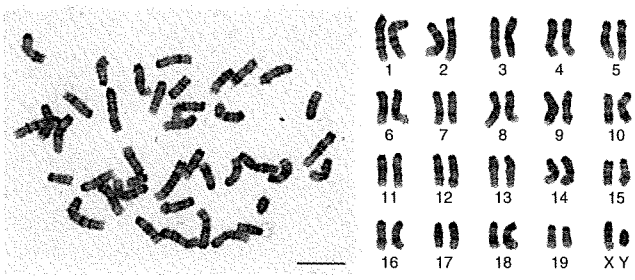

Figure 2 G-banding chromosome spread (left) and karyotype (right) from live fetus skin cells derived from spermatozoa pre-treated with DTT for 60 min. Bar=10 μm.

Sperm DNA provides half of an embryo's genetic source. Therefore, chromosomal damage of spermatozoa might induce defective zygotic gene activation that begins at the middle of the one-cell stage and bursts into the two-cell stage (Minami *et al.* 2007). Therefore, detailed observation of embryos derived from pre-treated spermatozoa should be carried out during pre- and post-implantation. The present results revealed that embryos derived from damaged (pre-treated) spermatozoa frequently developed up to the blastocyst stage with active mitosis (Table 2), in agreement with another study (Perez-Crespo *et al.* 2008). However, DNA-damaged spermatozoa reduced blastocyst formation depending on the disintegration level (Ahmadi & Ng 1999*a*, 1999*b*). These observations might suggest that the types of chromosomal damage generated in our study were insignificant for embryonic development during the pre-implantation stage. However, there was a contradiction between the percentage of blastocyst formation and that of chromosomal aberration of the blastocysts: the percentage of chromosomal aberration in DTT for 60 min was higher (66.7%; Table 3). In consequence, post-implantation development of the embryos in DTT treatment for 60 min was impaired, in agreement with previous studies (Montag *et al.* 1997, Stern *et al.* 1999, Benchaib *et al.* 2003, Tateno & Kamiguchi 2007), where it was shown that zygotic/embryonic chromosomal damage was a serious problem in ongoing pregnancy. These facts clearly demonstrate that a competent embryo is the result of chromosomal integrity – not developmental capacity – up to the blastocyst stage.

Previous studies demonstrated that DNA double-strand breaks were restored (Kanaar *et al.* 1998), and that oocytes were capable of repairing sperm DNA lesions until the first mitotic division (Generoso *et al.* 1979, Genescà *et al.* 1992) or during subsequent embryonic development (Generoso *et al.* 1979, Ahmadi & Ng 1999*b*). Therefore, from the results in Table 3, it was possible to interpret that embryos derived from spermatozoa treated with MBCD, LL, or TX were restored during embryonic development, while the damage generated by DTT for 60 min was beyond the repair potential of oocytes. The number of chromosomal aberrations per ICSI oocyte injected with spermatozoa pre-treated with these chemicals (0.46–5.36; Table 1) may support this interpretation. In other words, 'minor' chromosome damage in zygotes is repairable during the pre- and post-implantation stages. For example, the minor chromosome aberration in ICSI oocytes of the 60-min DTT group was 66% (Table 1), and some of these aberrations could be repaired. Therefore, the percentage of fetuses that could be obtained was 20% or more, although the incidence of chromosomal damage in ICSI oocytes was higher (83.0%). On the other hand, the severe chromosome aberration in ICSI oocytes in the 30- and 60-min DTT groups might have resulted in the wide range in the rates of live fetuses.

Post-implantation development of ICSI embryos derived from pre-treated spermatozoa was thus acceptable, except in the 60-min DTT treatment group. As reported previously (Morozumi *et al.* 2006), ICSI embryos derived from LL-treated spermatozoa had higher developmental competence in the post-implantation stage, suggesting that the acrosomal contents might hinder embryonic development in this phase. Interestingly, a higher developmental competence was also observed in DTT treatment for 30 min in the present study. However, it was not clear from the results how this higher developmental competence occurred. It might have been induced by the reducing disulfide bond and membrane disruption (Ock *et al.* 2003) in DTT treatment. Nevertheless, the appropriate duration of DTT treatment is likely <30 min, as supported by our previous study in pig (Watanabe & Fukui 2006).

The present results provide an additional possibility that the previous application of sperm pre-treatment into bull (Galli *et al.* 2003) and boar (Watanabe & Fukui 2006, Watanabe *et al.* 2009) spermatozoa might have induced critical damage in their chromosomes, since the success of sperm pre-treatment was decided only by monitoring embryonic development up to the blastocyst stage. In livestock, however, sperm pre-treatment during ICSI procedures would be very advantageous due to low fertilizability of the oocytes in these animals. Our goal is to establish the most appropriate method of sperm pre-treatment in order to improve the production of normal embryos and offspring mediated by ICSI.

In conclusion, *in vitro* incubation and chemical antagonism during sperm pre-treatments induced chromosomal aberrations in ICSI oocytes. Despite the chromosomal damage, the resultant embryos developed to the blastocyst stage. However, the ICSI oocytes derived from spermatozoa pre-treated with DTT for 60 min contained massive chromosome aberrations and decreased embryonic development during post-implantation. Thus, the present sperm pre-treatment before ICSI was not always effective, and fetal development

was not in line with blastocyst development. Given these issues, the use of sperm pre-treatment for the ICSI procedure needs to be reconsidered.

Materials and Methods

Reagents and media

All chemicals were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) unless otherwise stated. The culture medium used for the mouse oocytes after ICSI was Chatot–Ziomek–Bavister (CZB; Chatot *et al.* 1989) supplemented with 5.56 mM D-glucose and 4 mg/ml BSA (fraction V; Sigma–Aldrich). Mouse oocyte collection and microinjection were performed in modified CZB supplemented with 20 mM Hepes-Na, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol (cold water soluble; Sigma–Aldrich) in place of BSA (H-CZB). Mouse spermatozoa were collected in a modified TYH medium (Toyoda *et al.* 1971) supplemented with 20 mM Hepes, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol in place of BSA (H-TYH). The pH value of both H-CZB and H-TYH was adjusted to ~7.4. The chemicals (MBCD, LL, TX, and DTT) for sperm pre-treatment were from Sigma–Aldrich. For culture of skin cells derived from fetuses, DMEM (Sigma–Aldrich) supplemented with 1% penicillin–streptomycin solution (Sigma–Aldrich) and 10% FCS (Gibco-BRL) was used.

Animals

All animals were purchased from CLEA Japan, Inc (Tokyo, Japan). The oocytes and spermatozoa were collected from B6D2F1 mice. ICR mice were used as surrogate mothers. All experiments were performed according to the Guiding Principles for the Care and Use of Research Animals of Obihiro University of Agriculture and Veterinary Medicine.

Preparation of oocytes and spermatozoa for ICSI

B6D2F1 female mice, 7–11 weeks of age, were superovulated by i.p. injection of 10 IU eCG (Asuka Pharmaceutical, Tokyo, Japan) followed by injection of 10 IU hCG (Asuka Pharmaceutical) 48 h later. The oocytes recovered from oviducts between 14 and 16 h after hCG injection were denuded of their cumulus cells by treatment with 0.1% (w/v) bovine testicular hyaluronidase (Sigma–Aldrich) in H-CZB. The denuded oocytes were repeatedly rinsed in CZB medium and kept at 37 °C under 5% CO_2 in the same medium until ICSI. Spermatozoa were collected from the cauda epididymis of 7–12-week-old male mice. The collected spermatozoa were immediately used for the experiments.

Sperm pre-treatment

In MBCD treatment, the spermatozoa were kept in H-TYH containing 0.75 mM MBCD for 90 min (Choi & Toyoda 1998) at 37 °C. The treated spermatozoa were washed twice by centrifugation at 300 ***g*** for 5 min in H-TYH. A small amount (1–2 μl) of the sperm suspension was transferred into a droplet (5 μl) of H-TYH containing 10–12% polyvinyl pyrrolidone (PVP; molecular weight: 360 000; Nacalai Tesque, Kyoto, Japan). Motile spermatozoa were collected and used for ICSI.

The sperm pre-treatment procedure followed that done in a previous study (Morozumi *et al.* 2006). Namely, a motile spermatozoon was collected by a micromanipulator under an inverted microscope and transferred into the droplets of H-TYH containing 0.02% of LL, 0.02% of TX, or 5 mM DTT. After treatment, the spermatozoa were recovered and washed twice in a fresh H-TYH droplet with PVP. The duration of each treatment was 1 min (LL and TX) and 10, 30, or 60 min (DTT).

ICSI procedures

As the control, motile spermatozoa without any treatment and cultivation were used for ICSI. Before injection, a batch of 15 oocytes was transferred into a droplet (5 μl) of H-CZB, which had been beside a sperm-containing droplet in an ICSI chamber covered with paraffin oil (Merck). A control or treated spermatozoon was aspirated into the injection pipette tail first, and the tail was cut at the mid-piece by applying several piezo pulses. The tail-cut spermatozoon was then individually injected into a mouse oocyte according to the method of Kimura & Yanagimachi (1995). The series of experiments for ICSI were completed within 30 min of sperm pre-treatment.

Culture of oocytes injected with spermatozoa

The injected oocytes were washed with CZB and cultured in a droplet (30 μl) of the same medium covered with paraffin oil at 37 °C under 5% CO_2 in air. The oocytes that had a second polar body and two pronuclei after 5–6 h of ICSI were considered to be normally fertilized. The developmental stage of the embryos was observed at 24 and 96 h after ICSI.

Chromosome preparation of fertilized eggs and blastocysts

After 6–8 h of ICSI, the oocytes were transferred to CZB containing 0.02 μg/ml vinblastine sulfate to inhibit the first cleavage division. At 19–21 h after ICSI, they were treated with 0.5% protease (Kaken Pharmaceuticals, Tokyo, Japan) in Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS to digest zona pellucida. They were then kept in a solution consisting of equal volumes of 1% (w/v) sodium citrate and 30% (v/v) FCS (hypotonic solution) for 10 min at room temperature. These samples were prepared by the gradual fixation/air drying method (Mikamo & Kamiguchi 1983). The slides were stained with 2% Giemsa (Merck) in buffered saline (pH 6.8) for 10 min. Chromosomal aberrations were then examined without distinguishing between paternal and maternal nuclei. The detected aberrations were judged as paternal in origin because mouse oocytes rarely have chromosomal aberrations (Tateno & Kamiguchi 2002). Furthermore, the metaphase plates were classified as normal (Fig. 3A), minor (1–9 chromosome breaks in a metaphase plate; Fig. 3B) and severely (more than ten chromosome breaks in a metaphase plate; Fig. 3C) aberrant as reported by Kaneko & Nakagata (2005).

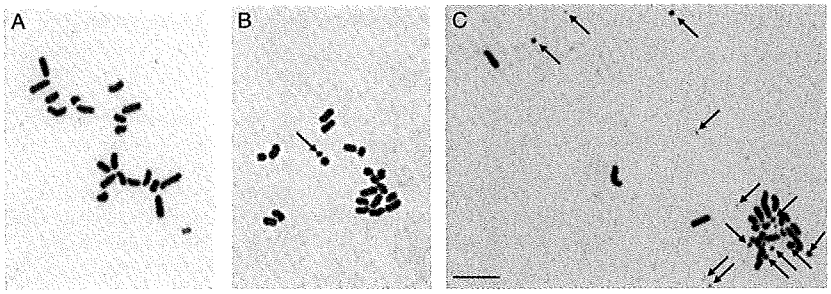

Figure 3 Types of chromosomal spread of mouse oocytes (first mitotic stage) injected with spermatozoa. (A) Normal chromosome spread. Twenty intact chromosomes are seen. (B) Minor chromosome aberration. Chromosome fragmentation is seen (arrow). (C) Severe chromosome aberration. Arrows indicate the representatives of chromosome and chromatid fragmentations. Bar=10 μm.

After culture for 96 h, the developed blastocysts were incubated in CZB containing 0.04 μg/ml colcemid (Sigma–Aldrich) for 3 h and were treated with 0.5% protease followed by a hypotonic solution for 2–3 min. Thereafter, the embryos were fixed and stained as described above. The number of cells was counted, and the mitotic index, an indicator of cell growth, was defined as the ratio between the number of mitotic and total number of cells (McCauley *et al.* 2003). Intact and non-overlapping chromosome spreads (range: 1–8 cells in an embryo) were examined for analysis. In the present study, an embryo containing cell(s) with structural aberration and/or abnormal ploidy (haploid, mixoploid, and polyploid) was judged as aberrant (Fig. 4).

Examination of post-implantation development and fetal chromosome analysis

Two-cell embryos were transferred into the oviducts of ICR pseudopregnant females at 8–14 weeks of age. Recipients were killed on day 16 of pregnancy, and the number of implantation sites and live fetuses were recorded. Live fetuses were randomly selected for examination of their chromosome integrity. After the fetal skin cells were cultured for 3–5 days under 5% CO_2 in air at 37 °C, they were treated with 0.05 μg/ml colcemid for 2–3 h. The cells recovered by treating trypsin–EDTA solution (Gibco-BRL) were kept in 0.075 M KCl in distilled water. They were then fixed with a methanol–acetic acid (3:1) mixture on a glass slide. The G-banded chromosome slides were obtained by a routine trypsin digestion method. The karyotype was determined according to previous reports (Nesbitt & Francke 1973, Cowell 1984).

Visualization of the effects of sperm pre-treatments

Functional changes of pre-treated spermatozoa (at least 100 cells in each group) were monitored by fluorescent dye as described below.

Sperm membrane cholesterol of the MBCD-treated spermatozoa was labeled with 0.1 mg/ml filipin (Cayman Chemical, Ann Arbor, MI, USA) for 30 min at room temperature in the dark. The spermatozoa were then smeared on glass slides and examined by fluorescence (UV excitation filter: UV-2A, Nikon, Tokyo, Japan). The fluorescence intensity was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

Spermatozoa pre-treated with LL and TX were mixed in 95% ethanol and kept for 30 min at 4 °C. Thereafter, the spermatozoa were smeared and the sperm acrosomes stained by 0.1 mg/ml FITC-PNA (Vector Laboratories, Inc., Burlingame, CA, USA) for 10 min at room temperature in the dark. The samples were sealed by Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc.) and examined by fluorescence (B excitation filter: B-2A, Nikon).

Spermatozoa pre-treated with DTT were smeared on glass slides and fixed in a methanol–acetic acid (3:1) mixture. After fixation, the slides were stained with 0.2% acridine orange (Sigma–Aldrich) for 5 min at room temperature and examined by fluorescence (B excitation filter).

Statistical analysis

All experiments were repeated for 3–5 times. The χ^2 or Fisher's exact probability test was used for analyses of the rates of chromosomal aberration and developmental competence.

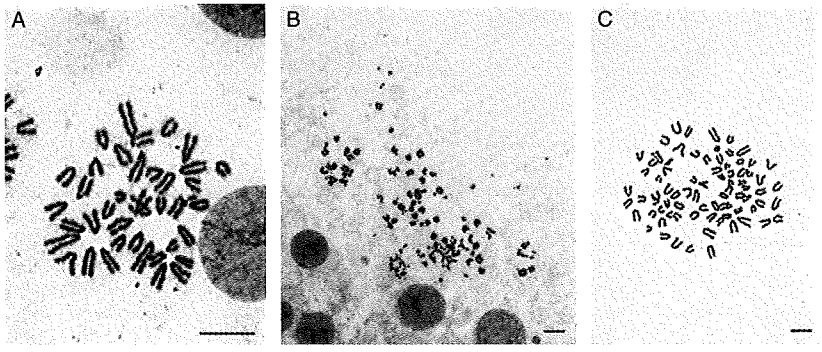

Figure 4 Types of chromosomal spread in mouse blastocysts. (A) Normal diploid cell. (B) Anomalous cell with ploidy and structural aberrations. (C) Polyploid cell. Bar=10 μm.

The cell numbers of the blastocysts and mitotic index were analyzed by Steel's test. All data were compared to the control group. In DTT treatment, the analysis was also performed within a subgroup. Differences were considered significant when the P value was <0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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RESEARCH NOTE

Vitrification of canine cumulus–oocyte complexes in DAP213 with a cryotop holder

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Abstract
Purpose The effects of the cryoprotectant and the container (holder) used for the vitrification of canine germinal vesicle stage oocytes were examined to improve the cryopreservation method for canine oocytes and embryos.
Methods Canine cumulus–oocyte complexes (COCs) were collected from ovaries, and were vitrified with E30S (30% ethylene glycol and 0.5 M sucrose) or DAP213 (2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol) solution held by a cryotube or cryotop sheets. After warming, the oocytes were stained with propidium iodide for the assessment of their plasma membrane integrity.
Results In all the vitrification groups, more than 65% of the vitrified oocytes displayed a normal morphology (E30S-top, 65.6%; DAP-tube, 67.3%; DAP-top, 80.0%). However, when assessed by propidium iodide staining, the viability of oocytes in the DAP-top group (43.6%) was higher than that in the E30S-top group (21.3%, $P < 0.05$). Furthermore, the viability of the oocytes in the DAP-top group (43.6%) was higher than that in the DAP-tube group (4.1%, $P < 0.05$).
Conclusions These results suggest that a combination of DAP213 as the cryoprotectant and a cryotop sheet as the holder improved viability after the vitrification of canine oocytes at the germinal vesicle stage.

Keywords Cryopreservation · Cryotop · DAP213 · Dog · Oocyte

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Introduction

Assisted reproductive techniques (ARTs), such as in vitro maturation (IVM) of oocytes, in vitro fertilization, and the culture and cryopreservation of embryos have only limited success in canine species when compared to these ARTs used in other animals. However, the above ARTs can still be useful; for example, for the improved breeding of experimental, companion, and working dogs, including guide dogs for the blind. Although guide dogs make a remarkable contribution to the improvement of the quality of life of blind people across the world, many countries suffer from an acute shortage of guide dogs. For example, in Japan current figures indicate that approximately 1,000 dogs are actively engaged in guiding blind people, but this number is extremely low in light of the demand, which is estimated to range from 4,800 to 7,800. Successful application of ARTs would help make it possible to overcome this problem. In this regard, there are several reports on IVM of oocytes, culture of embryos [1, 2], and cryopreservation of oocytes in dogs [3]. Proper storage of oocytes at the germinal vesicle (GV) stage in advance is an effective means of prospective animal production in dogs whose gamete availability is restricted, because the maturation rate of canine oocytes is still low at present. Vitrification has been widely applied to the cryopreservation of mammalian oocytes and embryos. In mice, cryopreservation of embryos by a vitrification method utilizing a cryotube with DAP213 solution (2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol) has been successfully demonstrated [4]. Moreover, this method was found to be applicable to the vitrification of canine ovarian tissues, including oocytes suitable for banking [5]. On the other hand, porcine oocytes were vitrified successfully using a cryotop sheet following exposure to E30S vitrification

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solution (30% ethylene glycol containing 0.5 M sucrose) [6]. Canine oocytes are similar to porcine oocytes in diameter and in the characteristic that they have a high cytoplasmic lipid content; the procedure of vitrification in porcine oocytes may therefore be suitable for canine oocytes. However, it is unclear whether these procedures are appropriate for the vitrification of canine oocytes. High cooling and warming rates are required to prevent ice crystal formation and to reduce chilling injury when oocytes and embryos are cryopreserved by vitrification. In an effort to improve the cooling and warming rates required during vitrification, modification of the methods has been undertaken, especially in the development of various containers, such as open-pulled straws [7], cryoloops [8], and cryotops [9]. Recently, we have shown that canine oocytes can be vitrified with DAP213 solution and E30S solution [3]. In the present study, the effect of the container (holder) on the vitrification of canine germinal vesicle (GV) stage oocytes in DAP 213 solution and E30S solution was examined, with the aim of further improving the cryopreservation method for canine oocytes and embryos. The viabilities of vitrified oocytes were assessed by their plasma membrane integrity, because efficient culture conditions for maturation have not yet been established for canines. Additionally, we examined differences in the cooling and warming rates between the use of a cryotop and a cryotube.

Materials and methods

Collection of cumulus oocyte complexes (COCs)

Ovaries within the ovarian bursa from 66 bitches (1–8 years) in diestrus stages were collected at veterinary clinics and transported to the laboratory in a thermos flask containing sterile saline at approximately 37°C. Each ovary was cleaned of fat and blood vessels and placed in a Petri dish containing TCM199 medium (Gibco-Invitrogen Life Technologies, NY, USA) supplemented with 10% fetal calf serum, 100 units/mL penicillin G potassium (Meiji, Tokyo, Japan), and 100 μg/mL streptomycin sulfate (Meiji), for further dissection. Ovarian tissue was sliced with a surgical blade (Feather, Osaka, Japan) repeatedly to collect COCs. Only COCs with more than two layers of cumulus cells and homogeneous dark cytoplasm 100 μm or more in diameter were used in this study. All chemicals were purchased from Sigma Chemical (St. Louis, MO, USA) except for those specifically described.

The tissues and cells derived from 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.

Vitrification and warming

DAP213 solution

The COCs were washed with phosphate-buffered medium (PB1) [10] containing 1 M dimethyl sulfoxide (DMSO) at room temperature (23 ± 2°C). The COCs were transferred into a 1 mL cryotube (Nalge Nunc International, Tokyo, Japan) containing 5 μL of 1 M DMSO, which was then placed in ice water for 5 min to allow the DMSO to thoroughly bathe the COCs. Subsequently, 95 μL of DAP 213 solution [4], maintained at 0°C, was added to each cryotube. After the cryotubes had been placed in ice water for 5 min, they were plunged directly into liquid nitrogen and stored until use (DAP-tube group). In another series of experiments, COCs were placed on a cryotop sheet (Kitazato Supplies, Tokyo, Japan) [9] after exposure to 1 M DMSO for 5 min and DAP 213 solution for 5 min, each, and immediately plunged into liquid nitrogen (DAP-top group). For warming, in the DAP-tube group, the samples were taken from the liquid nitrogen and allowed to stand at room temperature (23 ± 2°C) for 60 s, and then diluted with 900 μL of PB1 medium (37°C) containing 0.25 M sucrose. In the DAP-top group, the cryotop holder with COCs was transferred from liquid nitrogen (LN_2) into PB1 medium (37°C) containing 0.25 M sucrose. The recovered COCs were transferred to PB1 medium and washed 5 times.

E30S solution

The COCs or embryos were exposed to PB1 containing 5%, 10%, and 20% ethylene glycol (EG), and 30% EG containing 0.5 M sucrose for 5, 2, 2, and 1 min, respectively, at room temperature (23 ± 2°C) [5]. They were then placed on a cryotop sheet, and the cryotop was immediately plunged into liquid nitrogen (E30S-top group). The COCs were warmed at 37°C, and the cryoprotectants were removed in a step-wise manner at 37°C: the cryotop holder was transferred from LN_2 into PB1 containing 0.5 M sucrose, and the COCs that were recovered from the cryotop sheet were transferred to PB1 with a sequential series of 0.5, 0.25, and 0.125 M sucrose, 1 min in each solution at 37°C, and finally transferred into PB1 for 5 min at 37°C.

Plasma membrane integrity of vitrified—warmed GV oocytes and the kinetics of the sample temperature in the vitrification procedures

Immediately after warming and the removal of the cryoprotectants, the vitrified COCs were observed morphologically, and the oocytes were denuded of cumulus cells in PB1, using a fine-bore pipette, by repeated aspiration and