

—Technical Note—

## Artificial Insemination with Canine Spermatozoa Frozen in a Skim Milk/Glucose-Based Extender

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**Abstract.** Due to the recent outbreak of avian influenza, transportation of frozen canine semen with egg yolk has been sharply restricted. Thus, there is urgent need to develop a novel egg yolk-free extender for freezing canine spermatozoa. In the present study, the effect of using skim milk/glucose (SG)-based extender without egg yolk on the motility and fertilizing capacity of canine spermatozoa frozen-thawed in the presence of glycerol was examined. There was a tendency for the proportion of motile spermatozoa exposed to SG-based extender for 3 h to be higher than that exposed for 1 h, but the difference was not significant. The motility and other viability parameters of canine spermatozoa after thawing were similar to those obtained with an egg yolk-based extender. When spermatozoa frozen with SG-based extender containing glycerol after 3 h exposure were transcervically inseminated into 2 recipient bitches, a total of 6 pups were obtained. These results suggest that a simple extender composed of skim milk, glucose and glycerol is useful for cryopreservation of canine spermatozoa, which may contribute to improved exchange of genetic material and efficient production of companion and working dogs, such as guide dogs for the blind.

**Key words:** Canine, Cryopreservation, Skim milk/glucose-based extender, Spermatozoa

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Although freezing of canine semen and insemination of canine bitches with frozen-thawed semen is not as commonly used as in bovine and equine animals, successful artificial insemination with frozen canine semen has been well documented [1] since the first conception in 1969 [2]. Cryopreservation of canine spermatozoa offers potential exchange of genetic material, and thus may lead to improvement in the breeding management programs used to produce working dogs. In particular, in guide dog colonies, application of transcervical artificial insemination using frozen canine semen is anticipated to assist with meeting the demand for adequate supply of guide dogs for the blind. Egg yolk is the most commonly used compound in canine semen extenders for protection of spermatozoa from cold shock and disruption during the freezing and thawing process [1]. However, due to a recent outbreak of avian influenza and its triggering of growing concern throughout the world, transportation of frozen or chilled semen exposed to egg yolk has become extremely difficult. Several countries have, in fact, prohibited export and import of canine frozen semen that contains egg yolk. Thus, it is an urgent matter to develop a novel semen extender without egg yolk for use in freezing of canine spermatozoa. As an alternative compound to egg yolk, skim milk seems to be especially suitable as a semen extender in canine species, since a skim milk extender is the most commonly used extender for mouse [3] and goat [4] sperm. We report here successful artificial insemination with canine spermatozoa frozen in a solution contain-

ing skim milk, glucose and glycerol.

### Materials and Methods

#### Collection of ejaculated semen

The ejaculates from a total of five male Labrador Retrievers with proven fertility in natural mating were collected by digital manipulation into sterile tubes (Corning, Corning, NY, USA). The first and third fractions (seminal plasma) of the ejaculate were discarded. Only 3–4 ml of the sperm-rich second fraction of the ejaculates was collected for the experiments.

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

#### Preparation of semen extenders

For a skim milk/glucose (SG)-based extender, 30 mg/ml of skim milk (232100; Difco, Le Pont de Claix, France) and 0.3 M glucose (041-00595; Wako, Osaka, Japan) were dissolved in water for embryo transfer (W1503; Sigma-Aldrich, St. Louis, MO, USA) at 60 C, and then the solution was centrifuged at 10,000 g for 15 min at room temperature. The supernatants were filtered (25CS045AS; Advantec, Tokyo, Japan) and used as the SG-based extender.

As a control, an egg yolk-Tris-citrate-glucose (EY) extender composed of 20% [v/v] egg yolk, 24 mg/ml Tris(hydroxymethyl)aminomethane (252859; Sigma), 14 mg/ml citric acid monohydrate (035-03495; Wako), 0.8 mg/ml glucose, 0.65 mg/ml

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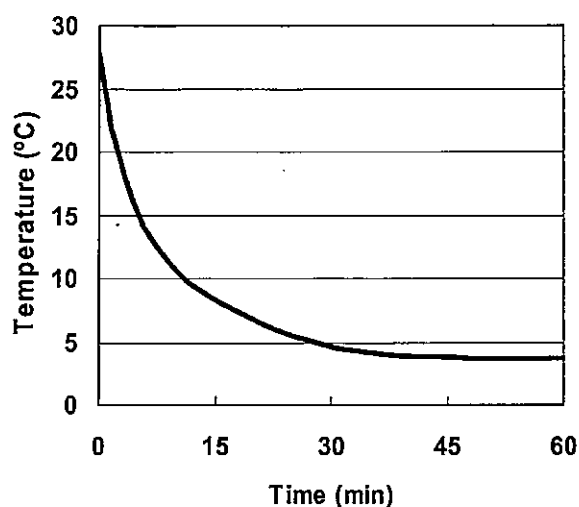


Fig. 1. Cooling rate of 1 ml of the skim milk/glucose-based extender at 4°C in a refrigerator. The temperature of the sample was measured using a thermo recorder.

penicillin G potassium (Meiji Seika, Tokyo, Japan) and 1 mg/ml streptomycin sulphate (Meiji Seika) was prepared as described previously by Rota *et al.* [5]. For the egg yolk preparation, the egg shell was cracked, and the egg yolk was passed from one shell to the other to remove most of the egg white, allowing the egg white to fall. The yolk was slowly transferred from the shell onto a paper towel and then was allowed to roll down the paper towel until it was dry and there was no remnant of egg white left. The yolk lost its glossy appearance and stuck to the paper towel when all the egg white had been removed. When the egg membrane was broken during the procedures described above, the entire egg was discarded. While holding the paper towel back, the egg membrane was punctured with a surgical blade (Feather safety razor, Osaka, Japan), and the contents were drained into a flask (Duran, Mainz, Germany). The flask was stored in a refrigerator for one to four days before use.

#### Semen dilution and freezing

The collected ejaculates were diluted with the SG or EY extender to give a sperm concentration of  $2 \times 10^8$  sperm/ml at room temperature and then cooled to 4°C in a refrigerator. The kinetic temperature of 1 ml of SG-based extender in a 1.5-ml microtube (Nippon Genetics, Tokyo, Japan) was monitored with a thermometer (EB22005; Chino, Tokyo, Japan). After addition of extender (total 0.5–1.0 ml) and cooling in the refrigerator (4°C) for 1 or 3 h, the equivalent volume of the second extender, which was the first extender supplemented with or without 14% (v/v) of glycerol (075-00616; Wako, Kanagawa, Japan) at 4°C, was added to the semen aliquots, and the semen samples were left at 4°C for 15 min. The diluted sperm suspension was loaded into a 0.25-ml straw (Fujihira, Tokyo, Japan). The straws were placed in an atmosphere of liquid nitrogen ( $LN_2$ ) vapor, i.e., placed horizontally 6 cm above the surface of  $LN_2$  in a closed styrene foam box (24.5 cm  $\times$  17.5 cm  $\times$  17.5

cm), retained there for 15 min and then plunged into the  $LN_2$ .

#### Post-thaw parameters examined

After thawing by immersing the straws in a water bath at 37°C for 60 sec, the content of each straw was expelled into a 1.5-ml microtube, and each sample was evaluated for the following parameters using a light microscope with the aid of a Computer Assisted Sperm Analysis (CASA) system (HTM-CEROS-S; Hamilton Thorne Research, Danvers, MA, USA): the proportion of total motile spermatozoa (TMS); the proportion of progressive motile spermatozoa (PMS); velocity average pathway (VAP) - the average velocity of the smoothed cell path in  $\mu\text{m}/\text{sec}$ ; the velocity straight line (VSL) - the average velocity measured in a straight line from the beginning to the end of track in  $\mu\text{m}/\text{sec}$ ; the curvilinear velocity (VCL) - the average velocity measured over the actual point-to-point track followed by the cell in  $\mu\text{m}/\text{sec}$ ; the amplitude lateral head (ALH) - amplitude of lateral head displacement in  $\mu\text{m}$ ; the beat cross frequency (BCF) - frequency of sperm heads crossing the sperm average path in Hertz; the straightness (STR) - the average value of the ratio VSL/VAP in percentage form (straightness estimates the proximity of the cell path to a straight line, with 100% corresponding to optimal straightness); and the linearity (LIN) - the average value of the ratio of VSL/VCL in percentage form (linearity estimates the proximity of the cell track to a straight line). The overall sperm population was subdivided into four categories: Rapid,  $\geq 25 \mu\text{m}/\text{sec}$ ; Medium,  $\geq 5 \mu\text{m}/\text{sec}$ ,  $< 25 \mu\text{m}/\text{sec}$ ; Slow,  $> 0 \mu\text{m}/\text{sec}$ ,  $< 5 \mu\text{m}/\text{sec}$ ; and Static,  $0 \mu\text{m}/\text{sec}$ .

#### Artificial insemination

To demonstrate the fertilizing capacity of the spermatozoa frozen in the SG-based extender containing glycerol after 3 h exposure, the post-thaw spermatozoa were transcervically inseminated into the uteri of two bitches (Labrador Retrievers). To estimate the LH surge, the plasma progesterone concentrations of the bitches were measured daily by enzyme-linked fluorescent assay (SV-5010, Spotchem Vidas; Arkray, Kyoto, Japan) after the appearance of a blood-tinged vaginal discharge and vaginal swelling. One ml of blood was collected from the anterior brachiocephalic vein and then was centrifuged to separate the plasma. The day when the plasma concentration of progesterone exceeded 2 ng/ml was estimated as the occurrence of the LH surge (defined as to Day 0) [6]. The bitches were inseminated with frozen-thawed semen on Days 5–8. Inseminations were performed on the bitches while they were standing and non-sedated. A catheter (8 Fr; Nippon Sherwood, Tokyo, Japan) equipped with a cystoscope for human use (Karl Storz, Tuttlingen, Germany) was inserted into the *corpus uteri* through the cervical canal [7]. Then, 2 ml of the post-thaw semen ( $2 \times 10^8$  spermatozoa) was inseminated through the catheter. Care was taken to avoid backflow of the semen, so the catheter was withdrawn one minute after the insemination and the hind quarters of the bitch were kept elevated for 5 min. Conception was diagnosed by Doppler ultrasonography (VPU-011A; Toshiba, Tokyo, Japan). Unsuccessful pregnancy after experimental artificial insemination by frozen-thawed spermatozoa exerts considerable influence on both the planning for and production of guide dogs in guide dog associations. Thus, insemination of

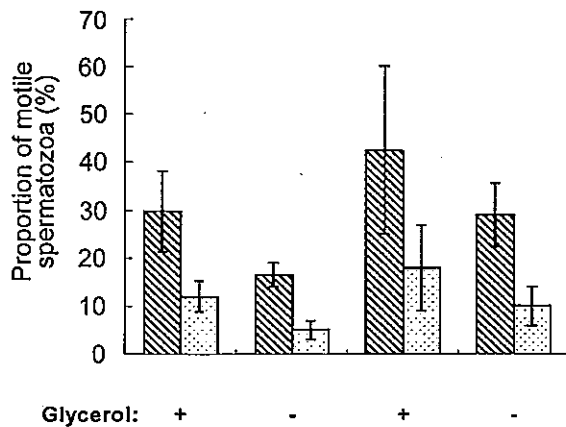


Fig. 2. Effect of semen exposure with or without glycerol on the proportion of total (slanted line bar) and progressively (stippled bar) motile spermatozoa after freezing and thawing. Experiments were replicated two or three times to examine the sperm motility using ejaculates of ICH or NAN. There was no significant difference between the groups with and without glycerol or cooling at 4 C for 1 and 3 h ( $P > 0.05$ ).

freshly ejaculated semen from different dogs was performed to avoid failure of conception in one bitch. The bitch was inseminated with the cryopreserved and fresh semen on Days 5 and 6, respectively. Another bitch was inseminated with only the cryopreserved semen on Days 6, 7 and 8. Paternity for the delivered pups was examined using microsatellite markers, as described previously [8].

#### Statistical analysis

Data were compared using the *t*-test and the StatView software (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant at a level of  $P < 0.05$ .

#### Results

When 1-ml of the SG-based extender was cooled to 4 C, the temperature of the sample reached 4 C within 60 min. However, there was a tendency for the proportion of total motile spermatozoa in each of the samples exposed for 3 h in the case of the SG-based extender to be higher than that in the samples after 1 h, but the difference was not significant (Fig. 2). Addition of glycerol to the SG-based extender was remarkable in that in terms of the resulting motility of the cryopreserved canine semen. The results for the glycerol added groups were higher in both the proportion of total motile spermatozoa and progressive motility of spermatozoa compared with the no glycerol groups, although the difference was not significant (Fig. 2).

When 8 ejaculates from 5 dogs were frozen-thawed after 3 h exposure to the SG and EY extenders containing glycerol, as shown in Fig. 3, the proportion of total motile spermatozoa of the samples in the SG and EY extenders ranged from 25–89% (average:  $58.8 \pm 8.6$ ) and 13–90% (average:  $57.1 \pm 10.1$ ), respectively. Thus, the effect of the SG extender for cryopreservation of canine

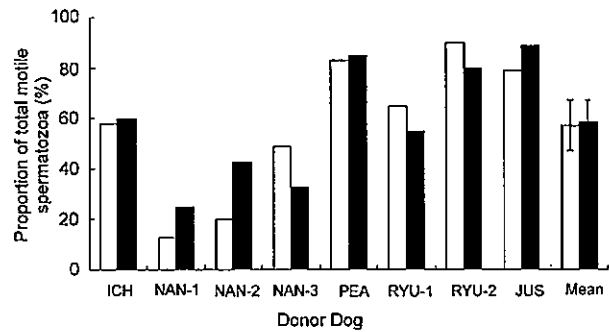


Fig. 3. The proportion of total motile spermatozoa frozen with the skim milk/glucose (SG)-based extender (black bar) and egg yolk (EY)-based extender (white bar).

spermatozoa was similar to those obtained with the EY extender (Fig. 3). Other parameters of motility for frozen-thawed spermatozoa in the SG extender were similar to the corresponding parameters in the EY extender (Fig. 4).

Spermatozoa frozen in the SG-based extender containing glycerol after 3 h exposure were transcervically inseminated, resulting in the delivery of 6 pups from 2 recipient bitches (Table 1).

#### Discussion

Although egg yolk extender is the most commonly used extender for freezing canine sperm, the process for preparing the extender involves complicated procedures including storage for one to four days prior to use in addition to microbiological problems. Moreover, there are considerable individual differences in the fertilizing capacity of cryopreserved canine spermatozoa frozen in egg yolk-based extender. Thus, an improved system for cryopreservation of canine spermatozoa is required for successful breeding programs in companion and working dog colonies. Although skim milk is itself an extract from biological products (similar to egg yolk), skim milk is commercially available as a reagent and widely used as a cryoprotective additive in mouse [3] and goat [4] spermatozoa. In our preliminary experiment, in which the most suitable concentration of skim milk in terms of the effect on canine sperm motility was determined after freezing and thawing, 30 mg/ml was found to be the most effective concentration in terms of the kinematic parameters from among concentrations of 15, 30 and 60 mg/ml (data not shown). The results in Figs. 1 and 2 suggested that it may be necessary to expose the SG-based extender for 3 h at 4 C, although the temperature of the sample reached 4 C within 60 min. In addition, sufficient exposure time to the cryoprotectants may be a critical factor for the viability of frozen canine spermatozoa. Thus, we utilized 30 mg/ml of skim milk as a component of semen extender and 3 h as the exposure time for the SG extender. When 30 mg/ml of skim milk, 0.3 M glucose and 7% (v/v) of glycerol were provided as cryoprotectants, the motility and other related sperm viability parameters of canine spermatozoa after thawing were similar to those obtained with the EY-based extender (Figs. 3 and 4). Rota *et al.* [9] have similarly shown that

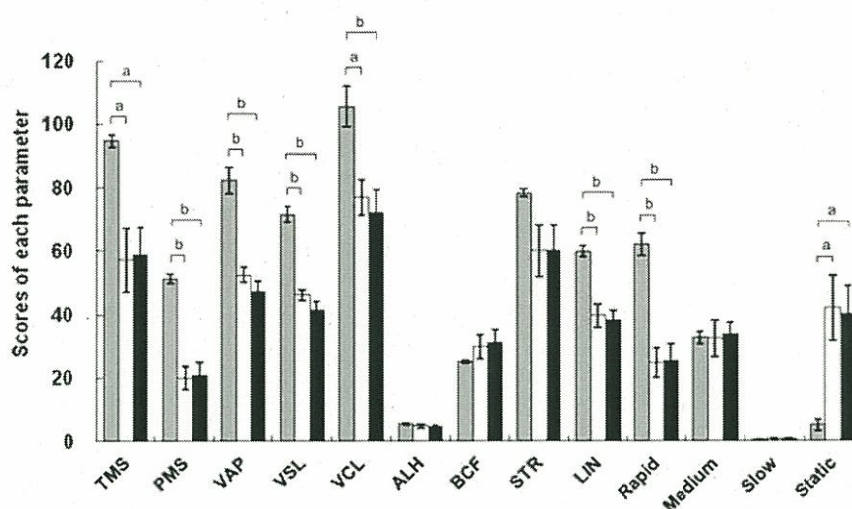


Fig. 4. Sperm motility parameters of fresh spermatozoa (gray bars) and spermatozoa frozen with skim milk/glucose-based (black bars) and egg yolk-based extender (white bars). TMS: The proportion of total motile spermatozoa. PMS: the proportion of progressively motile spermatozoa. VAP: velocity average pathway. VSL: velocity straight line. VCL: curvilinear velocity. ALH: amplitude lateral head. BCF: beat cross frequency. STR: straightness. LIN: linearity. The overall sperm population was subdivided into four categories: Rapid,  $\geq 25 \mu\text{m}/\text{sec}$ ; Medium,  $\geq 5 \mu\text{m}/\text{sec}$ ,  $< 25 \mu\text{m}/\text{sec}$ ; Slow,  $> 0 \mu\text{m}/\text{sec}$ ,  $< 5 \mu\text{m}/\text{sec}$ ; and Static,  $0 \mu\text{m}/\text{sec}$ . Experiments were replicated four or eight times to examine the sperm motility in the ejaculates of two (ICH and NAN) or five (ICH, NAN, PEA, RYU and JUN) male Labrador Retrievers in the fresh or cryopreserved groups, respectively. Significantly different between the three groups at  $P < 0.05$  (a) and  $P < 0.01$  (b).

Table 1. Results of artificial insemination with canine spermatozoa frozen in a skim milk/glucose-based extender

Bitch no.	No. of pups Delivered	Treatment	
		Cryopreserved	Fresh
1*	5	1	4
2**	5	5	—

\*Insemination with cryopreserved and fresh semen on Days 5 and 6, respectively. \*\*Insemination with cryopreserved semen on Days 6, 7 and 8.

the use of skim milk in extenders for freezing canine semen results in sperm motility and viability after thawing comparable to that obtained using a Tris-based buffer with egg yolk, although they did not reportedly determine the fertilizing capacity of the cryopreserved spermatozoa by artificial insemination.

In the bull, it has been reported that egg yolk protects sperm function by preventing the binding of sperm to the major seminal plasma proteins, thereby preventing seminal plasma protein-mediated stimulation of lipid loss from the plasma membrane [10]. As in the case of egg yolk, skim milk prevents the binding of seminal plasma protein to bull sperm and reduces sperm lipid loss while also maintaining sperm motility and viability during storage at 4 C [11]. On the other hand, since it has been shown that the fertilizing lifespan of sperm stored in milk or milk-based extenders does not exceed 12 h in the goat [12], further modification, such as antioxidant supplementation [13] or shortening of the exposure time in the extender before cryopreservation, might be required to prolong the

survival and fertilizing ability of frozen canine spermatozoa.

In conclusion, the results presented here clearly demonstrate that an effective, simple extender composed of skim milk, glucose and glycerol is available for the cryopreservation of canine spermatozoa as an alternative to extenders containing egg yolk, and this may contribute to both efficient exchange of genetic materials and production of guide dogs for the blind.

#### Acknowledgements

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