



NOTE

Theriogenology

Collection and frozen storage of semen for artificial insemination in red foxes (*Vulpes vulpes*)

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ABSTRACT. This study was conducted on red foxes to determine the appropriate voltage in electroejaculation for semen collection from stud males, and to confirm whether frozen semen with bovine semen extender can be used for artificial insemination. The proper load voltage for electroejaculation was 3–4 V based on semen collection rates and concentrations of spermatozoa. Frozen semen was prepared according to the known procedure for cows. In frozen-thawed semen, a relatively high conception rate (81.3%) was obtained in vixens, in which the optimum insemination time was detected by vaginal electrical resistance. These findings demonstrate that the restricted condition for semen collection by electroejaculation with cryopreservation of semen using bovine semen extender can be applied to artificial insemination of red foxes.

KEY WORDS: artificial insemination, cryopreservation, electroejaculation, red fox

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There is less information about fertility and puberty of red foxes (*Vulpes vulpes*) in Japan because they are raised only in zoos. In this situation, artificial insemination is essential for maintaining their genetic diversity and facilitating reproductive ability [1–5]. To perform artificial insemination, it is crucial to establish an effective and safe method for collection and frozen storage of spermatozoa of sufficient quality from breeding males. In countries where fur industries are well developed, frozen-thawed semen with the Tris-buffered extender as a diluent [4] has been widely applied to artificial insemination of foxes. However, it is cumbersome for practitioners in the veterinary field to prepare the semen extender in advance for foxes. Previously, the semen extender for cows (bovine semen extender) was reported to serve as the diluent for cryopreservation in brown bears [6]. Thus, we focused on the commercially available bovine semen extender from each Prefectural Livestock Experiment Station in Japan. For semen collection from stud male foxes, an electroejaculation unit utilized with bears [6] and giant pandas [10] was also used in this investigation.

The present study was performed in red foxes to determine the appropriate voltage for electroejaculation for semen collection from stud males and to confirm whether the bovine semen extender can be applied to artificial insemination. First, proper load voltage in electroejaculation was assessed in males using semen collection rates and concentrations of spermatozoa. Next, the viability of spermatozoa in frozen-thawed semen prepared from stud donors was examined, and the conception rate with litter size was explored.

Healthy red foxes maintained at the Zao Fox Village (Miyagi, Japan), aged 27.5 ± 1.7 (mean \pm SEM, range: 22–58) and 39.1 ± 2.9 (35–58) months and weighing 8.6 ± 0.2 (8.0–10.0) and 9.7 ± 0.2 (8.0–11.4) kg for females ($n=16$) and males ($n=71$), respectively, were used in the study. Foxes were considered “healthy” based on their appetites, conditions of feces and urine, appearance of the fur, and clinical observations. They were given 300–500 g mixed feed containing dog food and equal parts fish, horse, and beef meals once daily (total energy: approximately 150 kcal/100 g), and tap water *ad libitum*. Animals were identified individually with microchips and kept in individual outdoor wire cages (90 \times 120 \times 60 cm) during the experiment. The male fox was fasted for at least 20 hr before semen collection. All procedures were conducted in accordance with the animal welfare rules of the Zao Fox Village based on the Guidelines for Animal Experimentation [8].

Seventy-one males were premedicated intramuscularly with xylazine hydrochloride (1.6 mg/kg, ZENOAQ Co., Fukushima,

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Table 1. Components of the semen extenders I and II

Components	Molar concentration / unit / weight percent	Extender I	Extender II
Tris	mM	130	130
Citrate	mM	45	45
Lactose	mM	40	40
Raffinose	mM	50	50
Fructose	mM	85	85
Penicillin G	IU/l	0.6×10^6	0.6×10^6
Streptomycin	g/l	0.6	0.6
Egg yolk	v/v	20%	20%
Glycerol	v/v	-	13%

Japan) before electroejaculation, and placed in a right lateral recumbency position. The penis was cleaned with a physiological saline solution, and feces in the rectum were removed manually. The electroejaculation unit (one channel type for mammals, Fujihira Industry Co., Ltd., Tokyo, Japan) was composed of a wave-form generator, ammeter, and probe. The probe rod (30 cm long and 19 mm diameter) was lubricated sparingly and inserted to a depth of approximately 12 cm into the rectum. The electrical stimuli at a frequency of 60 Hz were applied in five sets of 1 sec duration each, with a 1 sec rest between the sets (regarded as "one cycle"). Proper load voltage was determined using stepwise increases in the voltage from 2 to 5 V in two to five cycles. The threshold of peak voltage was determined by the physical response of rear leg extension and adduction. As indicators for proper voltage, the semen collection rate (=number of males from which semen was obtained / total number of males used) was calculated. The duration from premedication to the completion of all procedures was approximately 20 min.

Using fresh semen samples collected from 20 males, ejaculate volumes, concentrations, and viability of spermatozoa were measured in February and March each year during 2011–2013. Most of the males utilized were imported as original stud foxes from Norway and their male offspring were also used in this study. Semen samples were considered largely uncontaminated with urine, which was judged by the lack of yellow color. The ejaculate volume was measured by a micropipette. Concentrations of spermatozoa were counted microscopically using a Thoma-Zeiss counting chamber (Hem cytometer, Fujihira Industry). The viability defined as motility of spermatozoa was assessed microscopically at a magnification of $\times 20$ –400 using a Horwell chamber (Fujihira Industry) placed on a warmer (Microwarm Plate MP-1000, Kitazato Corp., Fuji, Japan) at 38°C. Then, it was scored using a viability index according to a classic textbook [9] regarding bovine theriogenology. In brief, the criteria of the viability index was as follows: +++ (score 100), vortex motility with vigorous forward progressive movement; ++ (score 75), active forward progressive motility; + (score 50), slight forward progressive motility; \pm (score 25), pendular motility without forward movement; and – (score 0), no motility in any field examined. The score for each individual was recorded as the value of triplicate measurements.

In preparation of frozen samples, the bovine semen extender (Table 1) consisting of extenders I and II was provided by the Miyagi Prefectural Livestock Experiment Station (Osaki, Japan). Frozen semen was prepared according to the procedure for bears [6] using the bovine semen extender. In brief, ejaculate was diluted 1:5 (v/v) with the extender I at 37°C, and gradually cooled to 4°C over a 60 min period. Subsequently, cooled semen was fully diluted by adding extender II in a stepwise manner at 4°C to give a final count of 100×10^6 cells/ml [4, 5]. In these cases, the final calculated glycerol concentration was approximately 6%. Then, the semen was loaded into a 0.5 ml plastic straw (type 133, Fujihira Industry) after being equilibrated at 4°C for 5 min and the tip of the straw was heat-sealed. The straws containing diluted semen were placed horizontally on a straw rack in a freezing container and cooled by holding 5 cm above liquid nitrogen (–196°C) for 5 min, and 4 cm for 5 min. Then, they were immersed in liquid nitrogen and stored for 8 days to 25 months. The viability index of spermatozoa was assessed again within 1 min after rapid thawing of frozen semen in a water bath at 37°C for 1 min.

Estrus in vixens was assessed by monitoring vaginal electrical resistance (VER) when a well-swollen vulva was observed. VER was measured by an ovulation detector (Draminski Dog Ovulation Detector; Draminski, Olsztyn, Poland), which has become a routine technique in modern fox breeding [7]. Based on the result of the preliminary study, one unit in the VER measurement used in this study corresponded to one ohm (Ω) in the VER determined by the VER equipment provided from another manufacturer (SI-LI 3 D type, A/S Lima, Co., Sandnes, Norway).

In artificial insemination, 16 vixens were inseminated twice at 24 hr intervals. Briefly, each vixen under conscious conditions was given frozen-thawed semen at a volume of 0.5 ml (spermatozoa counts: 50×10^6 cells) per insemination on the first and second days after a single peak of VER was higher than 350 units. The insemination was conducted by using a fox insemination device (TSK Laboratory Japan, Tochigi, Japan) inserted deeply into the cervix. It took a few minutes from restraint of the vixen to semen injection.

Numerical data and viability indices of spermatozoa were expressed as the mean \pm standard error of the mean (SEM). Statistical evaluation was analyzed using the Steel test (among three groups). A probability level of $P < 0.05$ indicated statistical significance.

In electroejaculation, when determining proper load voltage using 71 males by stepwise increases in voltages, semen specimens were successfully obtained from 47 males (semen collection rate: 66.2%). Details of the semen collection rate were 0% (0/71),

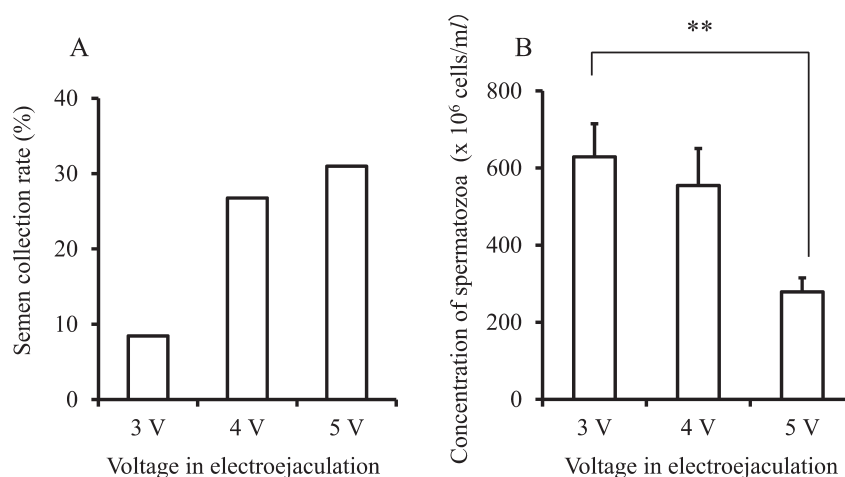


Fig. 1. Semen collection rate (A) and concentrations of spermatozoa (B) on various load voltages in electroejaculation using 71 male foxes. The number of foxes enabling semen collection was 6, 19, and 22 males in 3 V, 4 V, and 5 V loads, respectively. ** $P < 0.01$ (Steel test).

8.5% (6/71), 26.8% (19/71), and 31.0% (22/71) in load voltages of 2 V, 3 V, 4 V, and 5 V, respectively (Fig. 1A). The 2 V load was not sufficient to obtain semen. There was no difference in the semen volume among the 3 V ($408 \pm 47 \mu\text{l}$), 4 V ($418 \pm 32 \mu\text{l}$), and 5 V ($409 \pm 40 \mu\text{l}$) loads. The concentration of spermatozoa with 3 V was significantly ($P < 0.01$) greater than that with 5 V (Fig. 1B), although no statistical difference was noted between the 3 V and 4 V or 4 V and 5 V loads. The 5 V load occasionally evoked physical responses in certain foxes. Based on these results, loads of 3–4 V were used for semen collection. Although we could not collect semen in 24 of 71 males used, the reason may be at least in part explained by sexual immature status or technical errors, such as unsatisfied sedation or mis-insertion depth of the probe rod into the rectum.

Twenty of the aforementioned 25 males (6 plus 19 males) loaded with 3 V and 4 V were selected as stud donors based on concentrations and viability indices of spermatozoa. In 20 stud donors, the ejaculate volume of semen was $410 \pm 20 \mu\text{l}$, the concentrations of spermatozoa were $435 \pm 49 \times 10^6$ cells/ml, and the score for the viability index of spermatozoa was 65 ± 5 . An effect of fox age on spermatogenesis was not observed in the present protocols. Moreover, it has been confirmed that the viability index of spermatozoa remains stable throughout cryopreservation periods of up to 25 months.

In frozen-thawed semen ($n=20$), the viability index of spermatozoa was 47 ± 3 , exhibiting 72.3% in the calculated post-thaw viability index recovery (=mean score of frozen-thawed semen / mean score of fresh semen). This recovery rate appeared to be within the range observed for other species [6, 10], although there were large differences in evaluation procedures, breeding conditions, and management factors.

The conception rate using frozen-thawed semen was 81.3% (13 of the 16 vixens). This rate was nearly consistent with cumulative artificial insemination data [1, 4, 5] in red foxes or silver foxes, which belong to the same subspecies as red foxes. The gestation periods ranged from 52 to 56 days (52.7 ± 0.2 days), and the litter sizes consisted of 3 to 4 cubs. Both gestation period and litter size obtained in this study agreed with previous reports [1, 4]. The litter size in this study using captive red foxes was less than that (5 to 8 cubs) in farmed blue foxes [2–5]. Neither abortion nor dystocia was video-recorded during the pregnant to delivery periods. Further studies are required to compare differences in reproductive profiles between artificial insemination and natural mating in captive red foxes.

In conclusion, these findings demonstrate that the restricted conditions for semen collection by electroejaculation with cryopreservation of semen using bovine semen extender can be applied to artificial insemination of red foxes.

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