Cryopreservation of the Semen Collected by Electroejaculation from the Hokkaido Brown Bear (*Ursus arctos yesoensis*)

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ABSTRACT. Four adult Hokkaido brown bears were used as semen donors, and semen characteristics were examined before freezing and after thawing. A total of 10 electroejaculates were diluted with Tris-egg yolk extender and cooled to 4°C over 90 min. Spermatozoa were equilibrated with 4.7% glycerol for 80 min. Semen packed in 0.25 ml plastic straws were frozen with liquid nitrogen vapor. Percentages (mean ± SD) of motile and live sperm were 96 ± 2 and 86.5 ± 7.2% before freezing, and 43 ± 5 and 67.4 ± 3.9% after thawing, respectively. Although the number of progressively motile sperm after thawing varied among samples ($1.8 \pm 1.2 \times 10^8$ cells/ejaculate), frozen semen in the present study might serve for artificial insemination.

KEY WORDS: brown bear, freezing, spermatozoa.

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The Hokkaido brown bears are found only in Hokkaido Island, Japan, and their genetic diversity is known to be low [15]. An assisted reproductive technology like artificial insemination could contribute to the maintainance of their genetic diversity. We previously reported a successful semen collection of the Hokkaido brown bear by electroejaculation [7]. Semen cryopreservation is an essential procedure for assisted breeding; however, there are only a few reports on cryopreservation of the bear semen [2, 9]. The objective of the present study was to examine the potential of a freezing procedure used for bull semen with a modification in glycerol concentration.

Four adult males, aged 7 to 16 years and maintained at Noboribetsu Bear Park (Noboribetsu, Japan), were used as semen donors. They were kept in male groups and separated from females. A total of 10 trials of electroejaculation were conducted during the breeding season (May to July) in 1996 and 1997.

Electroejaculation was conducted as described previously [7]. Briefly, bears were immobilized with tiletamine HCl-zolazepam HCl (Zoletil; Virbac S. A., Carros, France) in 8 trials, and with midazolam (Dormicum; F. Hoffman-La Roche Ltd., Basel, Switzerland) and ketamine HCl (Ketalar; Sankyo Co., Ltd., Tokyo, Japan) in 2 trials. After immobilization, a handmade bipolar rectal probe with two ring electrodes was inserted into the rectum. The electrical stimulation with 60 Hz alternative current, sine wave and 5 V was repeated till ejaculates were obtained. The semen was assessed by determining ejaculate volume, pH, sperm concentration and the percentage of abnormal sperm as described elsewhere [7]. The percentage of viable spermatozoa was determined by an eosin-nigrosin staining method [7] before and after freezing. Small aliquot of raw and fro-

zen-thawed semen was taken for the assessment of sperm motility. Percentage of sperm in each type of progressive motility status was calculated under a light microscope using a scale of 0 to 4: 0, no movement; 1, slight side-to-side movement with no forward progression; 2, side-to-side movement, slow and steady forward progression; 3, steady, moderate forward progression; 4, steady, rapid forward progression. The total percentages of the sperm scaled 1 to 4, and scaled 3 and 4 were designated as motile and progressively motile, respectively. Ejaculates were kept for 30 to 60 min in a water bath at 37°C until dilution.

Tris-buffered egg yolk solutions for bovine semen freezing (Table 1) provided from Japan Holstein Breeding Service Co., Ltd. (Kitahiroshima, Japan) were used as semen extenders. Ejaculate was diluted 1:1 (v/v) with Extender I at 37°C and gradually cooled to 4°C over 90 min. Cooled semen was then fully diluted by adding Extender II in a step-wise manner at 4°C; 1/10, 2/10, 3/10 and 4/10 of Extender II was added every 20 min to give a final glycerol concentration of 4.7%. The fully diluted semen was packed in a 0.25 ml plastic straw (I.M.V., L'Aigle, France) and the tip of the straw was heat-sealed. The straws were placed horizontally on a rack in a Styrofoam box and were cooled in a step-wise manner as shown in Fig 1. They were then immersed in liq-

Table 1. Components of semen extender

Components	(conc.)	Extender I	Extender II
Tris	(mM)	160	130
Citrate	(mM)	50	10
Raffinose	(mM)	40	30
Lactose	(mM)	90	70
Fructose	(mM)	40	30
Penicillin G	(IU/l)	1×10^6	1×10^6
Streptomycin	(g/l)	1	1
Egg yolk	(v/v)	15%	15%
Glycerol	(v/v)	-	14%

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Fig. 1. A) Freezing container used for a step-wise cooling of the straws; plastic straws containing diluted semen were cooled by holding the rack 30 cm above liquid nitrogen (LN) for 2 min, 4 cm for 2 min, and 2 cm for 6 min. The distance between the straw rack and surface of LN was adjusted using the wire attached to the rack without opening the box lid. B) A cooling curve obtained using the above described procedure. Cooling rates were $9.8 \pm 1.8^\circ\text{C}/\text{min}$ and $46.6 \pm 3.4^\circ\text{C}/\text{min}$ (mean $\pm$ SD, $n=5$) from 4°C to -7.5°C and from -7.5°C to -100°C , respectively. Ten minutes after the initiation of cooling, the straws were immersed in LN.

uid nitrogen (LN) and stored for 6 to 58 days. The frozen semen was thawed by holding the straw in a water bath at 37°C for 1 min. Percentages of motile and viable spermatozoa were calculated by the following formula: the value of frozen-thawed semen/that of fresh semen $\times 100$.

As shown in Table 2, characteristics of fresh ejaculates

(ejaculate volume, pH, sperm concentration and the percentage of abnormal sperm) varied among samples. The motility and viability of fresh and frozen-thawed spermatozoa were shown in Table 3. The percentage of abnormal sperm increased to $21.7 \pm 9.7\%$ (ranged from 7.9 to 33.1%) after thawing, and detached head and bent tail were most frequently observed in the frozen-thawed samples. Mean percentages of motile and viable frozen-thawed spermatozoa collected in 1997 were higher than those in 1996 (Student's *t*-test, $p < 0.05$). Although the mean recovery rate of viable spermatozoa was not different between in 1996 and 1997, that of motile spermatozoa was significantly higher in 1997. The higher recovery rate in sperm motility can be attributed to an improvement in semen handling techniques in 1997. The temperature fluctuation during semen dilution and packing into straws, which might relate to the loss of motility, could be diminished.

Further analyses were undertaken using the samples obtained in 1997. There was no significant correlation between both motility and viability of frozen-thawed spermatozoa and any characteristics of fresh semen (Tables 2 and 3). The mean percentage of motile frozen-thawed spermatozoa in the present study ($43 \pm 5\%$) was comparable to that collected by electroejaculation in giant panda ($< 55\%$) [14], red-wolf ($< 40\%$) [5] and Tibetan black bear ($0\text{--}60\%$) [9], that collected by epididymal mincing in Cantabric brown bear (25%) [2], and that collected by artificial vagina in dog ($< 50\%$) [8, 12].

As shown in Table 4, proportion of progressively motile spermatozoa largely decreased after thawing. Total numbers of progressively motile sperm per ejaculate after thawing (0.5 to 3.2×10^8 cells) were within the range that was needed for intrauterine insemination in dog (0.3 to 2.8×10^8 cells) [16, 17], and were similar to the lower limit that could lead a pregnancy in dog by vaginal insemination (1 to 20×10^8 cells) [4, 10, 11, 16]. In dog, much higher number of frozen-thawed motile spermatozoa (20×10^8 cells) was required for vaginal insemination to obtain conception compared with intrauterine insemination (2.8×10^8 cells) [16];

Table 2. Characteristics of electroejaculates in the brown bears

Sample ^{a)}	Bear	Ejaculate		Sperm conc. ($\times 10^8$ cells/ml)	% of abnormal sperm
		Volume	pH		
9601	A	3.8	7.4	4.3	13.7
9602	A	1.5	7.4	21.8	7.7
9603	A	7.6	7.3	9.8	15.4
9604	B	2.5	7.4	1.6	11.7
9701	A	3.6	7.3	3.6	6.0
9702	A	5.4	7.2	2.8	8.2
9703	B	4.1	7.4	3.7	14.1
9704	B	5.5	7.4	1.5	8.0
9705	C	2.8	7.0	1.2	23.1
9706	D	0.2	7.4	9.0	7.7
Mean $\pm$ SD		3.7 ± 2.1	7.3 ± 0.1	5.9 ± 6.3	11.5 ± 5.2

a) Sample numbers starting with 96 and 97 were obtained in 1996 and 1997, respectively.

Table 3. Comparison of fresh and frozen-thawed sperm motility and viability

Sample	Bear	% of motile sperm			% of viable sperm		
		Fresh	Frozen -thawed	Recovery ^{a)}	Fresh	Frozen -thawed	Recovery ^{a)}
9601	A	93	20	21.6	88.9	63.7	71.7
9602	A	95	20	21.1	96.4	56.6	58.7
9603	A	93	47	50.1	79.9	60.3	72.9
9604	B	90	20	22.2	82.2	64.6	78.6
Mean±SD		93±2	27±13	28.8±14.2	86.8±7.4	61.3±3.6	70.5±8.4
9701	A	95	45	47.4	83.6	71.2	85.1
9702	A	95	43	44.7	91.7	70.2	76.6
9703	B	99	40	40.4	91.7	67.9	74.1
9704	B	93	40	43.6	81.7	69.9	86.2
9705	C	95	40	42.1	76.0	63.2	83.2
9706	D	97	53	54.7	94.5	62.2	65.8
Mean±SD		96±2	43±5*	45.5±5.1*	86.5±7.2	67.4±3.9*	78.5±7.9

Values with asterisks significantly differ from those in 1996 ($p < 0.05$).

a) The value of frozen-thawed semen/that of fresh semen $\times 100$.

Table 4. Total number and percentage of progressively motile spermatozoa

Sample	No. ^{a)} and (%) of progressively motile sperm			
	Fresh		Frozen-thawed	
9701	10.2	(80)	3.2	(25)
9702	12.0	(80)	3.0	(20)
9703	13.8	(90)	2.3	(15)
9704	5.2	(63)	0.9	(11)
9705	2.3	(70)	0.6	(18)
9706	1.7	(85)	0.5	(25)
Mean $\pm$ SD	7.5 $\pm$ 5.2	(78 $\pm$ 10)	1.8 $\pm$ 1.2	(19 $\pm$ 6)

a) Values ($\times 10^8$ cells/ejaculate) were calculated by the formula: % of progressively motile sperm/ $100 \times$ sperm concentration of ejaculate $\times$ ejaculate volume.

however, an acceptable conception rate (85%) was obtained after two intrauterine inseminations of only 0.30 to 0.35 $\times 10^8$ motile frozen-thawed spermatozoa [17]. Although the number of progressively motile sperm required for successful artificial insemination of the bear is unknown, the bear semen frozen by the method described here might be served for intrauterine insemination.

We adjusted the final glycerol concentration to 4.7% by diluting the fresh semen with the same volume of Extenders I and II. Around 7% of glycerol has been employed in the freezing of bovine semen [1]. Optimal glycerol concentration varies among animal species. Although the optimal glycerol concentration for canine sperm cryopreservation still remains to be determined and various concentration was acquired [3, 6, 13, 16], 3 to 4% of glycerol has been successfully used for sperm freezing to lead pregnancy after artificial insemination with the frozen-thawed semen [12]. Since Ursidae and Canidae are taxonomically close to each other, we used a lower concentration (4.7%) of glycerol. Anel *et al.* [2] reported that semen obtained from the epididymis of

a dead brown bear could be frozen using TEST-fructose extender with 20% egg yolk and 4% glycerol. Post-thaw progressive motility in their study (15%) is similar to our result (19%).

The present study first reported a successful frozen storage of the semen in the Hokkaido brown bear, using a simple freezing method without special equipment. Total number of progressively motile spermatozoa after thawing was within the range for the successful intrauterine insemination in dogs. However, the reduction in the proportion of motile sperm suggested the necessity of further investigation to improve frozen-thawed semen quality and to validate the fertilizability of frozen-thawed semen of the bear.

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