



Semen collection by urethral catheterization and electro-ejaculation with different voltages, and the effect of holding temperature and cooling rate before cryopreservation on semen quality in the Japanese macaque (*Macaca fuscata*)

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ABSTRACT. In the Japanese macaque, semen has been collected by electro-ejaculation (EE), using the higher voltage stimuli compared to other species including genus *Macaca*. Semen coagulates immediately after ejaculation, which makes difficult to produce high-quality semen for artificial insemination. Recently, semen collection using urethral catheterization (UC) has been reported in carnivore and this technique may allow semen collection without coagulation in a less invasive manner. Further, the temporal preservation temperature and cooling rate of semen during cryopreservation affect post thawing sperm quality. In this study, to improve semen quality and quantity, as well as the animal welfare, semen collection was performed by EE with high (5–15 V) or low (3–6 V) voltage, UC and a combination of the two (EE-UC). It has been suggested that a high voltage is necessary for semen collection, but 10 V stimulation was effective enough and 15 V is for additional sperm collection. Also, liquid semen was collected by EE-UC and this could increase the total number of sperm. Further, to improve the post thawing sperm motility, semen was kept at four temperatures (4, 15, 25 and 37°C) for 60 min, and processed with two cooling procedures (slow cooling before second dilution and fast cooling after second dilution). Holding semen at 25°C and fast cooling after the second dilution maintained progressive motile sperm rate. The present results will contribute to the improvement of semen collection and animal welfare of Japanese macaques.

KEY WORDS: cryopreservation, electro-ejaculation, Japanese macaques, semen collection, urethral catheterization

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The Japanese macaque (*Macaca fuscata*) is popular as a zoo animal and are typically kept in troops that consist of both males and females. High fertility and uncontrolled breeding have caused overpopulation and a decrease in genetic diversity in Japanese macaque troops [1, 18]. Reproductive management by artificial insemination (AI) with frozen semen has been widely used in domestic animals [12, 28, 34] and in some zoo animals [7, 23]. In captive Japanese macaque, AI using preserved semen of selected males under strict pedigree management can solve the genetic diversity problem. In addition, limiting individuals used for

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reproduction with controlled infertility treatment can reduce the number of newborns.

There are few previous studies that reported AI in the Japanese macaque using fresh semen and leading to live births [44, 45]. Intrauterine insemination using fresh sperm achieved pregnancy, but semen injection into the uterus was difficult in the Japanese macaque due to the complex structure of the cervical canal [45]. The quality of fresh semen collected by the current electro-ejaculation (EE) method is sufficient for intrauterine insemination, but not for intracervical insemination [45].

In a previous report [26, 46] the voltage applied in the Japanese macaque for EE (5–20 V) was higher than in other species including the genus *Macaca* (2–8 V): the howler monkey (*Alouatta caraya*) [4, 48], the stump-tailed macaque (*Macaca arctoides*) [11], the Goeldi's monkey (*Callimico goeldii*) [2], the brown bear (*Ursus arctos*) [13], and the Siberian tiger (*Panthera tigris altaica*) [8]. EE using a rectal probe can cause pain and heat injury [16] and higher voltages increase the pain [9]. Therefore, sperm collection at a low voltage should be considered in terms of animal welfare in captive Japanese macaque. Furthermore, semen coagulates immediately after ejaculation due to secretions from accessory glands [26, 46]. In feline species, it has been reported that semen can be collected by urethral catheterization (UC) after sedation with an α 2-adrenergic agonist, which causes an influx of semen into the urethra via contraction of the ductus deferens [3, 24, 50]. Since semen collected by UC is assumed to contain lower or no accessory gland secretions, semen can be collected without coagulation in a less invasive way.

On the other hand, AI using cryopreserved semen has not been reported in the Japanese macaque, though there are some studies in which succeeded such as the rhesus macaque (*Macaca mulatta*) [10] and the cynomolgus macaque (*Macaca fascicularis*) [43]. Torii *et al.* [46] achieved high motility of frozen-thawed semen in the Japanese macaque, but a limited number of sperm selected by the swim-up method and density gradient centrifugation were cryopreserved in their protocol, in which the number of sperm may be insufficient for AI. Therefore, improvement in semen cryopreservation is essential to ensure an adequate number of progressive motile sperm for AI.

In the Japanese macaque, coagulated ejaculates are incubated at 37°C for 30 min for liquefaction [46]. After incubation, it takes 30–60 min for semen evaluation and adjust semen concentration of diluted semen. The optimal temperature for temporal preservation in liquid form (24–72 hr) differs among species (bull [30]: 15°C, human [41]: 23°C, stallion [12]: 4–10°C, boar [34]: 15–17°C, ram [22, 33]: 5°C). To maximize the post-thawed semen quality, the effect of the temporal holding temperature before the cryopreservation process needs to be investigated in the Japanese macaque. Further, it is beneficial to change the semen cryopreservation protocol, since a faster cooling rate to around 0°C after addition of a cryoprotectant improved post-thawed sperm motility in rhesus macaque [25].

In the present study, the efficacy of semen collection using different voltages of EE and UC application were evaluated to improve the semen collection method. Furthermore, to improve the post-thawed sperm condition, the effect of the semen holding temperature and changes in the cooling process before cryopreservation were examined.

MATERIALS AND METHODS

Animals

Thirteen male Japanese macaques (*Macaca fuscata*) aged 8–20 years old kept at the Primate Research Institute, Kyoto University, were used in the present study. All monkeys were kept in individual cages and fed on pellets for monkeys (Primate Diets AS, Oriental Yeast Co., Ltd., Tokyo) and a small amount of snacks (e.g. sweet potatoes, bananas, apples and peanuts) with water supplied *ad libitum*. All experiments were approved by the Animal Welfare and Animal Care Committee, Primate Research Institute, Kyoto University (Nos. 2012-142, 2013-089, 2014-043, 2015-037, 2016-013).

Anesthesia

The monkeys were anesthetized with a combination of ketamine hydrochloride (5.0 mg/kg, Ketamine Injection 5% Fujita, Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) and medetomidine hydrochloride (50.0 μ g/kg, Medetomidine Injection Meiji, Fujita Pharmaceutical Co., Ltd.), or a combination of ketamine hydrochloride (5.0 mg/kg), medetomidine hydrochloride (25.0 μ g/kg) and midazolam (25.0 μ g/kg, Midazolam Injection 10 mg Sandoz, Sandoz K. K., Tokyo, Japan). After the examination, atipamezole (Mepatia injection Meiji, Fujita Pharmaceutical Co., Ltd.) was administered intramuscularly at dose of 125.0 μ g/kg for the two drug, and 62.5 μ g/kg for the three drug anesthetizations to antagonize the effects of medetomidine hydrochloride.

Electro-ejaculation (EE)

For EE, a handmade rectal probe with two longitudinal electrodes (Fig. 1) connected to an electro stimulator (Fujihira Industry Co., Ltd., Tokyo, Japan) was used. After anesthesia, the monkey was laid in a lateral position and the penis extending beyond the prepuce was washed with physiological saline. Then, the tip of penis was held inside a 50-ml polypropylene conical centrifuge tube (352070; Becton Dickinson, Franklin Lakes, NJ, USA). The rectal probe was lubricated with jelly (K-Y jelly, Johnson & Johnson Inc., New Brunswick, NJ, USA) and inserted 8–10 cm into the rectum. A series of electrical stimuli were applied 10 times in a 3-sec-on/ 3-sec-off pattern as described previously [8]. After semen collection, an equivalent volume of Tes Tris Egg-yolk medium (TTE) [21, 37] was added (first dilution) and the semen was kept at 37°C for 30 min for liquefaction (Fig. 3). After removing the coagulum, diluted semen was centrifuged at 500 \times g for 10 min to concentrate the semen sample. The resulting sperm pellet was resuspended with TTE after removal of the supernatant for adjusting sperm concentration to 20–100 \times 10⁶ sperm/ml and subjected to the following process.

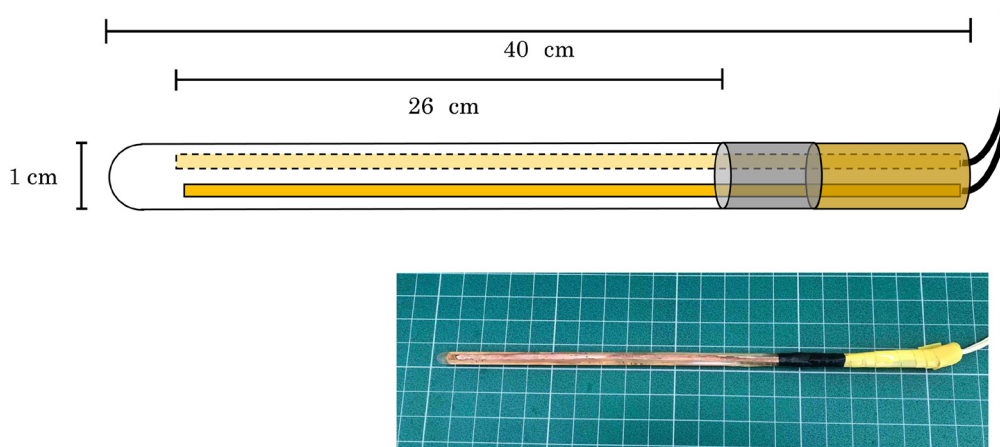


Fig. 1. Rectal probe with two longitudinal copper electrodes on both sides. Grip is insulated with plastic tape. Total length: 40 cm, diameter: 1 cm, electrode length: 26 cm, electrode width: 0.4 cm.

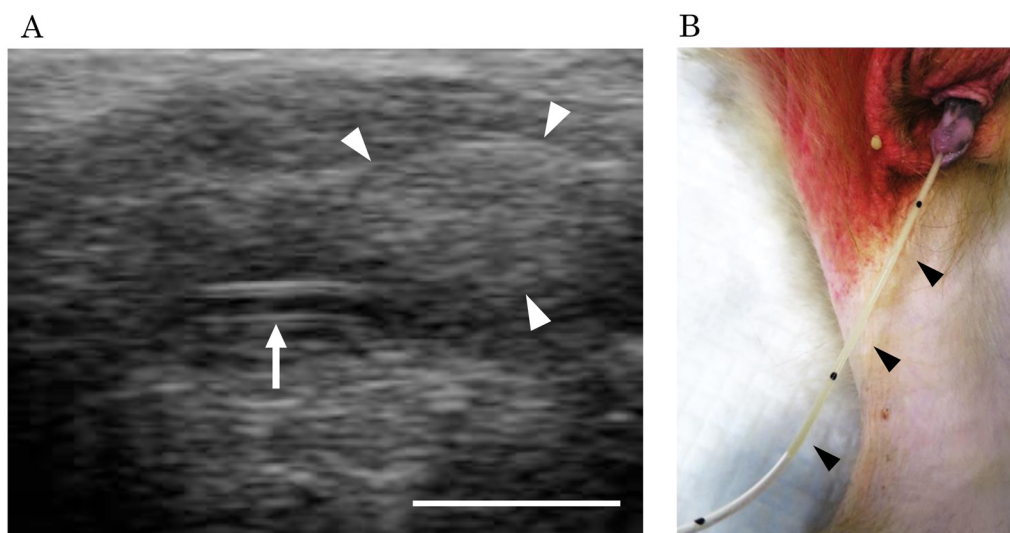


Fig. 2. Semen collection by urethral catheterization. **A:** Sagittal ultrasound image of urethral catheterization. The tip of the catheter (arrow) was inserted until it approached the prostate (white arrowheads). Left side is caudal. Bar=1 cm. **B:** Liquid form semen collected in the catheter removed from the urethra (black arrowheads).

Urethral catheterization (UC)

For UC, a 10-MHz probe (HLV-375M; Honda Electronics Ltd., Toyohashi, Japan) attached to ultrasound device (HS-1500V; Honda Electronics Ltd.) was introduced into the rectum of each anesthetized monkey laid in a lateral position to visualize the prostate. A 6 Fr polyvinyl chloride catheter (46006, Atom Medical Corp., Tokyo, Japan) was inserted from the external urethral opening until the tip reached the prostate (17–25 cm: Fig. 2A). After closing the cap of the catheter, it was retracted slowly from urethra (Fig. 2B). Liquid form semen was expelled into 1.5 ml tube and equal volume of pre-warmed TTE was added (first dilution). It was kept at 37°C until next process. If UC was performed after EE, it was separately treated from UC alone and defined as EE-UC in the present study.

Semen evaluation

Sperm concentration was examined by using hemocytometer (DHC-N01, NanoEnTek, Seoul, Korea) after dilution with distilled water containing 0.3% calf serum. Total sperm number was calculated with the concentration and semen volume. After the semen sample was diluted to $5\text{--}10 \times 10^6/\text{ml}$, the sperm motility was evaluated by light microscopy using 37°C prewarmed counting chamber (SC-20-01-04B, Leja, Nieuw-Vennep, Netherlands) by two of the authors at the same time according to a previous study with slight modification [37]. Briefly, sperm were graded into five classes by their motility and scored from 4 to 0 (4: highly active progression, 3: active progression, 2: sluggish progression, 1: barely moving, 0: immotile) and their percentages were calculated. Sperm scored 3 to 4 were evaluated as progressive motile sperm, and the sum of the percentage of sperm scored as 3 and 4 was

defined as the progressive motile sperm rate.

Besides subjective evaluation by a practitioner, motility of frozen-thawed semen was also examined with a Computer Assisted Sperm Analysis (CASA) system (SMAS, DITECT Corp., Tokyo, Japan). Briefly semen were diluted into 10×10^6 /ml with Dulbecco's phosphate buffered saline (PBS) and 3 μ l of semen sample was applied to the counting chamber. Over 200 sperm in more than three fields of view per one sample was examined for the following parameters by the CASA system: straight line velocity (VSL, μ m/sec), curvilinear velocity (VCL, μ m/sec), average path velocity (VAP, μ m/sec), amplitude of lateral head displacement (ALH, μ m), beat-cross frequency (BCF, Hz) and linearity (LIN, %). CASA recorded 150 frames per second.

Viability and acrosomal integrity were examined for frozen-thawed semen with the modified method of Kanno *et al.* [17]. Stock solution of Hoechst 33342 (H33342: Molecular Probe, Eugene, OR, USA), propidium iodide (PI: P4170, Sigma, St. Louis, MO, USA) and fluorescein peanut agglutinin FITC conjugate (FITC-PNA: FL-1071, Vector Laboratories, Tokyo, Japan) was added to PBS to give final concentrations of 0.8, 10 and 25 μ g/ml, respectively. An equal volume of fluorescence containing PBS and semen were mixed and incubated for 10 min at 37°C in the dark. Stained semen were examined using a fluorescence microscope (ECLIPSE Ci, NIKON, Tokyo, Japan) with an attached triple-band filter (DAPI/FITC/TRITC, NIKON). Over 200 sperm per sample was examined and sperm which was stained with neither PI nor FITC-PNA was evaluated as viable sperm with an intact acrosome.

Semen cryopreservation and thawing

Semen with over 20% of progressive motile sperm evaluated after resuspension subsequent to the first dilution and centrifugation for semen collected by EE or after the first dilution for semen collected by UC were subjected to cryopreservation regardless of collection method. The semen samples were randomly allocated to one of the two groups after sperm evaluation and centrifugation at 25°C (after 30–60 min of holding time, Fig. 3). In Group 1, the semen samples were cooled slowly. For slow cooling, a 1.5-ml tube containing the semen sample was immersed into 15–20 ml of water at 25°C held in a 50-ml conical tube, and then transferred to a refrigerator. The temperature of the water in the 50-ml tube surrounding the 1.5-ml tube was monitored every 10–15 min using a thermometer during refrigeration until they were cooled to 4–6°C within 60 to 90 min (slow cooling). The cooling time varied according to the difference in volume of water in the 50-ml tube and sample contained in the 1.5-ml tube which caused a difference in the height of the tube immersion. Furthermore, the cooling performance of the refrigerator varied daily, which may have been caused by the presence of other stuff stored together with the samples from this study. After cooling, TTE containing 10% of glycerol equivalent to semen volume was divided into four portions and added at five min intervals (second dilution). Five min after the final portion was added, semen samples were packed into 0.25 ml straws. In Group 2, after temporal holding at 25°C for no more than 45 min, TTE containing 10% of glycerol equivalent to semen volume was added in the same way as Group 1 (second dilution). After the second dilution, semen samples were aspirated to 0.25 ml straws, then put into plastic bags and immersed in 25°C water in a styrofoam box. The water temperature was decreased to 5°C at 20 min after starting the cooling procedure (fast cooling). Every 5 min, water temperature was reduced by 5°C and this process repeated total four times until temperature decreased to 5°C. During the temperature reducing process, crushed ice was added to the water in the box to reduce the temperature within first min, so semen samples were maintained in reduced temperature

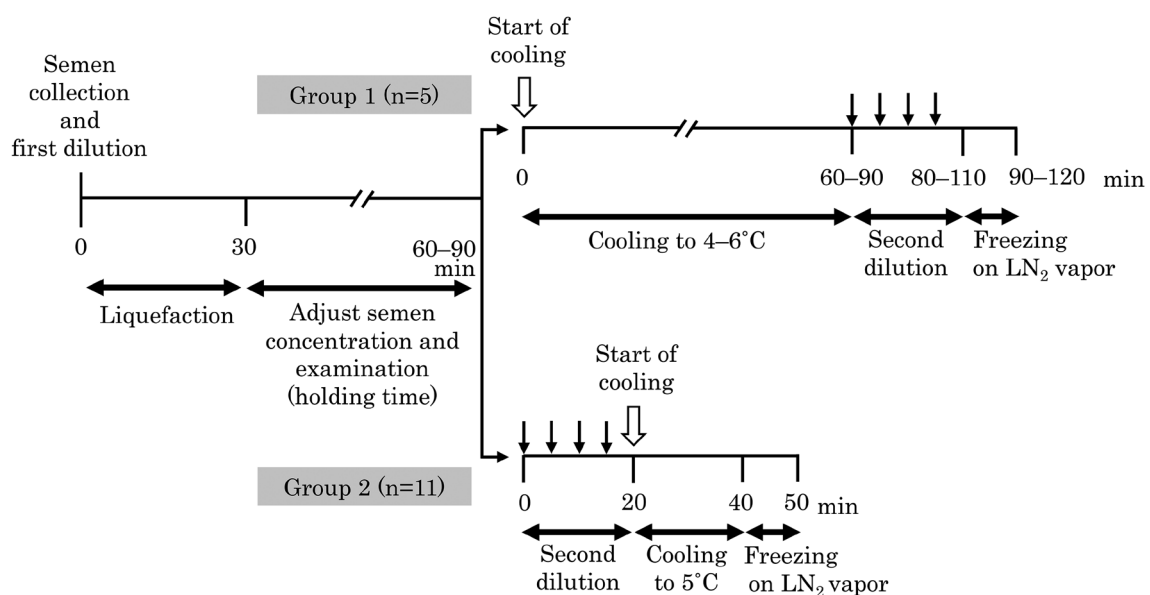


Fig. 3. Schedule of semen processing from collection to cryopreservation. Semen were cryopreserved in two procedures (Groups 1 and 2). Liquefaction was omitted for semen samples collected in liquid form. First dilution: addition of extender equivalent to semen sample volume. Second dilution: addition of extender containing 10% of glycerol at five min intervals (four times addition: short arrows). Group 1: semen sample was cooled slowly (60–90 min) after the second dilution. Group 2: semen sample was cooled quickly (20 min) before the second dilution. LN₂: liquid nitrogen.

for 4 min until next process. In both groups, after cooling and the second dilution, packed semen samples were placed in liquid nitrogen vapor (LN₂) 4 cm above the surface for 10 min. Then, the straws were immersed in liquid nitrogen for further storage. For thawing, the straws were immersed in 37°C water for 40 sec.

Study design

1. *Efficacy of semen collection by different methods:* Each monkey was subjected to one of five different combinations and order patterns of the semen collection methods (Table 1). The semen collection procedure was divided into 3 collection method groups: UC, EE, and UC after EE (EE-UC) (Table 1). Any coagulum and liquid collected by EE and liquid collected by UC, was defined as semen. If any spermatozoa were confirmed in the collected semen, it was defined as semen containing sperm. The semen collection rate and semen containing sperm collection rate were calculated as the number of successes in collection divided by the number of trials. The semen collection rate, semen containing sperm collection rate, total sperm number, and progressive motile sperm rate were evaluated. EE and EE-UC groups were further divided into two groups according to the voltage of electro stimulation: low and high voltage groups (Table 1).

For the low voltage group, electro stimulus started from 3 or 4 V and the voltage was increased by 1 V between series up to 5 or 6 V (3–4–5 V series and 4–5–6 V series). For the high voltage group, electro stimulus started from 5 V and the voltage was increased by 5 V up to 15 V (5–10–15 V series). Electro stimulation was sometimes stopped between the voltage increases when it was estimated that there would be one hour from the start of anesthesia until the next series. The semen collection rate, semen containing sperm collection rate, total sperm number, and progressive motile sperm rate were compared between the semen collection methods (UC, EE, and EE-UC), and also between the two voltage groups (low voltage and high voltage). In addition, the semen collection rate at each voltage was investigated along with the increase in the voltage in each voltage group.

2. *Effect of temperature during holding time on sperm characteristics:* Semen collected by high voltage EE and after the first dilution was aliquoted to four tubes at equal volume (0.1–0.5 ml) at room temperature. Samples were allocated four different temperatures (4°C: refrigeration temperature, 15°C: referencing appropriate temperature of boar [34], 25°C: room temperature, 37°C: referring to the body temperature of the Japanese macaque [31]). Tubes were placed in a refrigerator and incubator to keep them at 4°C or 37°C, respectively. The other tubes were placed in a heat and cool dry bath incubator at 15 and 25°C. Progressive motile sperm rate at 0, 30 and 60 min from the start of the holding time were evaluated. The semen samples were warmed to 37°C immediately before evaluation.

3. *Effect of cooling procedure on sperm characteristics:* Five and 11 samples were allocated to Group 1 (slow cooling before the second dilution) and Group 2 (fast cooling after the second dilution), respectively (Fig. 3). Progressive motile sperm rate was evaluated before freezing, immediately before LN₂ and post thawing. Before freezing represents before cooling in Group 1 and before the second dilution in Group 2 following holding time. Immediately before LN₂ represents just before freezing with LN₂. The semen samples were warmed to 37°C immediately before evaluation.

Statistical analysis

The semen collection rate among the three collection method groups (UC, EE, and EE-UC) and between the two voltage groups (low voltage and high voltage) were compared by the Fisher's exact test with the Bonferroni correction. Total sperm number and progressive motile sperm rate between EE and EE-UC were analyzed with the Wilcoxon's rank sum test. The semen collection rates at each stimulation voltage within each voltage group were compared by the Fisher's exact test with the Bonferroni correction. The progressive motile sperm rate after 30 and 60 min of holding time among four temperatures were analyzed with the Steel-Dwass test. Effects of cooling procedure on progressive motile sperm rate between immediately before LN₂ and post thawing were analyzed by the Wilcoxon's rank sum test. Effect of sperm motility analyzed by the CASA system and the rate of viable sperm with intact acrosomes examined by fluorescent staining were compared with the Student's *t*-test. All analysis was performed by JMP pro16 (SAS Institute, Cary, NC, USA) except for manual calculation of the Bonferroni correction. Total sperm number and progressive motile sperm rate are shown as the median (range). Values analyzed with the CASA or fluorescent staining are shown as the mean ± SD.

RESULTS

Efficacy of semen collection by different methods

The collection rates of semen and semen containing sperm in the three collection methods are shown in Table 2. Semen and

Table 1. Semen collection patterns and study groups

Semen collection pattern	Study group		
	UC (n=9)	EE (n=49)	
		Low (n=15)	EE-UC (n=44)
		High (n=34)	Low (n=14)
			High (n=30)
Pattern 1 (n=1)	UC (n=1)		
Pattern 2 (n=1)	UC (n=1)	→ EE (n=1)	
Pattern 3 (n=7)	UC (n=7)	→ EE (n=7)	→ UC (n=7)
Pattern 4 (n=37)		EE (n=37)	→ UC (n=37)
Pattern 5 (n=4)		EE (n=4)	

Semen collection was performed by five different combination and order patterns of semen collection methods. Study groups were divided into three groups; urethral catheterization (UC), electro-ejaculation (EE) and UC performed after EE (EE-UC) groups. Further, UC and EE-UC groups were divided into two groups according to the voltage of electro stimulation (Low and High). Low: monkeys in low voltage group were stimulated with 3–4–5 V series or 4–5–6 V series. High: monkeys in high voltage group were stimulated with 5–10–15 V series. Semen collected at each phase was allocated to three study groups (UC or EE, or EE-UC).

Table 2. Semen collection rate and sperm condition in different collection groups

Group		Semen collection rate (%)	Semen containing sperm collection rate (%)	Total sperm number ($\times 10^6$)	Progressive motile sperm rate (%)
UC		33.3 (3/9) ^a	0.0 (0/9) ^a	Not collected	Not collected
EE	Total	79.5 (39/49) ^b	65.3 (32/49) ^b	19.6 (0.01–450.0) (n=29)	39.5 (0.0–80.0) (n=32)
	Low	53.3 (8/15)	20.0 (3/15)	101.0 (85.0–130.0) (n=3)	50.0 (50.0–60.0) (n=3)
	High	91.1* (31/34)	85.2* (29/34)	12.0 (0.01–450.0) (n=26)	30.0 (0.0–80.0) (n=29)
EE-UC	Total	84.1 (37/44) ^b	77.3 (34/44) ^b	9.8 (0.05–672.5) (n=33)	40.0 (0.0–80.0) (n=34)
	Low	71.4 (10/14)	50.0 (7/14)	10.5 (0.06–170.0) (n=7)	50.0 (0.0–80.0) (n=7)
	High	90.0 (27/30)	90.0* (27/30)	8.7 (0.2–672.5) (n=26)	40.0 (0.0–80.0) (n=27)

UC: urethral catheterization, EE: electro ejaculation, EE-UC: UC performed after EE. Low: monkeys in low voltage group were stimulated with 3–4–5 V series or 4–5–6 V series. High: monkeys in high voltage group were stimulated with 5–10–15 V series. ^{a,b} Values with different letters differ significantly among UC, total of EE and total of EE-UC ($P < 0.05$, Fisher's exact test). * Values with superscripts differ significantly between low and high voltage groups in each procedure group ($P < 0.05$, Fisher's exact test). Values of total sperm number and progressive motile sperm rate are shown as the median (range).

the semen containing sperm collection rate in the UC group were significantly lower than those in the EE and EE-UC groups, and these were not significantly different between the EE and EE-UC groups. In the UC group, a small amount of slightly clouded liquid was collected, but it did not contain any sperm (only epithelial cells). On the other hand, a milky or slightly whitish liquid containing sperm was collected in the EE and EE-UC groups (Fig. 2B). There were three cases where semen was collected by EE-UC without any ejaculation by EE. Total sperm number and the progressive motile sperm rate were not different between the EE and EE-UC groups (Table 2). Comparing the total sperm number between EE and the sum of EE and EE-UC in totals of Pattern 3 and Pattern 4 (n=44, Table 1), the sum of EE and EE-UC was 36.3×10^6 (0.06 – 672.5×10^6), which was higher than 4.7×10^6 (0 – 450×10^6) in EE only ($P < 0.05$).

The semen collection rate and semen characteristics in the low and high voltage groups are shown in Table 2. Sperm were collected by EE-UC without ejaculation by EE in one and two trials in the low and high voltage groups, respectively. The semen collection rate was significantly higher in EE in the higher voltage group ($P < 0.05$). The semen containing sperm collection rate in both EE and EE-UC were significantly higher in the high voltage group ($P < 0.05$). Although statistical analysis were not performed due to big difference in number of the samples between the voltage groups, median values were similar in the total sperm number and progressive motile sperm rate, except total sperm number in EE. In high voltage group, range of value relatively wide in comparison with low voltage group.

The semen collection rates at each voltage in the low and high voltage groups are shown in Fig. 4. Semen were collected when stimulated by 4 and 5 V, and there was no significant difference in the semen collection rate between them (Fig. 4A). Semen was not collected by 6 V. In five out of seven monkeys subjected to 6 V stimulation, semen was also not collected by other voltages. In the high voltage group, semen were collected at all voltages. Semen collection rates were higher at 10 V (78.1%, 25/32) and 15 V (80.8%, 21/26) than at 5 V (26.4%, 9/34) and did not differ between 10 V and 15 V (Fig. 4B). Two out of 21 monkeys those could collect semen by 15 V, ejaculation occurred only at 15 V.

Effect of temperature during holding time on sperm characteristics

Changes in the progressive motile sperm rate at each holding time temperature after 30 and 60 min are shown in Fig. 5. There was no significant difference between holding temperature after 30 min. After 60 min of holding, the progressive motile sperm rates at 25°C and 37°C tended to be higher than 4°C ($P < 0.10$).

Effect of cooling procedure on sperm characteristics

As shown in Fig. 6, the progressive motile sperm rate in Group 2 (fast cooling after the second dilution) was significantly higher than in Group 1 (slow cooling before the second dilution) at immediately before LN₂, but there was no difference between groups at after thawing. Regarding the post-thaw motility evaluated by the CASA system, VSL and ALH of spermatozoa in Group 2 were higher than those of Group 1, whereas there was no difference in VCL, VAP, BCF and LIN between the groups (Table 3). The percentage of viable sperm with intact acrosomes after thawing was $9.6 \pm 5.1\%$ in Group 1, which is not significantly different from $11.9 \pm 6.9\%$ in Group 2.

DISCUSSION

The age of the monkeys used in the present study ranged from 8–20 years old, which was assumed not to affect semen collection since the testis at these ages are sufficiently matured and not degraded by aging [27]. The present study is the first trial using UC for semen collection in primates. In fact, semen with spermatozoa could not be collected from the Japanese macaques using the UC protocol in felids [3, 24, 50]. It is thought that the following three factors are involved in the regulation of semen collection by UC: adrenergic innervation [35, 47, 50], dose of medetomidine [6] and the type of drug used for pharmacological stimulation [19, 40, 47]. The release of sperm from the epididymis and/or ductus deferens to the urethra by administration of a α 2-adrenergic agonist

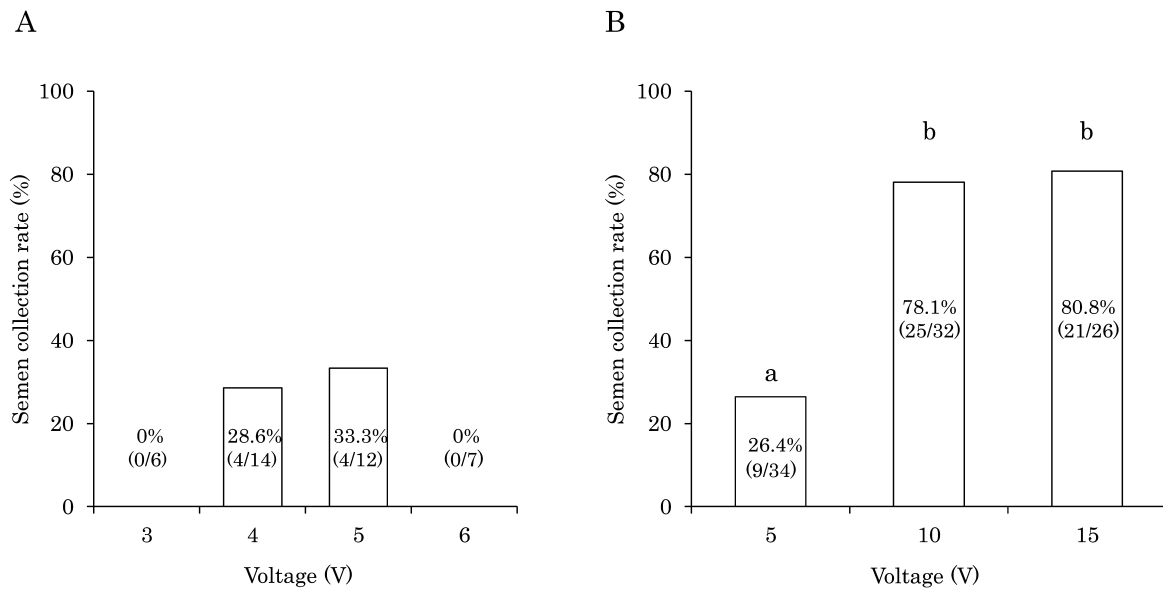


Fig. 4. Semen collection rate at each voltage in the two different voltage groups. **A:** Low voltage group. **B:** High voltage group. ^{a, b} Bars with different letters significantly differ ($P < 0.05$, Fisher's exact test).

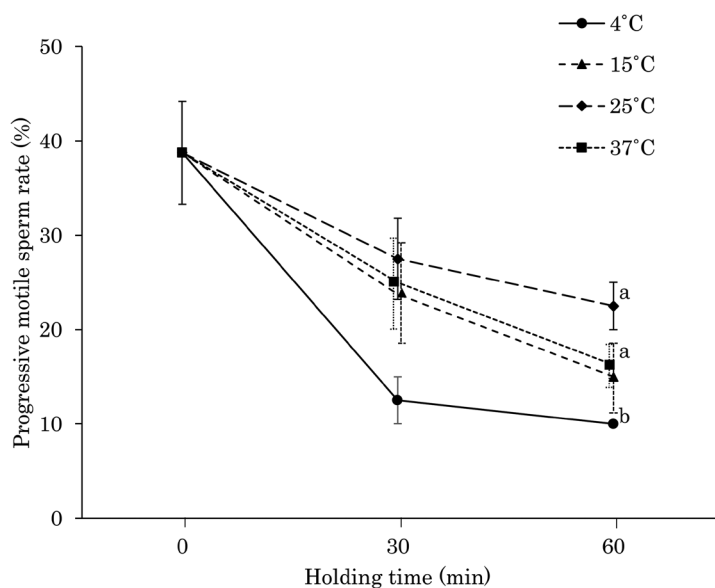


Fig. 5. Changes in the progressive motile sperm rate in four different holding temperature conditions. ^{a, b} Values with different letters tended to be different between temperatures at the same holding time ($P < 0.10$, Steel-Dwass test).

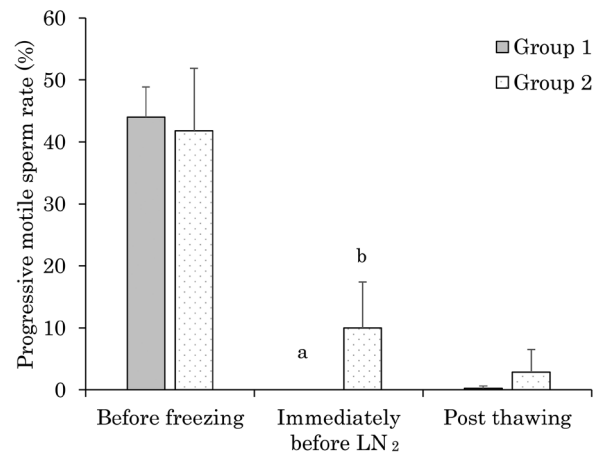


Fig. 6. Progressive motile sperm rate between different cooling procedures during cryopreservation. Group 1: semen was cooled slowly (60–90 min) before the second dilution. Group 2: semen was cooled quickly (20 min) after the second dilution. Before freezing: before cooling (Group 1) or the second dilution (Group 2) after temporal holding. Immediately before LN₂: just before freezing with liquid nitrogen. ^{a, b} Values with different letters differ significantly between cooling groups within each process ($P < 0.05$, Wilcoxon's rank sum test).

was reported [25, 50]. Although adrenergic innervation in the epididymis and/or ductus deferens was reported previously in cats [14, 35] and macaques [20, 29], the details regarding the reactivity against $\alpha 2$ -adrenergic agonists remains unclear. Prochowska *et al.* [35] reported that $\alpha 2$ -adrenergic receptor expression in the reproductive tract was higher in male cats than rats. It was also reported that the dose of adrenaline that causes contraction of the ductus deferens in Japanese macaques was lower than in rabbits and higher than guinea pigs [29]. These variations in adrenergic reactivity among species, can explain the differences in semen emission in the Japanese macaque. In this study, a dose of medetomidine (25 or 50 $\mu\text{g}/\text{kg}$) was used for immobilization. It was reported that a higher dose of medetomidine (130 $\mu\text{g}/\text{kg}$) increased semen quality compared with a lower dose (50 $\mu\text{g}/\text{kg}$) in UC in cats [6]. Examining the effect of higher doses of medetomidine is needed in further studies on the Japanese macaque. On the

Table 3. Characteristics of post thawed spermatozoa cryopreserved by different cooling procedures evaluated by the computer assisted sperm analysis system

Cooling procedure group	VSL (µm/sec)	VCL (µm/sec)	VAP (µm/sec)	ALH (µm)	BCF (Hz)	LIN (%)
Group 1 (n=5)	7.0 ± 3.4 (1.8–11.1)	79.4 ± 23.4 (52.5–115.3)	18.1 ± 7.7 (11.4–30.4)	1.4 ± 0.4 (1.1–2.1)	5.2 ± 1.2 (3.6–7.2)	10.1 ± 5.0 (3.3–17.1)
Group 2 (n=11)	17.7 ± 8.3* (7.1–33.0)	101.7 ± 18.0 (73.2–132.0)	27.1 ± 9.4 (12.6–45.3)	2.2 ± 0.3* (1.8–2.8)	6.2 ± 1.4 (4.0–8.9)	15.7 ± 6.0 (7.4–24.3)

VSL: straight line velocity, VCL: curvilinear velocity, VAP: average path velocity, ALH: amplitude of lateral head displacement, BCF: beat-cross frequency, LIN: linearity. Group 1: semen was cooled slowly (60–90 min) before the second dilution. Group 2: semen was cooled quickly (20 min) after the second dilution. Values are shown as the mean ± SD (range). * Values differ significantly between cooling procedures ($P < 0.05$, Student's *t*-test).

other hand, a highly selective $\alpha 2$ -agonist (dexmedetomidine) increased the total sperm number in UC in cats [40]. Detomidine and xylazine, which are other $\alpha 2$ -agonists, were used for pharmacological semen collection from stallions in combination with imipramine and oxytocin [5, 47]. The sympathetic effect of imipramine [15, 36] and promotion of sperm fluid from the epididymis with oxytocin [32] may assist semen collection. Therefore, UC protocols using other pharmacological stimulations should be investigated since the effect of these drugs is unclear for the Japanese macaque.

Liquid form semen was collected using a combination of EE and UC (EE-UC), although sperm could not be collected by UC alone. Most of the semen collected by EE immediately coagulates due to the effects of seminal vesicle and prostate secretions [26, 46], but the Japanese macaque ejaculates can be separated into fluid and coagulated portions [42]. Since it is thought that a considerable number of sperm in the fluid portion remain in the urethra after ejaculation by EE, total number of collected sperm can be increased by adding UC protocol following EE.

By low voltage electro stimulation (3–6 V), semen was collected in about half of the trials, but sperm were collected from only 20% of them. Since no semen were collected by 6 V stimulation, increasing the voltage to 6 V after stimulating with 5 V is insufficient to obtain additional semen. Therefore, there might be no difference between stimulation at 5 V and 6 V regarding the strength for inducing ejaculation in Japanese macaques. Further, sperm were collected in only about half of the EE-UC trials. Although the stimulation patterns and wide targeting stimulation sites with longitudinal electrodes in this study were the same with other macaque studies using low voltage [11], a higher voltage was needed. The reason for this difference is uncertain, but one possible reason is body size differences. In primates, a higher voltage tends to be used for EE of larger species like humans [16] and great apes [38, 49], and the Japanese macaques are larger or slightly larger (body weight: 10 kg or more) than other monkeys (1–10 kg) in which EE was performed using lower voltages (2–8 V) [2, 4, 11, 48]. In the present study, we tried up to 15 V in the high voltage group, but the semen collection rate was not different between 10 V and 15 V. These results suggest that stimulation of up to 10 V, which is lower than previous reports (10–15 V [26], 5–20 V [46]), is effective enough for semen collection in the Japanese macaque. However, 15 V stimulation was required to collect semen in a few cases. Further, 15 V stimulation gives us additional semen ejaculation following 10 V stimulation. Therefore, it is up to how many sperm will be required and necessity of collection from specific individual to perform 15 V stimulation following 10 V. Previously, electro stimulation of 2–8 V achieved a high sperm collection rate in other primates [2, 4, 11, 48]. A lower voltage is desirable for animal welfare since pain and heat injury are adverse effects of EE [16]. Further investigation of voltages between 6–10 V may lower the required voltage for EE in the Japanese macaque.

A wide range of temperatures (5–23°C) for temporal preservation (24–72 hr) among different species has been reported [12, 22, 30, 33, 34, 41]. After 60 min of holding time, the progressive motile sperm rate tended to be higher at 25°C and 37°C than 4°C. A low holding temperature at 4°C could cause damage due to cold shock [12, 34]. Although there was no significant difference between 25°C and 37°C at 60 min, value at 25°C was higher than at 37°C. This may be effected by increase of sperm metabolic rate at 37°C [41]. For Japanese macaque semen, 25°C appears to be a suitable temperature, as indicated in the present study, to minimize the effect of metabolism and cold shock.

In the present study, the progressive motile sperm rate and percentage of viable sperm with intact acrosomes after thawing was not different between cooling methods. Although LIN is not different between the two cooling methods, VSL and ALH were higher when the semen was cooled quickly (20 min) after the second dilution, which is advantageous. Furthermore, fast cooling after the second dilution improved the progressive motile sperm rate at immediately before LN₂. Addition of a cryoprotectant before cooling will provide suitable time for equilibration and dehydration, although the second dilution was usually performed after cooling above 0°C in previous studies [39, 46]. Martorana *et al.* [25] improved post-thawed sperm motility by shortening the duration of cooling to 20 min from 2 hr in a previous report [39]. Therefore, both the order of the second dilution and a cooling rate above 0°C contributed to the improvement. In a previous study, highly motile sperm were selected using density gradient centrifugation for cryopreservation [46]. On the other hand, the cryopreservation media (TTE) were used for the first dilution and washing to make the cryopreservation more convenient in the present study, and we found that fast cooling improve the progressive motile rate during cryopreservation.

In this study, it was shown that UC after administration of medetomidine (25 or 50 µg/kg) was not effective to collect semen containing sperm in the Japanese macaque, but EE-UC can increase the number of sperm by collecting semen remaining in the

urethra after EE. Further, the present study indicates that a lower voltage (10 V), compared to previous studies using up to 20 V [26, 46], is sufficient for EE in the Japanese macaque but 15 V can provide additional semen collection. It was also shown that holding at 25°C and short-term cooling after the second dilution in the freezing process can contribute to cryopreservation of Japanese macaque sperm. Although, the fresh semen quality collected in this study was sufficient for AI with intra uterine insertion [45], in a future study, it will be necessary to improve sperm motility together with acrosomal and plasma membrane integrity after cryopreservation in order to achieve effective AI. Since the progressive motile sperm rate after holding at two temperatures (15 and 37°C) was not significantly different from that at 25°C, which was used in the present study for cryopreservation, the temperature effect on cryopreservation should be further investigated in future studies.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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