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-Technology Report-

Title: The effects of frequent electroejaculation on the semen characteristics of a captive Siberian tiger (*Panthera tigris altaica*).

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Running head: FREQUENT ELECTROEJACULATION IN A TIGER

Abstract. Artificial insemination (AI) can help to avoid inbreeding and genetic degeneration for sustaining genetically healthy populations of endangered species in captivity. Collection of a sufficient quantity of viable sperm is an essential first step in the AI process. In the present study, we examined the effects of frequent electroejaculation on semen characteristics in a Siberian tiger. We collected semen in all 17 trials during 6 breeding seasons (6 years). The mean number of sperm and the percentage of motile sperm were 294.3 ± 250.2×10^6/ejaculate and 82.4 ± 11.4%, respectively. The number of motile sperm tended to increase during frequent electroejaculation in the same breeding season. Semen collection by electroejaculation can be performed effectively up to the fourth sequential ejaculate, which contained the most sperm in the study. In conclusion, frequent collection of sperm by electroejaculation from tigers may be effective for collection of a large number of motile sperm.

Key words: Frequent electroejaculation, Semen quality, Tiger

Text

The Siberian tiger (*Panthera tigris altaica*) is a critically endangered species, and the wild population is estimated to be approximately 360 individuals in the world according to a report of the International Union for Conservation of Nature and Natural Resources in 2011 [1]. On the other hand, zoos throughout the world maintain 466 Siberian tigers in captivity [2]. Therefore, it is necessary for zoos to develop effective reproduction methods and contribute to *ex situ* conservation for the Siberian tiger. In domestic animals, artificial insemination (AI) is usually used commercially. The AI technique can help to avoid inbreeding and genetic degeneration for sustaining a genetically healthy population of Siberian tiger, because it makes it possible to transfer genes among populations. But the success rate of AI remains inconsistent for many wild felidae including the tiger [3]. Although a great deal of effort has been made to develop AI methods in the tiger [4, 5], only three pregnancies have been reported by two research groups using laparoscopic intrauterine [6, 7] and intravaginal AI [8]. In the domestic cat, it was clarified that the sperm number required in fresh semen for pregnancy was 8×10^6 for intrauterine AI [9] and 80×10^6 for intravaginal AI [10]. In the tiger, intrauterine AI using 62.5-340×10^6 motile sperm for 9 tigers resulted in no pregnancy [4], although offspring were obtained using 16.8×10^6 (intrauterine) [6] and 500×10^6 motile sperm (intravaginal) [8]. Although the optimal number of sperm for pregnancy by AI in the tiger is not clear, studies concerning improvement of the semen collection protocol to obtain as many viable sperm as possible in order to achieve successful AI are necessary.

To obtain semen in the tiger, the electroejaculation method developed by a research group at the Smithsonian's National Zoological Park [5, 11] has been used in many reports [4, 6, 8, 12-18]. However, the quantity and quality of semen collected by this method from 14 males (23 ejaculates) were variable as follows: seminal volume, 0.8 to 14.0 ml; number of motile sperm, 2.2 to 644.0×10^6; motility of sperm, 50 to 90%; abnormality of sperm, 8.0 to 91.0% [5]. This electroejaculation

regimen was composed of 3-stimulus series using 3 to 6 V [5, 11]. Therefore, semen could be collected as several ejaculates by this regimen. However, the ejaculation pattern and characteristics of each ejaculate were not reported. In the domestic cat, it has been reported that the volume and the number of sperm were different in each ejaculate when electroejaculation with stepwise increases in voltage (1, 2, 4 and 8 V) was used [19], and that the semen volume increased as the electroejaculation trial was repeated [20].

In the present study, we tried to collect semen from a male Siberian tiger during 6 breeding seasons (2002 to 2008). To clarify the changes in the characteristics of semen in each ejaculate and within/between reproductive seasons, we examined the sperm number and quality, testicular volume and serum testosterone and cortisol concentrations. For collecting sperm with good quality, urine contamination, which is known to cause a decrease in sperm quality [21], is a common problem in the cat [19, 20] and tiger [11]. To avoid urine contamination or decrease its effects, we drained urine before electroejaculation and replaced it with Ham's F10 medium.

In all 17 trials during the 6 breeding seasons, we could collect semen from a male tiger. Each trial included 2 to 6 ejaculates of semen. As shown in Table 1, the characteristics of the collected semen without urine contamination were within the range described by Wildt *et al.* [5] and similar to mean values reported by Howard [11]. The influence of tiger age on spermatogenesis was not observed in the present study, since a large quantity of viable sperm (267.2×10^6) was obtained at 11 years of age in February, 2008 (Fig. 1). There were no obvious relationships between testicular volume, steroid hormone concentrations and semen characteristics in the present study. The testicular volume during the experimental period was within the range (22.6 to 41.5 cm^3) reported previously [5]. The mean concentration of serum testosterone and cortisol were 1.9 ng/ml (range: 1.2 to 4.4 ng/ml) and 22.6 ng/ml (range: 15.6 to 30.1 ng/ml), respectively. The concentration of serum testosterone was similar to those reported in the previous studies [17, 22],

and the serum cortisol level was lower than that in a previous study [22]. Our results indicate that the frequent anesthesia and electrical stimulation did not become a problematic stress in the tiger.

Urine contaminations occurred in 4 out of 17 trials. Semen pH and the percentage of motile sperm were decreased by urine contamination ($p < 0.05$) (Table 1). It was reported that sperm lose their motility completely when mixed with fresh urine [21]; however, sperm contaminated with urine showed various motilities (10 to 70%) in the present study. This suggests that ejaculated sperm are partially protected from changes in pH and osmolarity by replacement of urine with Ham's F10 before electroejaculation.

Although there were large variations in the number of progressively motile sperm in each breeding season, the number tended to be increased by frequent electroejaculation in the same breeding season except for in 2007 to 2008 (Fig. 1). This result suggests that the frequent electroejaculation of a male tiger in a breeding season stimulates spermatogenesis or increases the sensitivity to electroejaculation, although the reason why the numbers of sperm fluctuated in the 2007 to 2008 breeding season is not clear. In a previous study [20], the semen volume tended to increase during frequent semen collection in the cat (once a week for 2 weeks, followed by a week of rest; a total of 22 collections over 32 weeks). Pineda et al. [20] concluded this trend might indicate increase in the sensitivity of the accessory sex gland by repeated electrical stimulation, although the number of sperm was not affected ($p > 0.05$). The reason for the discrepancy in sperm number between the present and previous studies is not clear. In a future study, we should examine the effect of frequent semen collection on number of sperm using other male tigers.

As shown in Fig. 2, the mean cumulative number of progressively motile sperm obtained by the fourth ejaculate was more than 200×10^6, which is sufficient for pregnancy by intrauterine AI in the tiger (16.8×10^6 motile sperm) [6]. Also, we speculate that 200×10^6 sperm can be used for intravaginal AI, since intravaginal AI with 80×10^6 sperm [10], tenfold the number used for

109 successful intrauterine AI (8×10^6) [9], achieved pregnancy in the domestic cat. The sperm number
110 in each sequential ejaculate was similar ($p = 0.72$); however, the standard errors of the first and
111 fourth ejaculates (50.1×10^6 and 76.0×10^6, respectively) were relatively large compared with those of
112 the second and third ejaculates (21.6×10^6 and 6.4×10^6, respectively). This means that sperm were
113 obtained constantly in the second and third ejaculates and that the most sperm were included by the
114 fourth ejaculate. In Table 2, the relationship between timing of collection of each ejaculate and
115 series of electrical stimuli is shown. There were only 6 out of 14 trials (42.9%) in which fourth
116 ejaculates were collected during three stimulus series. In the first trial of the present study, the first
117 ejaculate of semen was not collected until performance of additional series. This suggests that
118 frequent electrical stimuli are necessary to enhance the sensitivity to electroejaculation and that
119 modification of electroejaculation technique may be needed depending on individual males.
120 In conclusion, large numbers of viable sperm from a male tiger were constantly collected
121 by electroejaculation, and frequent semen collection (once a month for several months) may be
122 effective for increasing the sensitivity to electrical stimuli and the number of progressively motile
123 sperm. For effective and safe semen collection, we recommend that electroejaculation in the tiger
124 performed up to collection of the fourth sequential ejaculate. Replacement of urine in the bladder
125 with Ham's F10 medium before electroejaculation may decrease damage of sperm caused by urine
126 contamination. In a future investigation with collected semen, we should study the protocol for
127 AI and semen cryopreservation in the tiger.
128
129 **Methods**
130 A male mature Siberian tiger that was being kept at Asahiyama Zoological Park and
131 Wildlife Conservation Center (Asahikawa, Japan), studbook no. 4264, and that was born in captivity
132 in 1996 and weighed 131 to 176 kg was used in this study for 6 breeding seasons (2002 to 2008).

This study was conducted in accordance with the Hokkaido University guidelines for the care and use of animals. The tiger was fed once daily, 2 to 4 kg of horse meat, chicken and pig bone, with a fasting day every week. Water was available *ad libitum*. The tiger was food fasted for at least 24 hr before each study. Water was provided up until the morning. For semen collection, the male was intramuscularly premedicated with 0.6 mg/kg xylazine hydrochloride (Celactal, Bayer Ltd., Leverkusen, Germany) in the first three trials or 50 μg/kg medetomidine hydrochloride (Domitor, Orion Corp., Espoo, Finland) in the other 14 trials and 0.15 mg/kg midazolam (Dormicum, Roche, Basel, Switzerland) in a blow dart using a blowpipe. After the animal was sedated, 5 mg/kg ketamine hydrochloride (Ketalar, Sankyo, Tokyo, Japan) was administered intramuscularly to complete anesthetic induction and immobilization. After semen collection, 100 μg/kg atipamezole hydrochloride (Antisedan, Orion Corp., Espoo, Finland) was administered intramuscularly to antagonize the effects of medetomidine hydrochloride. It took an average of 106 min from premedication to administration of the α_2-adrenoceptor antagonist atipamezole after all procedures were finished. The activeness and appetite of the tiger were smoothly recovered the day after all electroejaculation trials.

Before semen collection, each testicular size (length [a] × width [b] × height [c]) was measured with a caliper, and the testicular volume (cm^3) was calculated using a formula ($\pi/6 \times a \times b \times c$) [23]. The summed value for the testes was divided by 2 to provide the mean testicular volume. Bladder catheterization was performed to drain urine followed by replacement with 40-100 ml Ham's F10 medium (Invitrogen, Grand Island, NY, USA), and urine pH was evaluated immediately. Feces in the rectum were removed manually. A rectal ejaculatory probe (55 cm long and 3.7 cm in diameter) with three longitudinal electrodes (125 × 11 mm) was used for semen collection (Fig. 3). The distance between each electrode was 10 mm. The probe was connected to an alternatiing current, 60 Hz sine wave electroejaculator (1 channel type for mammals, Fujihira Industry Co., Ltd.,

157 Tokyo, Japan).

158 Semen collection trials were performed a total of 17 times at 1- to 4-month intervals
159 during November through April using the procedures described by a previous report [11]. Briefly,
160 the penis was washed up with physiological saline and covered with a 50-ml polypropylene conical
161 centrifuge tube (Becton Dickinson and Company, Franklin Lakes, NJ, USA) that had been warmed
162 to 37˚C in advance. The probe was lubricated with K-Y jelly (Johnson & Johnson Inc., New
163 Brunswick, NJ, USA) and inserted 22 cm into the rectum. The probe was directed ventrally so that
164 it would be directly over the male accessory sex glands. The electrical stimuli were applied
165 basically as 3 series in a 3-sec-on/3-sec-off pattern using 3 to 6 V; 30 (10 stimulations at 3, 4 and 5
166 V: first series), 30 (10 stimulations at 4, 5 and 6 V: second series), and 20 stimuli (10 stimulations at
167 5 and 6 V: third series), and a 3-min rest was permitted between each series as previously described
168 [5, 11]. In the present study, to prevent urine contamination and clarify the characteristics of each
169 ejaculate, the conical tube was replaced with a new tube when semen was ejaculated during 10
170 stimuli with the same voltage. In seven out of 17 trials, the total and motile sperm numbers in each
171 ejaculate were counted, and these data were used for the analysis of sperm number dynamics in
172 sequential ejaculates. In other trials, the volume of each ejaculate was checked, and numbers of
173 sperm were evaluated after mixing some ejaculates. If the number of collected sperm was low after
174 the 3-stimulus series, additional series of 10 to 50 electrical stimuli (4 to 6 V) were performed.
175 Collected semen was subjected to evaluation of its characteristics immediately after collection.
176 When semen was contaminated with urine, the samples with and without contamination were
177 evaluated separately. The sperm concentration was calculated by a hemacytometer. The
178 percentage of progressively motile sperm was assessed microscopically as described previously [11].
179 The viability of sperm was assessed using an eosin-nigrosin staining method [24]. Sperm
180 morphology was evaluated after fixing in 2.5% glutaraldehyde followed by staining with 3%

Giemsa's solution. For calculating the viability and morphological normality of sperm, more than 200 sperm per slide were observed.

Blood samples were collected into vacutainer glass tube by femoral vein venipuncture, and serum was separated by centrifugation and stored at -80˚C until assayed. Serum testosterone and cortisol were measured with a chemiluminescence immunoassay method as described previously [25].

The data were statistically analyzed using software (JMP version 8, SAS Institute Inc., Cary, NC, USA). The numbers of total and motile sperm in each ejaculates were compared by one-way analysis of variance. The characteristics of semen with and without urine contamination were compared by Student's *t*-test or Welch's *t*-test after examining the equality of variances using the F-test.

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Table 1. Semen characteristics collected by electroejaculation and the effect of urine contamination on sperm quality in a Siberian tiger

Items	Mean semen characteristics* (n = 17)	Characteristics of semen without or with urine contamination collected in the same trial (n = 4)	
		–	+
Volume (ml)	5.8 ± 2.7 (2.3 - 11.5)	3.7 ± 1.3 (2.7 - 5.7)	5.1 ± 2.2 (3.0 – 8.1)
pH	7.5 ± 0.2 (7.2 - 7.8)	7.5 ± 0.1[a] (7.4 - 7.6)	6.4 ± 0.2[b] (6.2 - 6.5)
No. of sperm ($\times 10^6$ cells/ejaculate)	294.3 ± 250.2 (13.8 - 1027.7)	241.5 ± 200.9 (34.0 - 473.1)	85.3 ± 49.8 (25.8 - 145.0)
No. of motile sperm ($\times 10^6$ cells/ejaculate)	255.6 ± 252.3 (9.7 - 919.8)	207.1 ± 169.0 (30.6 - 400.7)	33.4 ± 27.9 (5.2 - 63.3)
% of motile sperm	82.4 ± 11.4 (50.0 – 95.0)	87.3 ± 2.6[a] (84.7 - 90.0)	41.1 ± 30.5[b] (10.0 - 70.0)
% of live sperm**	86.3 ± 11.3 (54.0 - 98.5)	89.7 ± 6.5 (84.5 - 97.0)	60.5 (54.5, 66.5)
% of morphologically normal sperm	91.2 ± 3.5 (86.0 – 96.5)	NE	NE

Values are means ± SD (ranges).

[ab] Values with different superscripts differ significantly ($p < 0.05$).

* Values are calculated from semen excluding the ejaculates with urine contamination.

** Samples without and with urine contamination (3 and 2, respectively) are examined.

NE: not examined.

Table 2. Series of electrical stimuli* in which each ejaculate was collected

Ejaculates (total no. of trials**)	No. of trials collecting each ejaculate at			
	1st series	2nd series	3rd series	additional series
1st (17)	14	2	0	1
2nd (17)	4	11	1	1
3rd (16)	1	6	4	5
≥4th (14)	0	4	2	8

*A series was composed of more than 2 sets of 10 electrical stimuli.

** In three trials, electrical stimulation was halted at the second or third ejaculate because more than 200×10^6 sperm were collected.

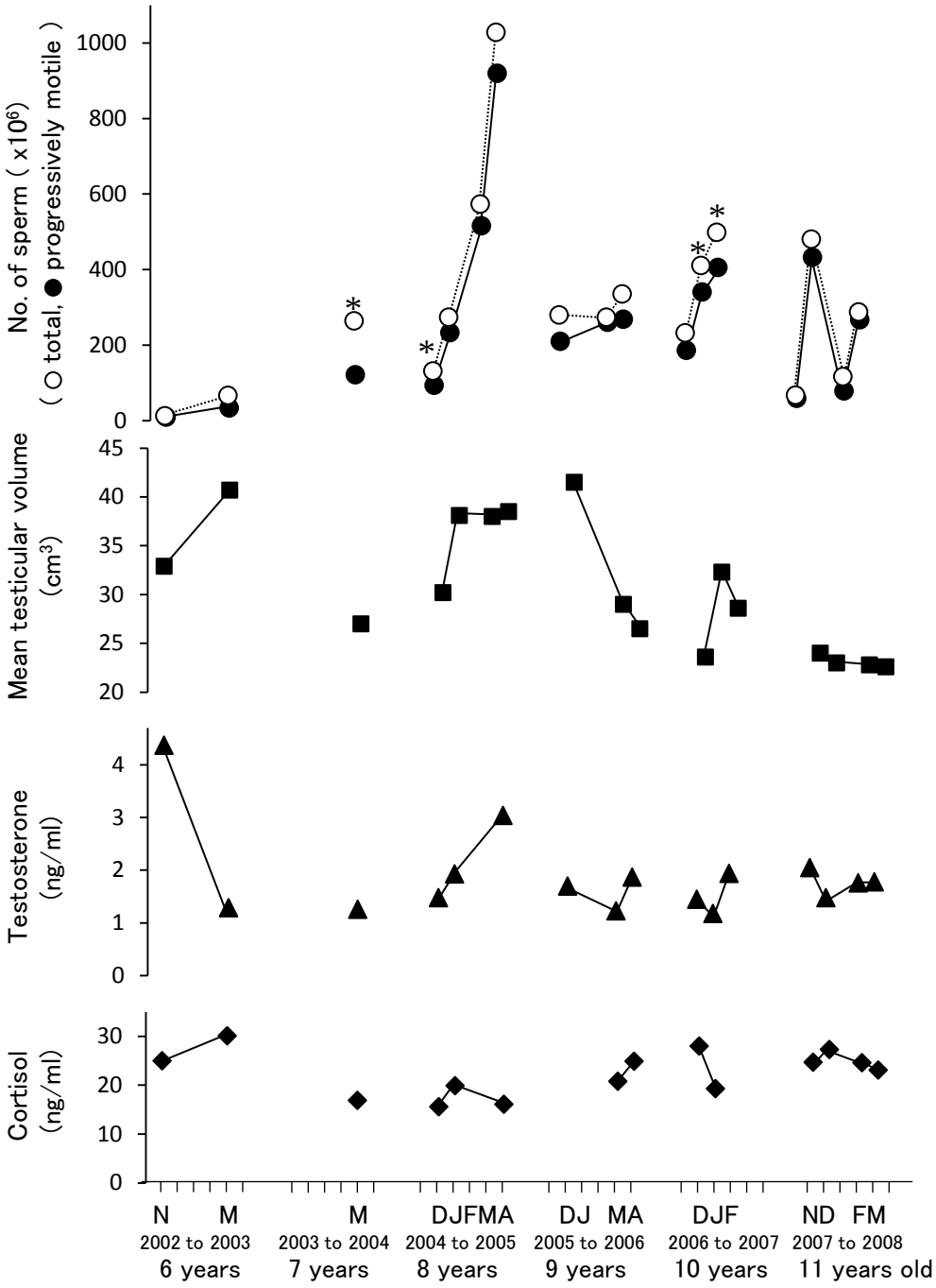


Fig. 1. Effect of frequent electroejaculation on number of sperm, testicular volume, serum testosterone and cortisol concentrations in each semen collection.

*Urine contaminations were observed.

N, November; D, December; J, January; F, February; M, March; A, April.

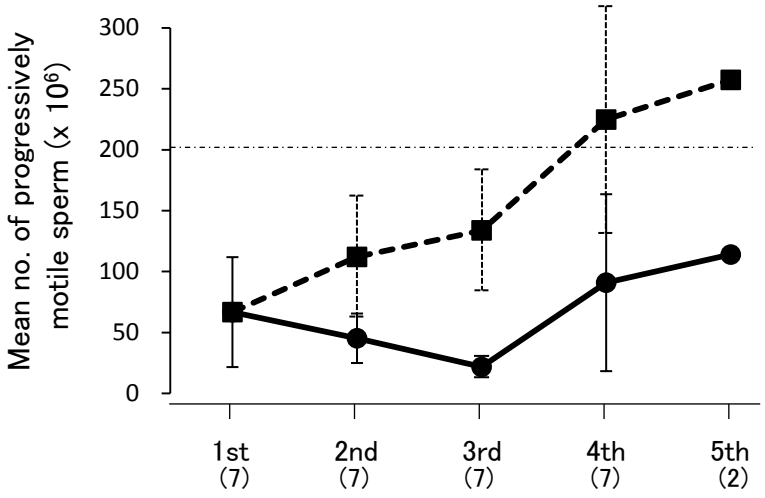


Fig. 2. Semen volume and number of progressively motile sperm in each ejaculate during electroejaculation in a Siberian tiger.
The solid line (—●—) shows the mean values in each ejaculate.
The dashed line (--■--) shows the average of cumulative values.
Error bars indicate the SEM.
Values in parentheses indicate the number of trials.
Data for semen with urine contamination were eliminated from this analysis.

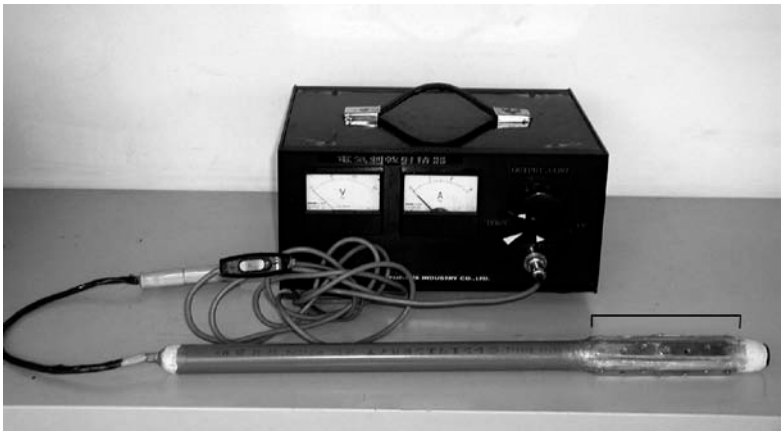


Fig. 3. The electroejaculator and rectal ejaculatory probe with three longitudinal electrodes.