The studies on establishment of artificial insemination protocol in the Japanese macaque

（ニホンザルにおける人工授精プロトコル

確立に向けた研究）

Noboru TAKAESU

CONTENTS

ABBRIVIATION

AI: artificial insemination

ALH: amplitude of lateral head displacement

BCF: beat-cross frequency

CASA: Computer Assisted Sperm Analysis

CL: corpus luteum

E_2: estradiol-17β

EE: electro-ejaculation

EE-UC: combination of EE and UC

FITC-PNA: fluorescein peanut agglutinin FITC conjugate

FSH: follicle stimulating hormone

GnRH: gonadotropin releasing hormone

hCG: human chorionic gonadotropin

LH: luteinizing hormone

LIN: linearity

LN_2: liquid nitrogen

P_4: progesterone

PBS: Dulbecco's phosphate buffered saline

PI: propidium iodine

SD: standard deviation

TTE: Tes Tris Egg-yolk medium

UC: urethral catheterization

VAP: average path velocity

VCL: curvilinear velocity

VSL: strait line velocity

NOTES

The contents of Chapter 1 have been accepted in the Journal of Veterinary Medical Science.

Takaesu N, Kanno C, Sugimoto K, Nagano M, Kaneko A, Indo Y, Imai H, Hirai H, Okamoto M, Sashika M, Shimozuru M, Katagiri S, Tsubota T, Yanagawa Y.
Semen collection by urethral catheterization and electro-ejaculation with different voltage, and effect of holding temperature and cooling rate before cryopreservation on semen quality in the Japanese macaque. J Vet Med Sci In press.

The contents of Chapter 2 have been accepted in Japanese Journal of Veterinary Research.

Takaesu N, Minami M, Nagano M, Kaneko A, Indo Y, Imai H, Hirai H, Sashika M, Shimozuru M, Okamoto M, Katagiri S, Tsubota T, Yanagawa Y.
Ovulatory follicle size investigated by ultrasonography and single artificial insemination with ovulation induction in the Japanese macaque. Jpn J Vet Res In press.

PREFACE

The Japanese macaque (*Macaca fuscata*) is endemic species in Japan and has a long history as a subject of biological research in Japan[87]. Especially, species-specific society of the troop have been investigated by many researchers who established the basement of primate research in Japan[22, 25, 82]. Furthermore, the Japanese macaque has been used as an experimental animal due to their physiological similarity to human. In studies of cranial nerve, pharmacology, disease model etc.[51, 60], therefore the Japanese macaque has contributed widely to development of research as an animal model for medical research. On the other hand, study of reproduction, especially artificial reproductive technology is limited. One of the reasons why artificial reproductive technique has not been developed is their huge fertility. In fact, population has increased and habitat area has spread in wild Japanese macaque, then agricultural damage by monkeys is serious in many local regions[14]. Also, overpopulation problem occurs in many Japanese zoos[3] since the Japanese macaque is popular as a zoo animal and exhibited in troop consisting of males and females to replicate the wild troop. In spite of seasonal breeding and small litter size (delivery of one offspring every two years[56, 59]), the population has been increased easily even in captive condition due to strong fecundity and uncontrolled breeding in the troops[3, 56]. Castration or vasectomy in males and implantation of progesterone releasing capsules in females have been widely used to prevent breeding, but they sometimes fail because of unintentional recanalization and removal of implants[3]. Furthermore, there is another major problem of captive Japanese macaque in zoos, namely, decreasing of genetic diversity[3]. In most species, exchange of individuals between zoos is chosen to keep genetic diversity. However, in the Japanese macaque, introduction of individuals between troops is difficult due to their clannish nature in which introduced animals are often attacked and killed[3]. History of the Japanese macaque rearing in Japanese zoos is very long, then reproduction in closed colony has been repeated for over three decades in many zoos[3]. Therefore, necessity of proper reproductive management of the Japanese macaque has been increasing. Artificial insemination (AI) using preserved semen of selected

males under strict pedigree management and well-planned contraception will be a solution[3, 32]. However, there are only a few reports about AI in the Japanese macaque[85, 86], thus problems to be solved remains both in males and females to establish AI protocol. Furthermore, to conduct AI in zoos there are some circumstances specific to zoo animals. First, more attention to animal welfare is required. Animals in zoos are usually kept for exhibition but not for experiments, their health condition are opened for many visitors and invasiveness should be minimized to conduct AI in zoos. Second, facility, equipment, staffs and budget for AI is usually limited even though reproductive management is one of important activities for zoos. Though AI protocol is usually improved to increase the pregnancy rate, the protocol for practical and routine use in zoos is also required to be less invasive in a fewer steps without expensive and special equipment. At last, species-specific structure of the Japanese macaque troop should be taken into account. There is a hierarchy in Japanese macaque troop, and capture and isolation of monkeys for AI cause the shuffle of the hierarchy and struggle involving the hierarchy[3]. Therefore, the terms of AI protocol should be shortened but long term isolation in laboratory was assumed in the previous protocol[86]. Furthermore, AI can contribute to conservation of wildlife, which is one of the most important roles of zoos. In fact, AI is performed in many endangered species in zoos for increase of individuals and maintaining of genetic diversity[2, 39]. Though AI protocol is different among species, it is similar among related species. For example, the difficulty of semen injection into the uterus due to the complex structure of the cervical canal[21, 86] is a subject to be solved commonly in genus *Macaca*. There are many endangered species related to the Japanese macaque in zoos[23, 27]. Improvement of AI technique in the Japanese macaque can apply for such related species, in which the reproduction does not often go smooth in captive condition. Therefore, this thesis also aims to contribute to *ex situ* conservation in future.

In chapter 1, as male side subjects, semen collection and preservation were investigated. In the Japanese macaque electro-ejaculation (EE) has been used for semen collection but an electro-stimulator for EE is not available in most zoos

because of expensive price and unusual tool. Furthermore, high voltage of stimulation (5–20 V) for EE in the Japanese macaque[42, 84] causes invasiveness. In this study use of lower voltage (3–6 V) was evaluated to reduce the invasiveness. Semen collection with urethral catheterization (UC) has been conducted in carnivores[5, 40, 94]. UC is semen collection method using urethral catheter under α_2-adrenergic agonist and does not need the electro stimulation. UC, which is less invasive procedure for semen collection without special equipment, is acceptable for zoos in terms of both cost and animal welfare. Exchange of cryopreserved semen for AI is an alternative to exchange of individuals to maintain genetic diversity. However, success of AI using cryopreserved semen has not been reported in Japanese macaque, since cryopreserved semen in previous cryopreservation procedure[84] seemed not to keep sufficient number of sperm for AI. To improve semen cryopreservation procedure, first, appropriate temperature for temporary preservation of semen in the Japanese macaque was investigated. Second, effect of cooling rate and the order of addition of the cryoprotectant before freezing of semen to the post thawed sperm characteristics was investigated.

In chapter 2, as female side subjects, ovarian monitoring using ultrasonography to detect ovulation and ovulation induction using gonadotropin releasing hormone (GnRH) were investigated. Ultrasonography can be an alternative to laparoscopy which was used in the previous reports for detection of ovulation[85, 86]. Ultrasonography is less invasive method due to omitting surgical procedure and anesthesia in laparoscopy. Ovulation induction enables to perform AI at appropriate timing since both sperm and oocyte meet with high fertility, and is effective for AI of single time. Ovulation induction omits repeated operation for detection of ovulation, and work for practical use to enable conducting AI on the zoo staff's side of schedule. The advantage of single AI is minimizing the isolation duration of monkeys from their troops for AI. Though frequent AI may increase pregnancy rate, considering performing in zoos, even a few days of isolation for AI can increase the risk of shuffle of hierarchy in the troop of monkeys. Single AI can reduce the risk of struggle due to isolation. As the last attempt of chapter 2, single

AI was performed with monitoring ovary using ultrasonography and ovulation induction using GnRH.

This study attempts to establish AI procedure in the Japanese macaque through the experiments in both male and female sides. A convenient and less invasive protocol of AI will contribute to improvement of animal welfare and the reproductive management of captive Japanese macaque in zoos.

CHAPTER 1

Semen collection by urethral catheterization and electro-ejaculation with different voltage, and effect of holding temperature and cooling rate before cryopreservation on semen quality in the Japanese macaque

Introduction

The Japanese macaque (*Macaca fuscata*) is popular as a zoo animal and 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[3, 32].

Reproductive management by artificial insemination (AI) with frozen semen has been widely used in domestic animals[19, 45, 63] and in some zoo animals[15, 39]. In captive Japanese macaques, AI using preserved semen of selected males under strict pedigree management can solve the genetic diversity problem. In addition, limiting individuals used for reproduction with controlled infertility treatment can reduce the number of newborns.

There are a few previous studies that reported AI in the Japanese macaque using fresh semen and leading to live births[85, 86]. Intrauterine insemination using fresh sperm achieved pregnancy. The quality of fresh semen collected by the current electro-ejaculation (EE) method is sufficient for intrauterine insemination, but not for intracervical insemination[86].

In a previous report[42, 84] 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*)[9, 89], the stump-tailed macaque (*Macaca arctoides*)[20], the Goeldi's monkey (*Callimico goeldii*)[4], the brown bear (*Ursus arctos*)[24], and the Siberian tiger (*Panthera tigris altaica*)[17]. EE using a rectal probe can cause pain and heat injury[29] and higher voltages increase the pain[18]. Therefore, semen collection at a low voltage should be considered in terms of animal welfare in captive Japanese macaque. Further, semen coagulates immediately after ejaculation due to secretions from

accessory glands[42, 84]. In feline species, it is 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[5, 40, 94]. 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 this succeeded such as the rhesus macaque (*Macaca mulatta*)[68] and the cynomolgus macaque (*Macaca fascicularis*)[83]. Torii et al. [84] achieved high motility of frozen-thawed semen in the Japanese macaque, but limited number of sperm selected by swim up method and density gradient centrifugation were cryopreserved in their protocol, in which number of sperm might be not sufficient 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[84]. 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[50]: 15˚C, human[80]: 23˚C , stallion[19]: 4–10˚C, boar[63]: 15–17˚C, ram[38, 61]: 5˚C). To maximize post thawed semen quality, the effect of the temporal holding temperature in 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 the rhesus macaque[41].

In the present study, efficacy of semen collection by different voltage of EE and UC application were evaluated to improve semen collection methods. Further, to improve 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 μg/kg, Medetomine Injection Meiji, Fujita Pharmaceutical Co., Ltd., Tokyo, Japan), or a combination of ketamine hydrochloride (5.0 mg/kg), medetomidine hydrochloride (25.0 μg/kg) and midazolam (25.0 μg/kg, Midazolam Injection 10mg Sandoz, Sandoz K. K., Tokyo, Japan). After the examination, atipamezole (Mepatia injection Meiji, Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) was administered intramuscularly at dose of 125.0 μg/kg for the two drug, and 62.5 μg/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. I-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[17]. After semen collection, an equivalent volume of Tes Tris Egg-yolk

medium (TTE)[37, 69] was added (first dilution) and the semen was kept at 37˚C for 30 min for liquefaction (Fig. I-3). After removing the coagulum, diluted semen was centrifuged at 500 × 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 × 10^6 sperm/ml and subjected to the following process.

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. I-2A). After closing the cap of the catheter, it was retracted slowly from urethra (Fig. I-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 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 semen sample was diluted to 5–10 × 10^6/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[69]. 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 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 Corporation, Tokyo, Japan). Briefly semen were diluted into 10×10^6 /ml with Dulbecco's phosphate buffered saline (PBS) and 3 μl 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, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), amplitude of lateral head displacement (ALH, μm), 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. [31]. 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 25μg/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. 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. I-3). In Group 1, the semen samples were cooled slowly. For slow cooling, a 1.5 ml tube contained semen sample was immersed into 15–20 ml of 25˚C water put in 50 ml conical

tube, then transferred to a refrigerator. The temperature of water in 50 ml tube surrounding 1.5 ml tube were monitored every 10–15 minutes by thermometer and whole tubes were placed in refrigerator 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 50 ml tube and sample contained in 1.5 ml tube which causes the difference in height of tube immersion. Further, cooling performance of refrigerator were changed day by day, may be due to amount of other stuff placed together with samples. 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 minutes, 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 for 4 min until next process. In both groups, after cooling and the second dilution, packed semen samples were placed in liquid nitrogen (LN_2) vapor 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 combination and order patterns of semen collection methods (Table I-1). The semen collection procedure was divided into 3 collection method groups: UC, EE, and UC after EE (EE-UC) (Table I-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. Semen collection rate and semen containing sperm collection rate were calculated as number of successes in collection divided by number of the 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 I-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 semen collection methods (UC, EE, and EE-UC), and also compared between 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[63], 25˚C: room temperature, 37˚C: referring to the body temperature of the Japanese macaque[52]). 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. Semen samples were warmed to 37˚C just 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. I-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 LN_2 represents just before freezing with LN_2. Semen samples were warmed to 37˚C just before evaluation.

Statistical analysis

The semen collection rate among three collection method groups (UC, EE and EE-UC), between two voltage groups (low voltage and high voltage) and among each stimulation voltage in low and high voltage groups were compared by 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. Semen collection rate at each stimulation voltage within each voltage group were compared by Fisher's exact test with the Bonferroni correction. 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 LN_2 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, 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 ± standard deviation (SD).

Results

1. 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 I-2. Semen and 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. I-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 I-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 I-1), the sum of EE and EE-UC was 36.3×10^6 ($0.06–672.5 \times 10^6$), which was higher than 4.7×10^6 ($0–450 \times 10^6$) in EE only ($P < 0.05$).

The semen collection rate and semen characteristics in the low and high voltage groups are shown in Table I-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 ($P < 0.05$). 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. I-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. I-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. I-4B). Two out of 21 monkeys in which semen could be collected by 15 V, ejaculation occurred only at 15 V.

2. 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. I-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 ($P < 0.10$).

3. Effect of cooling procedure on sperm characteristics

As shown in Fig. I-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 LN_2, 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 or LIN between the groups (Table I-3). The percentage of viable sperm with intact acrosomes after thawing was 9.6 ± 5.1% in Group 1, which is not significantly different from 11.9 ± 6.9% in Group 2.

Discussion

The age of monkeys used in this study were ranged 8–20 years old which assumed not affect semen collection, since testis of these age are sufficiently matured and not degraded by aging[43]. The present study is the first trial using UC for semen collection in primates. In fact, semen with spermatozoa could not be collected from Japanese macaques using the UC protocol in felids[5, 40, 94]. It is thought that the following three factors are involved in the regulation of semen collection by UC: adrenergic innervation[65, 88, 94], dose of medetomidine[13] and the type of drug used for pharmacological stimulation[35, 78, 88]. The release of sperm from the epididymis and/or ductus deferens to the urethra by administration of a α_2-adrenergic agonist was reported[65, 94]. Although adrenergic innervation in the epididymis and/or ductus deferens was reported previously in cats[26, 65] and macaques[36, 47], the details regarding the reactivity against α_2-adrenergic agonists remains unclear. Prochowska et al.[65] reported that α_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 in guinea pigs[47]. 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 μg/kg) was used for immobilization. It was reported that a higher dose of medetomidine (130 μg/kg) increased semen quality compared with a lower dose (50 μg/kg) in UC in cats[13]. Examining the effect of higher doses of medetomidine is needed in further studies on the Japanese macaque. On the other hand, a highly selective α_2-agonist (dexmedetomidine) increased the total sperm number in UC in cats[78]. Detomidine and Xylazine, which are other α_2-agonists, were used for pharmacological semen collection from stallions in combination with imipramine and oxytocin[10, 88]. The sympathetic effect of imipramine[28, 67] and promotion of sperm fluid from the epididymis with oxytocin[53] 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[42, 84], but the Japanese macaque ejaculates can be separated into fluid and coagulated portions[81]. 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 voltage to 6 V after stimulating with 5 V is not enough to obtain additional semen. Therefore, there might be no difference between stimulation by 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 monkey studies using low voltage[20, 89], 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[29] and great apes[70, 91], 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)[4, 9, 20, 89]. In the present study, I 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[42], 5–20 V[84]), is effective enough for semen collection in the Japanese macaque. However, 15 V stimulation was required to collect semen in a few cases. Therefore, to maximize the semen collection rate, 15 V of stimulation is suitable, but use of up to 10 V of stimulation may be effective in some specific case, in which reduction of invasiveness is required, for example semen collection from aged monkeys or when shortening anesthesia time is required. Previously, electro stimulation of 2–8 V achieved a high sperm collection rate in other primates[4, 9, 20, 89]. A lower voltage is desirable for animal welfare since pain and heat injury are adverse effects of EE[29]. 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[19, 38, 50, 61, 63, 80]. 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[19, 63]. 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 affected by increase of sperm metabolic rate at 37˚C[80]. 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 two cooling methods, VSL and ALH were higher when semen were cooled quickly (20 min) after the second dilution, which is advantageous to reaching destination. Furthermore, fast cooling after the second

dilution improved the progressive motile sperm rate at immediately before LN_2. 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[75, 84]. Martorana et al.[41] improved post thawed sperm motility by shortening the duration of cooling to 20 min from 2 hr in a previous report[75]. Therefore, both the order of the second dilution and a cooling rate above 0˚C contributed to the improvement. In previous study, highly motile sperm were selected by using density gradient centrifugation for cryopreservation[84]. On the other hands, cryopreservation media (TTE) were used for the first dilution and washing to make the cryopreservation more convenient in the present study. In this convenient method, I found that fast cooling improved the progressive motile rate during cryopreservation. However, the progressive motile sperm rate and results of CASA and fluorescence staining suggested quality of post thawed sperm was quite low and contribution to fertilization in AI could not be expected[30, 48, 68, 83]. It was reported that sperm motility decrease after coagulation and liquefaction[42], these may cause low quality of post thawed sperm in this study. Since liquefaction can be omitted in liquid form semen collected by EE-UC, more investigation about cryopreservation using EE-UC semen may affect post thawed sperm quality.

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[42, 84] is sufficient for EE in the Japanese macaque but 15 V can maximize semen collection rate. 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[86], 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 progressive motile sperm rate after holding in two temperatures (15 and 37˚C) were not significantly different from 25˚C which used in present study for cryopreservation, their

effect on cryopreservation better to be investigated in further study.

Summary

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 coagulate 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 thawed 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 maximize sperm collection rate. Also, liquid semen was collected by EE-UC and this could increase the total number of sperm. Further, to improve the post thawed 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). Though holding semen at 25˚C and fast cooling after the second dilution increased progressive motile sperm rate, the ranges of increase were small and post-thawed motility was not sufficient for AI. The present results will contribute to the improvement of semen collection and animal welfare of captive Japanese macaques.

Tables and Figures

Table I-1. Semen collection patterns and study groups.

Semen collection pattern	Study group		
	UC (n = 9)	EE (n = 49)	EE-UC (n = 44)
		Low (n = 15)	Low (n = 14)
		High (n = 34)	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 I-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 (analyzed by the Fisher's exact test, $P < 0.05$).

* Values with superscripts differ significantly between low and high voltage groups in each procedure group (analyzed by the Fisher's exact test, $P < 0.05$).

Values of total sperm number and progressive motile sperm rate are shown as the median (range).

Table I-3. Characteristics of post thawed spermatozoa cryopreserved by different cooling procedures evaluated by the CASA system.

Cooling procedure group	VSL (μm/sec)	VCL (μm/sec)	VAP (μm/sec)	ALH (μm)	BCF (Hz)	LI (%)
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)

CASA: Computer Assisted Sperm Analysis

VSL: straight line velocity, VCL: curvilinear velocity, VAP: average path velocity,

ALH: amplitude of lateral head displacement, BCF: beat-cross frequency, LI: 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 (analyzed by the Student's *t*-test, $P < 0.05$).

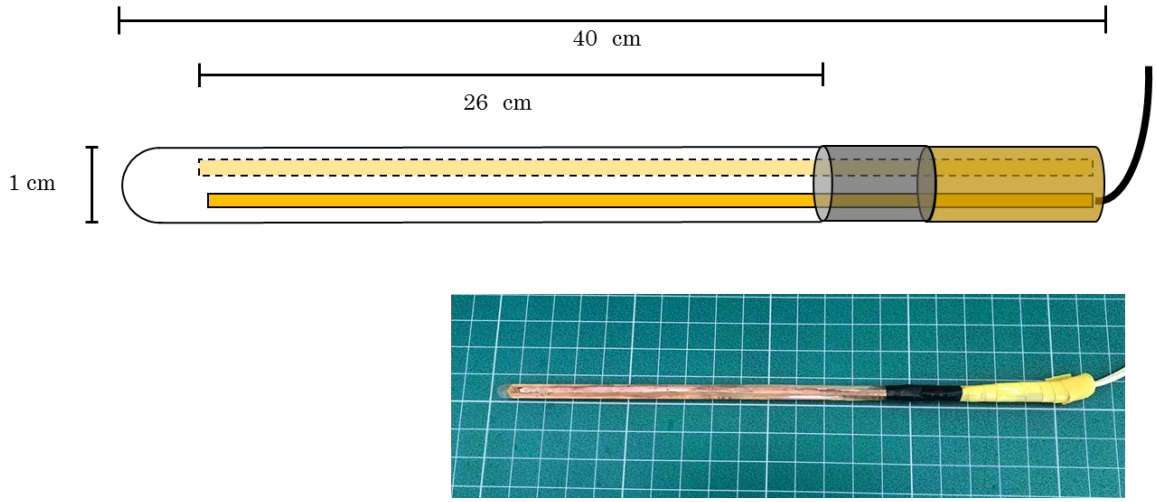
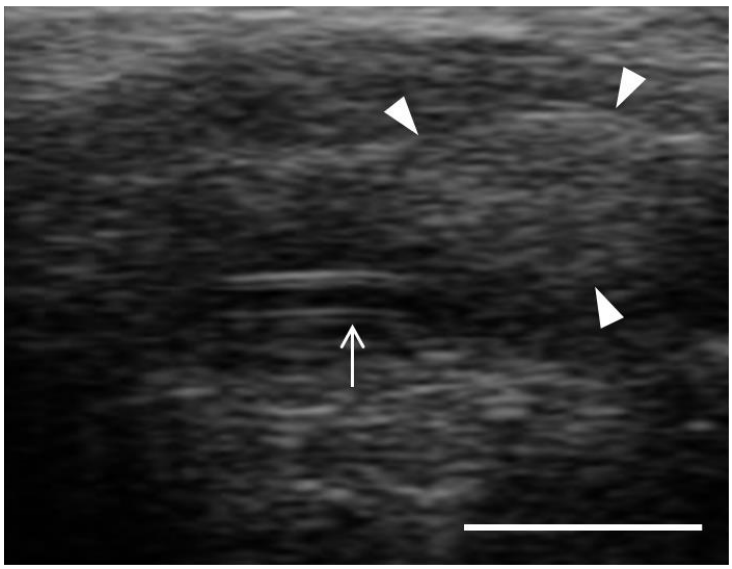


Fig. I-1. Rectal probe with two longitudinal copper electrodes on both sides. Grip is insulated with plastic tape.

Total length: 40 cm, Diameter: 1 cm, Electrode length: 26 cm, Electrode width: 0.4 cm

A



B

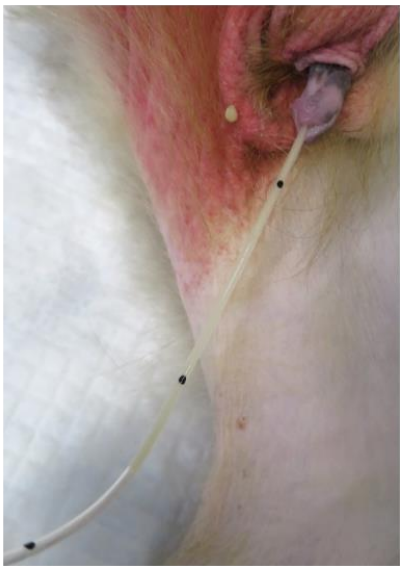


Fig. I-2. Semen collection by urethral catheterization.

A: Sagittal ultrasound image of urethral catheterization. The tip of the catheter (arrow) was inserted until it approached the prostate (arrowheads). Left side is caudal. Bar = 1cm

B: Liquid form semen collected in the catheter removed from the urethra

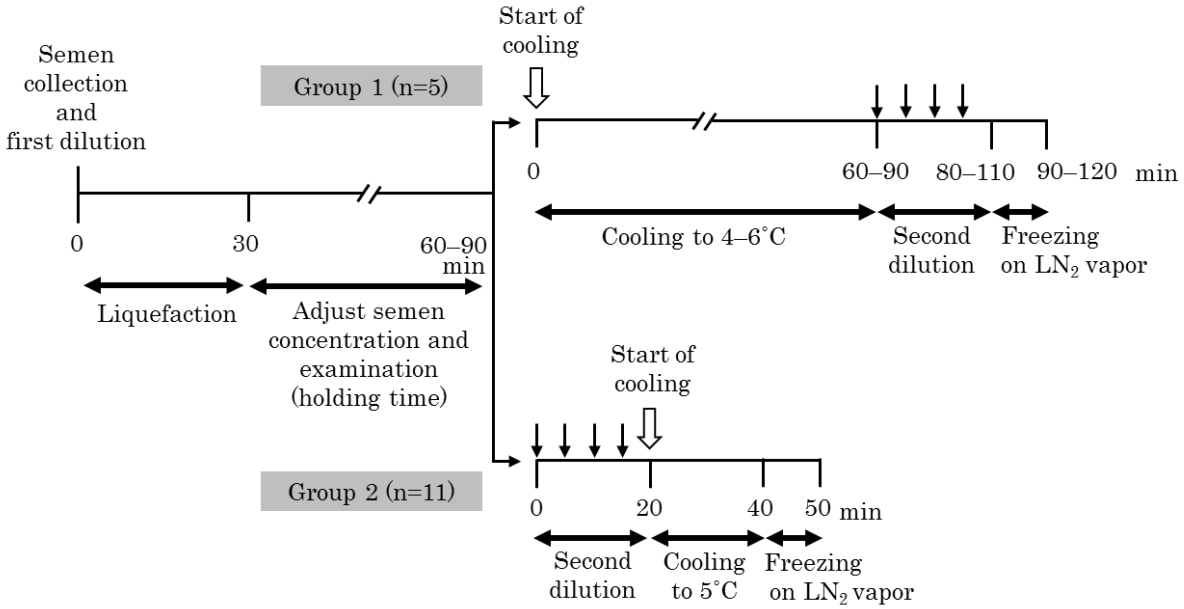


Fig. I-3. Schedule of semen processing from collection to cryopreservation. Semen were cryopreserved in two procedures (Groups 1 and 2). Liquefaction was omitted for semen samples collected in liquid form.

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

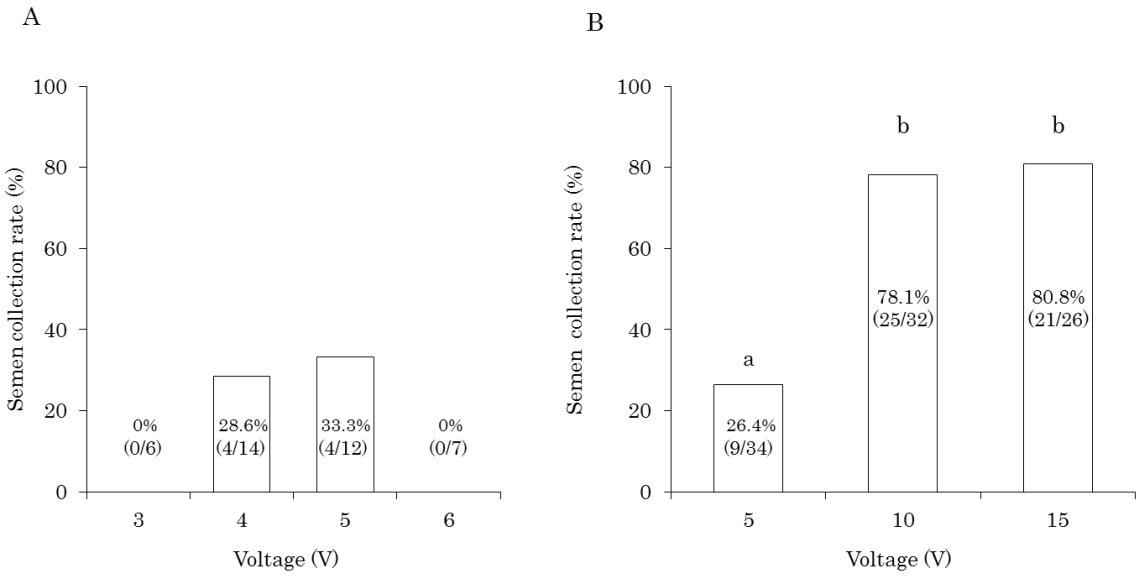


Fig. I-4. Semen collection rate at each voltage in the two different voltage groups

A: Low voltage group

B: High voltage group

[a, b]: Bars with different letters significantly differ (the $P < 0.05$, the Fisher's exact test)

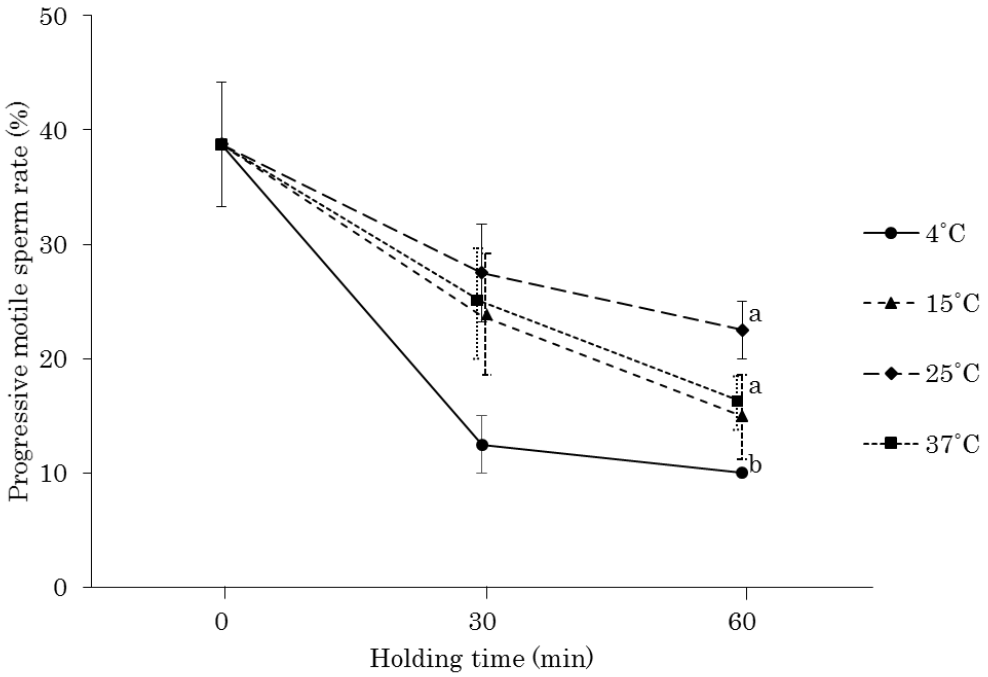


Fig. I-5. Changes in the progressive motile sperm rate in four different holding temperature conditions

[a, b]: Values with different letters tended to be different between temperature at same holding time ($P < 0.10$, the Steel-Dwass test)

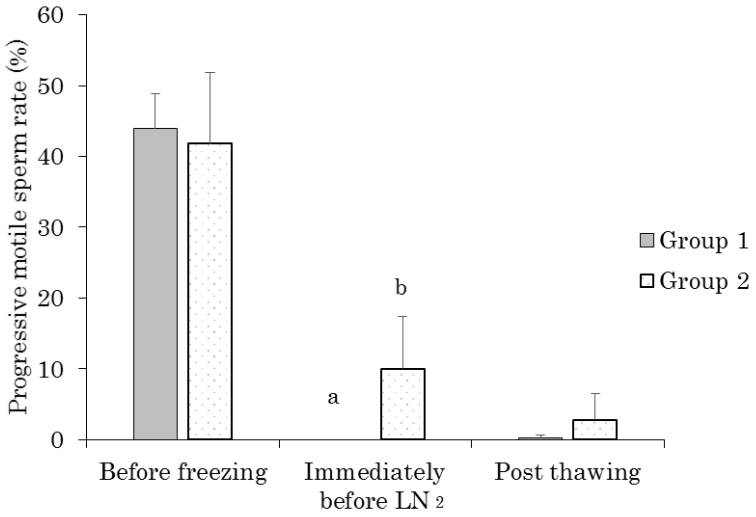


Fig. I-6. Progressive motile sperm rate between different cooling procedures during cryopreservation.

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 LN_2: Just before freezing with liquid nitrogen

[a, b]: Values with different letters differ significantly between cooling groups within each process ($P < 0.05$, the Wilcoxon's rank sum exact test)

CHAPTER 2

Ovulatory follicle size investigated by ultrasonography and single artificial insemination with ovulation induction in the Japanese macaque

Introduction

The Japanese macaque (*Macaca fuscata*) is a popular zoo animal and is usually kept as a troop including both males and females in order to imitate their natural habitat. However, their high fertility and mixed sex troop often result in overpopulation[3, 32]. Poor genetic diversity is also a common problem due to repeated reproduction in a closed colony of the Japanese macaque. For reproductive management, artificial insemination (AI) is a common technique in domestic animals[45] and it has been applied to zoo animals including primates[2, 39] to limited extent. Therefore, reproduction management applying AI should be evaluated in the Japanese macaque.

Successful AI has been reported in the Japanese macaque[86]. Laparoscopic ovarian observation and AI under anesthesia were repeated several times until ovulation[86], but this protocol is not suitable for practical and routine use in zoos due to its invasiveness. In one study, animals conceived following at least two successive AIs on the day before and on the day of ovulation[86]. Further, a single AI on the expected ovulation day[74] resulted in a pregnancy in the cynomolgus macaque (*Macaca fascicularis*). Therefore, to establish a single AI protocol, detection of timing of ovulation is important. The menstrual cycle is 25.3 ± 2.8 days[54] and ovulation timing ranges from Day 11 to 16 of the menstrual cycle[55] which can be estimated by monitoring plasma estradiol or urinal estrogen metabolites in the Japanese macaque[16]. However, repeated sample collection and measurement to monitor these hormones are difficult in zoos, where they are kept in troops. Therefore, AI in combination with hormonal treatment for ovulation induction could lead to a single AI protocol for the Japanese macaque.

For ovulation induction, information about the ovarian dynamics, including the size of ovulatory follicles and the time it takes the follicle to reach a possible ovulation size is needed. Nigi (1977)[54] reported morphological changes in follicles during the

course of ovulation by laparoscope in the Japanese macaque, but the sizes of ovulatory follicles were not examined. Ultrasonography is commonly used to investigate ovarian dynamics during the estrous cycle in domestic animals[64, 66], but it has never been performed in the Japanese macaque. In non-human primates, ultrasonographic observation of ovaries has been reported in some species to monitor their response to exogenous gonadotropin[7, 77, 93], but not in the Japanese macaque. Ovarian dynamics during the menstrual cycle have been investigated in the rhesus macaque (*Macaca mulatta*)[7, 46] and the cynomolgus macaque[90]. The mean diameter of ovulatory follicles before ovulation was about 6–7 mm and the follicular phase was about 14 days during a spontaneous cycle in rhesus and cynomolgus macaques. Since their menstrual cycle time and mean litter size (one) are almost the same with the Japanese macaque, the ovulatory follicle size might be similar to the Japanese macaque, but there may be differences because of their different body sizes (the Japanese macaque is larger than rhesus and cynomolgus macaques).

Ovulation induction using human chorionic gonadotropin (hCG) was previously reported in the Japanese macaque during the non-mating season under complete exogenous hormonal control of follicle development[85]. However, repeated use of hCG is known to produce antibodies, even in humans[1, 8, 76, 79], which can neutralize the hCG and prevent the ovulatory effect. Furthermore, acquiring anti-hCG antibodies may affect maternal physiology, since macaque CG cross reacts with anti-hCG antibodies[49]. On the other hand, gonadotropin releasing hormone (GnRH) has no antigenicity[6] and is used for ovulation induction in various domestic animals[12] without the detrimental effect of hCG. Further, the structure of GnRH is common among mammals[44, 73]. Therefore, the ovulatory effect of GnRH and the sensitive follicle size should be investigated in the Japanese macaque.

In the present study, to establish an AI protocol in the Japanese macaque, ovarian dynamics during the menstrual cycle were investigated by ultrasonography together with changes in ovarian steroid hormones. Further, the ovulatory effect of GnRH was evaluated, and a single AI was performed simultaneously.

Materials and methods

Animals

Six female (5–11 years old) and four male (11–17 years old) Japanese macaques (*Macaca fuscata*) kept at the Primate Research Institute, Kyoto University (Inuyama, Aichi 484-8506, Japan), were used in the present study. 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 supplemental food (sweet potatoes, bananas, apples and peanuts). Water was supplied *ad libitum*. Estrus behavior of monkeys was observed for 15 min twice a day (at 9:00 and 15:00). The start of a menstrual period was defined as a day when any menstrual blood spotted on the floor or inside the cage was observed and this day was determined to be Day 1 of menstrual cycle. All experiments were approved by the Animal Welfare and Animal Care Committee, Primate Research Institute, Kyoto University (No. 2013-089, 2015-037, 2016-013, 2017-047).

Ultrasonography of the ovaries and definition of ovulation

The ovaries were examined using an ultrasonography device (Prosound SSD-3500SX, Hitachi Aloka Medical, Ltd., Tokyo, Japan) attached to a convex transducer (UST-987-7.5, Hitachi Aloka Medical, Ltd.) via the abdominal wall without anesthesia. The monkey was restrained by a squeeze cage in the sitting position. The movies of the ovary were recorded on a PC attached to the ultrasonography device using image analyzing software (CyberLink PowerDirecter 10, CyberLink Corp., Tokyo, Japan). The number and size (mm) of follicles and corpora lutea (CLs), and their relative positions in the ovaries were recorded (Fig. II-1). The dominant follicle was defined as a follicle that reached the maximum diameter during the follicular phase. Ovulation was defined as the disappearance of the dominant follicle or a distinct reduction in the diameter of the dominant follicle. The day of ovulation was defined as the next day of the last observation of the dominant follicle before ovulation confirmation. Diameter of follicles are shown as the mean ± standard deviation (SD) (range).

Sample collection

Peripheral blood was collected from the cephalic vein using a syringe containing 15 μl/ml heparin. Collected blood was centrifuged at 1,100 × *g* for 20 min at 4˚C and the plasma was stored at -30˚C until assays. Feces were collected by placing a tray under the individual cage around 16:00 and feces on the tray were sampled at 9:00 the next day and stored at -30˚C.

Steroid hormone assay

Steroid hormone in plasma was extracted with diethyl ether and reconstituted with a buffer[92]. The feces were prepared as described previously[33] with slight modification. Briefly, the feces were dried in a drying oven at 100˚C for 24 hr and pulverized. Then, 0.1 g of the fecal powder was vortexed for 30 min in 5 ml of 80% methanol at room temperature and centrifuged at 4˚C and 1190 × *g* for 10 min to collect the supernatant used as the assay sample. Plasma and fecal concentrations of estradiol-17β (E_2) and progesterone (P_4) were determined using competitive double antibody enzyme immunoassays as previously described[92] with slight modification. Goat anti-rabbit serum (111-005-003, Jackson Immuno Research, Pennsylvania, USA), anti E_2 antibody (QF-121, Teikoku Hormone Mfg. Co., Ltd., Kanagawa, Japan), anti P_4 antibody (KZ-HS-P13, Cosmo Bio, Tokyo, Japan) and steroid hormone labeled by horseradish peroxidase were used. The intra and inter assay coefficients of variation were 4.0% and 5.1% for E_2 and 5.8 and 8.6% for P_4, respectively. The plasma hormone concentration was recorded as the quantity per 1 ml of plasma and fecal hormone was recorded as the quantity per 1 g of dried feces.

Anesthesia

Monkeys were anesthetized with a combination of ketamine hydrochloride (5 mg/kg Ketamine Injection 5% Fujita, Fujita Pharmaceutical Co., Ltd., Tokyo, Japan), medetomidine hydrochloride (25 μg/kg, Medetomine Injection Meiji, Fujita Pharmaceutical Co., Ltd., Tokyo, Japan), and midazolam (125 μg/kg, Midazolam Injection 10 mg Sandoz, Sandoz K. K., Tokyo, Japan). All the drugs for anesthesia were administered intramuscularly. After the examination, 0.0625 mg/kg atipamezole

(Mepatia injection Meiji, Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) was administered intramuscularly to antagonize the effects of medetomidine hydrochloride.

Semen collection and evaluation

Semen collection was carried out via electro-ejaculation in same method as in Chapter 1. 5–10–15 V of voltage series was used. After the collection, an equivalent volume of Tes-Tris Egg-yolk medium (TTE) to semen was added[37, 69]. In one case of semen collection (for AI in ID 2068 on January 15th, 2016), the remaining semen in the urethra after ejaculation was collected by inserting a catheter after electro ejaculation in same method as in Chapter 1 of urethral catheterization (UC). Liquid form semen in the catheter was expelled by flushing with 0.2 ml of TTE medium.

The semen fractions from all males collected on the same day were pooled and centrifuged at 500 × g. After removing most of the supernatant and coagulum, the sperm pellet was resuspended with a small amount of TTE medium and transferred to a 1.5 ml test tube. Total semen volume, sperm concentration, total sperm number and progressive motile sperm rate were examined in the same method as in Chapter 1. The semen were kept at 25˚C in a water bath for up to 1 hr until insemination.

Artificial insemination (AI)

The immobilized females were laid in an abdominal position and the peri-vaginal area was sterilized. Visualizing the external uterine orifice using a vaginal speculum, the tip of a custom-ordered 120 mm long stainless animal feeding needle, with a round tip of 0.9 mm in diameter was inserted into the uterine cavity with manipulation of the cervix by finger rectal palpation. Then, a syringe filled with diluted semen was connected to the needle and 100–150 μl of semen was inseminated. If insertion into the uterus was impossible due to a complex cervical structure, semen was inseminated into the cervical canal.

Study design

1. Ovarian and hormonal dynamics during the menstrual cycle and follicle size at

spontaneous ovulation

Two female monkeys were used to monitor ovarian and hormonal dynamics during the menstrual cycle (Table II-1). For 38 days from early November to the middle of December, ultrasonographic observation of ovaries and blood collection were done three times a week at one- or two-day intervals. During this period, feces were collected every day. Feces were also collected five days a week from the middle of October to early November prior to the ultrasonographic examination period. Further, changes in ovaries of three female monkeys were monitored from Day 9 to 16 of the menstrual cycle by ultrasonography with a zero to two days interval to confirm the ovulatory follicle diameter.

2. Ovulation induction by GnRH

Two female monkeys were used in three ovulation induction trials using GnRH (Table II-1). As shown in Table II-2, ultrasonographic ovarian observation and blood collection were performed daily or at intervals of one or two days from Day 7 to Day 16 of the menstrual cycle. Feces were collected daily during this study period for each monkey. A single dose (2 ml) of a GnRH analogue (100 μg as fertirelin acetate, Conceral injection, Nagase Medicals Co., Ltd., Hyogo, Japan) was administered intramuscularly from Day 10 to 13 of the menstrual cycle (Table II-2).

3. Artificial insemination (AI) with ovulation induction

A total of seven AI trials were performed for four female monkeys (Table II-1 and II-3). Ultrasonographic observation of ovaries was performed to confirm the presence of a dominant follicle of more than 5 mm in diameter prior to AI. A mixture of fresh semen collected from one to three monkeys on each AI day was used for insemination. Females were subjected to AI on Day 8–14 of the menstrual cycle and a single dose (2 ml) of GnRH was administered simultaneously for ovulation induction (Table II-3). The dominant follicle was observed on the day following AI using ultrasonography without anesthesia to confirm the ovulation day. Pregnancy was diagnosed by ultrasonography in monkeys in which menses was not observed after AI.

Results

1. Ovarian and hormonal dynamics during the menstrual cycle and follicle size at spontaneous ovulation

Ovarian and hormonal dynamics in two females monitored for changes during menstrual cycle are shown in Fig. II-2. One female showed two ovulations and another showed one. The inter ovulation interval of one female was 29 days with a 17-day luteal phase and a 12-day follicular phase. The plasma E_2 concentration showed high value one to three days before ovulation day (406–894 pg/ml). Although fecal E_2 showed a peak at ovulation in female No. 2140, this was uncertain in female No.2068. The plasma P_4 concentration increased to over 1 ng/ml at one to three days after the ovulation day and decreased sharply just before the first day of menses. Fecal P_4 concentration showed similar changes to plasma P_4 concentration. Before the first ovulation in each animal, there was no increase in fecal P_4 concentration. The dominant follicle of one monkey (ID 2068) was in preovulatory stage at the first observation and ovulated by examination two days later. Dominant follicle was detectable four days after the start of menstruation and development of the diameter (3.4–9.6 mm) were observed for 10 days. CLs were detectable by ultrasonography two to four days after ovulation. Decrease of the diameter of CL (9.5–6.1 mm) was observed two to four days before menses (ID 2068). Table II-2 shows changes in ovarian structure, including those of three females monitored at spontaneous ovulation as well as those monitored during the menstrual cycle. Spontaneous ovulation occurred from Day 12 to 14 of the menstrual cycle in four monkeys and the follicle diameter immediately before ovulation was 7.4 ± 1.5 mm (5.1–9.6 mm: in six ovulations).

2. Ovulation induction by GnRH

Changes in ovarian structure related to GnRH administration are shown in Table II-2. In all three trials, a dominant follicle was ovulated on the day after GnRH administration. The follicle diameter on the GnRH administration day was 5.8 ± 1.2 mm (4.7–7.4 mm: in three cases). CLs were detectable within one to three days after

ovulation in two cases.

Hormonal and ovarian dynamics of ovulation induced monkeys are shown in Fig. II-3. Plasma E_2 concentration increased in two out of three cases on the day of GnRH administration. In the other case, plasma E_2 concentration increased slightly after GnRH administration. Meanwhile, fecal E_2 concentration increased by GnRH administration in all three cases. Plasma P_4 concentration increased within three days after ovulation in two cases, but it did not increase at two days after ovulation, which was last day of monitoring. Fecal P_4 concentration dynamics were similar to those of plasma in two cases (2068, 2140-2), but not in one case (2140-1).

3. Artificial insemination (AI) with ovulation induction

Semen characteristics of monkeys inseminated via AI are shown in Table II-3. Over 14×10^6 progressive motile sperm were inseminated in each AI. The mean progressive motile sperm number was $49.0 \pm 26.8 \times 10^6$ ($14.8–225.0 \times 10^6$ sperms). Only one case that inseminated to the uterus became pregnant. At 71 days after AI, a fetus was confirmed by ultrasonography (Fig. II-4). However, there was no fetal heart beat and the fetal age was estimated to be 50 days old according to the biparietal diameter[57].

Discussion

This is the first study to monitor ovarian dynamics by ultrasonography in the Japanese macaque. The age of female monkeys in this study were ranged 5–11 years old, in which monkeys were sufficiently matured and not degraded by aging[56, 59]. During the breeding season, spontaneous ovulations were observed total of six times in four females, and the diameter of the dominant follicle just before ovulation was 7.4 ± 1.5 mm. This size was larger than the preovulatory dominant follicle in the rhesus macaque (5.9 ± 1.8 mm[7] , 5.3 ± 0.4 mm[46]) and the cynomolgus macaque (6.8 ± 0.1 mm)[90], also measured by ultrasonography. The maximum diameter of the dominant follicle was 9.6 mm in this study, while that of the rhesus macaque was 7 mm[46]. It seems that these differences occur due to the body size differences between the Japanese macaque (around 8 kg) and the other two species (around 5 kg). On the other hand, the minimum size of the

preovulatory follicle was 5.1 mm. This size was determined by observation with two days interval, which means ovulation could take place after follicular development during this interval. Therefore, follicle size of 5.1 mm could be a spontaneous preovulatory follicle size, further study is necessary to conclude the minimum size of preovulatory follicle. Spontaneous ovulation occurred between on Day 12 to 14 of the menstrual cycle, which was in the range of a previous study in the Japanese macaque[54].

Similar to a previous study that monitored the dynamics of steroid hormone in the Japanese macaque[55], the E_2 concentration reached a peak before ovulation and the P_4 concentration increased after ovulation. As in the previous report[16], plasma and fecal hormones showed similar dynamics during the menstrual cycle. Fecal E_2 concentration reached a peak on the day after the plasma E_2 peak. Ovulation occurred on the next day or three days after the plasma E_2 peak, while it occurred the same day or the day following the fecal E_2 peak. This was the same as a previous report showing that ovulation occurred within two days after the plasma E_2 peak[55]. In the present study, we could show the stability of monitoring steroid hormone dynamics, even the measuring methods and the period is different. Further, ovarian dynamics first time monitored by ultrasonography in Japanese macaque showed that the hormonal dynamics truly reflect the ovarian dynamics in this species.

In this study, fertirelin acetate was used as GnRH analogue. The structure of GnRH is common in mammals[44, 73], and fertirelin acetate sufficiently induces secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in cows[11]. Therefore, fertirelin acetate is competent in inducing ovulation in the Japanese macaque. In all cases of GnRH administration, ovulation was confirmed on the following day in the present study. Nigi and Torii (1991)[58] reported that ovulation occurs within 48 h after LH peak in the Japanese macaque, and within 3 hr at the earliest. GnRH could induce the ovulation of follicles of more than 4.7 mm in diameter. In the rhesus macaque, dominant follicle selection is completed by Day 3 of the follicular phase when the diameter is around 3 mm[7], which means the LH receptor is already expressed. In the present study, the smallest dominant follicle detectable by ultrasonography was 3.9 mm on Day 7 of the menstrual cycle. Therefore, the dominant follicle was probably selected

by that period and the follicle size indicates the presence of the LH receptor. Further, this suggests that a greater than 5 mm diameter follicle is sufficient to react to the LH surge induced by GnRH administration, followed by ovulation in practical settings. In the three subjects monitored for both ovarian and hormonal dynamics during ovulation induction, the plasma E_2 concentrations were high around the day of ovulation induction and it was similar in fecal hormone dynamics in the two subjects. Meanwhile, the plasma E_2 concentration was at the baseline level in the other subject and this was the opposite of fecal dynamics. One possible reason is that plasma E_2 concentration had already increased before the start of monitoring and spontaneous ovulation occurred, although a reduction in follicle size was only detected after GnRH injection as with ovulation. The E_2 peak to LH peak interval was reported to be 20 to 30 hr[58], so the maximum estimated interval from the E_2 peak to ovulation is about 78 h. Therefore, in this case, plasma E_2 concentration already increased before monitoring and we could only detect it by a fecal hormone assay due to a delay in the plasma dynamics; spontaneous ovulation had already taken place at the time of ovulation induction.

In three cases of AI on 2018/3/7 (Table II-3), semen mixture contained sperm collected from a male which is derived from same troop with recipient females. Since it was reported that reproduction in closed colony had been smoothly repeated[3], AI between males and females from same troop did not affect the fecundity. In the present study, semen was inseminated once with ovulation induction with over 14.0×10^6 progressive motile sperm. In a previous report on AI in the Japanese macaque, semen was inseminated two to three times per ovulation and $5.0–10.0 \times 10^6$ sperm was inseminated each time[86]. The pregnancy rate in the present study was 14.3% (1/7), while it was 50% (3/6) in the previous report[86]. Although the pregnancy rate was low, a single AI with ovulation induction was shown to be feasible to achieve pregnancy. The reason for the low pregnancy rate may be due to the site of insemination. The pregnancy rate of intracervical insemination of fresh semen was quite low in the Japanese macaque (0.0%: 0/3) and the rhesus macaque (33.3%: 1/3) compared to intrauterine insemination (100.0%: 3/3 and 57.1%: 8/14, respectively)[68, 86]. In macaque, the cervical canal is serpentine[21], so trans-cervical access to the uterus is difficult, especially in nulliparous

females. Therefore, I must try another method to inseminate into the uterus, such as transabdominal insemination[74] or retaining semen in the vagina using a special device[34].

Although semen was inseminated into the uterus, females did not become pregnant in three out of four cases. One of the possible reasons for this is timing of ovulation induction, since two cases were several days earlier considering the range of spontaneous ovulation (Day 10 to 14 of the cycle), even though the dominant follicles were ovulatory size. In the rhesus macaque, a follicle of 6 mm in diameter still showed enhanced growth with FSH and this improved the meiotic and developmental competence of oocytes[71]. For *in vitro* embryo production in the rhesus macaque, FSH priming before hCG administration enhances meiotic and developmental competence[72]. Therefore, a gap of several days between the spontaneous ovulation and ovulation induction by GnRH administration in the present study may have impaired oocyte potential by depriving them of a few days of exposure to FSH. Since studies about oocyte competence usually focus on gonadotropin-induced situations, further study investigating the spontaneous cycle is needed to clarify this issue. Further, the diameter of follicles at AI ranged 6.0–10.0 mm in present study. Although there is no previous study indicates preovulatory follicle size and developmental competence of oocytes inside the follicle in non-human primates, it was suggested that small preovulatory follicle size at GnRH administration decreased pregnancy rate in cattle[62]. Therefore, the effect of follicle size on fertility also should be taken into the account in further study.

Though repeated AI might increase the pregnancy rate, single AI was adopted in the present study. Considering practical use in zoos, minimizing the isolation duration of monkeys from their troop for AI is required, since even a few days of isolation can cause the change of hierarchy in the troop and struggle over the hierarchy whichever the isolated individual is male or female[3]. Therefore single AI should have been evaluated to minimize the risk of getting injuries caused by isolation.

In the present study, the ovulatory size of the dominant follicle during the spontaneous cycle was estimated to be 7.4 ± 1.5 mm in the Japanese macaque. Further, we found that a follicle size over 5 mm was enough to induce ovulation by GnRH administration. When we perform AI with ovulation induction, it should be done within

the period of spontaneous ovulation in the presence of an ovulatory size follicle along with intrauterine insemination.

Summary

In the Japanese macaque, artificial insemination (AI) has been successful, but it required repeated AI procedures two to three times a day under anesthesia until ovulation was confirmed by laparoscopy. To reduce this invasiveness, the ovulation timing and size of preovulatory follicle need to be elucidated. However, ovulatory follicle size is not well understood as monitoring ovarian dynamics using ultrasonography has never been performed in this species. Further, although ovulation induction facilitates establishing the AI protocol, the effect of gonadotropin releasing hormone (GnRH) on ovulation induction has not been studied. Monitoring of ovarian dynamics by ultrasonography of six spontaneous ovulations clarified that the diameter of the ovulatory follicle before ovulation was 7.4 ± 1.5 (5.1–9.6 mm) and that ovulation occurred from Day 12 to 14 of the menstrual cycle. The effect of GnRH on ovulation induction was monitored in three monkeys, and one day after GnRH administration ovulations were induced with follicles of diameter of 4.7–7.4 mm. Further, AI with ovulation induction by GnRH was performed seven times using four females. Semen were inseminated in the uterus in four cases and into the cervix in three between Day 8 to 14 of the menstrual cycle. Only one female inseminated in the uterus on Day 12 of the menstrual cycle became pregnant, but fetal development stopped at around 50 days of gestation. The present study investigated a less invasive single AI protocol with ovulation induction in the Japanese macaque, and the insemination technique needs to be developed further to improve the pregnancy rate.

Tables and Figures

Table II-1. Information on female monkeys and study items used.

Female	Breeding season	Age at research	Parity	Study purpose			
				Menstrual cycle	Spontaneous ovulation	Ovulation induction	Artificial insemination
2068	2013-2014	8	parous	○		○	
	2015-2016	10	parous				○*
	2016-2017	11	parous				○
2099	2013-2014	9	nulliparous		○		
2140	2013-2014	8	nulliparous	○	○	○*	
2249	2013-2014	5	nulliparous		○		
	2016-2017	8	nulliparous				○
	2017-2018	9	nulliparous				○
2366	2017-2018	7	nulliparous				○
2408	2017-2018	7	parous				○

○: Animal has used to investigate study items

*: Trials were performed twice during different menstrual cycles

If an animal was used for different purpose in the same breeding season, each test was performed at different times.

Table II-2. Day of ultrasonographic observation and diameter of follicles in ovaries during spontaneous and induced ovulation

Study purpose	Female	Breeding season	Day from menses										Diameter of follicle immediately before ovulation†
			7	8	9	10	11	12	13	14	15	16	
Menstrual cycle	2068	2013-2014											7.9‡
	2068	2013-2014			6.1		3.9		9.6			8.7	
	2140	2013-2014											5.8‡
Spontaneous ovulation	2140	2013-2014						8.5	ND			8.2	
	2249	2013-2014					5.1			ND		8.2	
	2099	2013-2014						7.6		ND	8.3		
Ovulation induction	2068	2013-2014	3.9	4.8	6.1	7.4*	6.1			8.7			
	2140	2013-2014					4.7	4.7*	4.0	5.6			
	2140	2013-2014		3.0		3.9			5.2*	3.5			
Artificial insemination	2068	2015-2016		6.0*,#	ND								
	2068	2015-2016			7.7*,#	5.0							
	2068	2016-2017						7.3*,#	ND				
	2249	2016-2017						7.6*,#	ND				
	2249	2017-2018								8.0*,#	ND		
	2366	2017-2018							10.0*,#	5.5			
	2408	2017-2018					10.0*,#	6.2					

The menstrual days with numbers or letters are days of ultrasonographic observation, while the number indicates the diameter (mm) of follicle or corpus luteum.

Shaded day indicates day of corpus luteum detection.

The day from menses with boxes indicate the estimated day of ovulation.

ND: No apparent structure was detected in ovaries.

*: Day of gonadotropin releasing hormone administration.

#: Day of artificial insemination.

†: No previous menses was observed as it was the first ovulation of the breeding season.

‡: The follicle size on the day preceding ovulation.

Table II-3. Condition of females on artificial insemination (AI) day, semen preparation for AI and the results

Female	Date of AI	Semen preparation for AI				Condition of female and AI procedure			Result
		Numbers of donor males	Semen concentrations ($\times 10^6$/ml)	Progressive motile sperm count ($\times 10^6$)	Progressive motile sperm (%)	Day of menstrual cycle at AI	Diameter of dominant follicle (mm)	Insemination site	
2068	2015/11/19	2	151	14.8	32	8	6.0	uterus	not pregnant
	2016/1/15	1*	210	61.0	55	9	7.7	uterus	not pregnant
	2017/2/6	2	900	27.0	30	12	7.3	uterus	pregnant
2249	2017/3/16	2	332	14.9	30	12	7.6	uterus	not pregnant
	2018/3/7	3	3000	225.0	15	14	8.0	cervix	not pregnant
2366	2018/3/7	3	3000	225.0	15	13	10.0	cervix	not pregnant
2408	2018/3/7	3	3000	225.0	15	11	10.0	cervix	not pregnant

Dominant follicle: The follicle of maximum size on AI day.

*: Semen was collected by electro-ejaculation and catheterization.

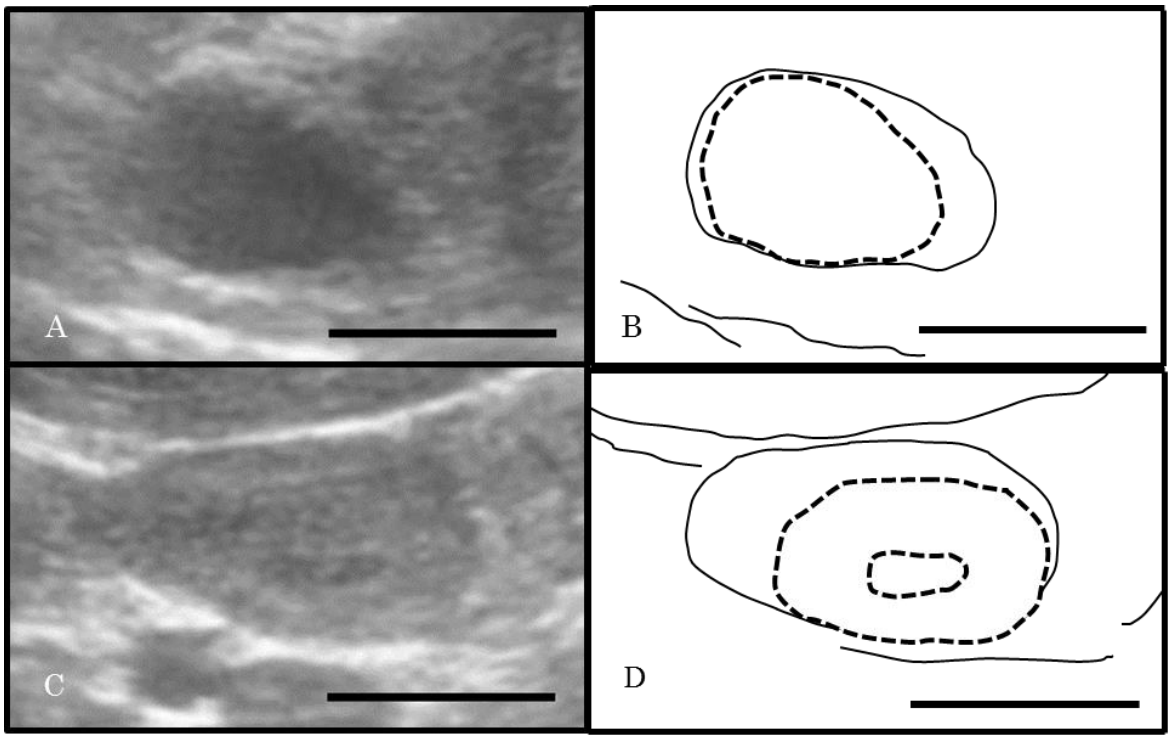


Fig. II-1. (A) an ultrasonographic image and (B) an illustration of an ovary with a dominant follicle at one day before ovulation. The diameter of follicle was 9.6 mm. (C) an ultrasonographic image and (D) an illustration of an ovary with a corpus luteum (CL) at six days after ovulation. The diameter of CL was 9.5 mm. Dotted line indicates structure (follicle or CL) in the ovary. bar = 10 mm

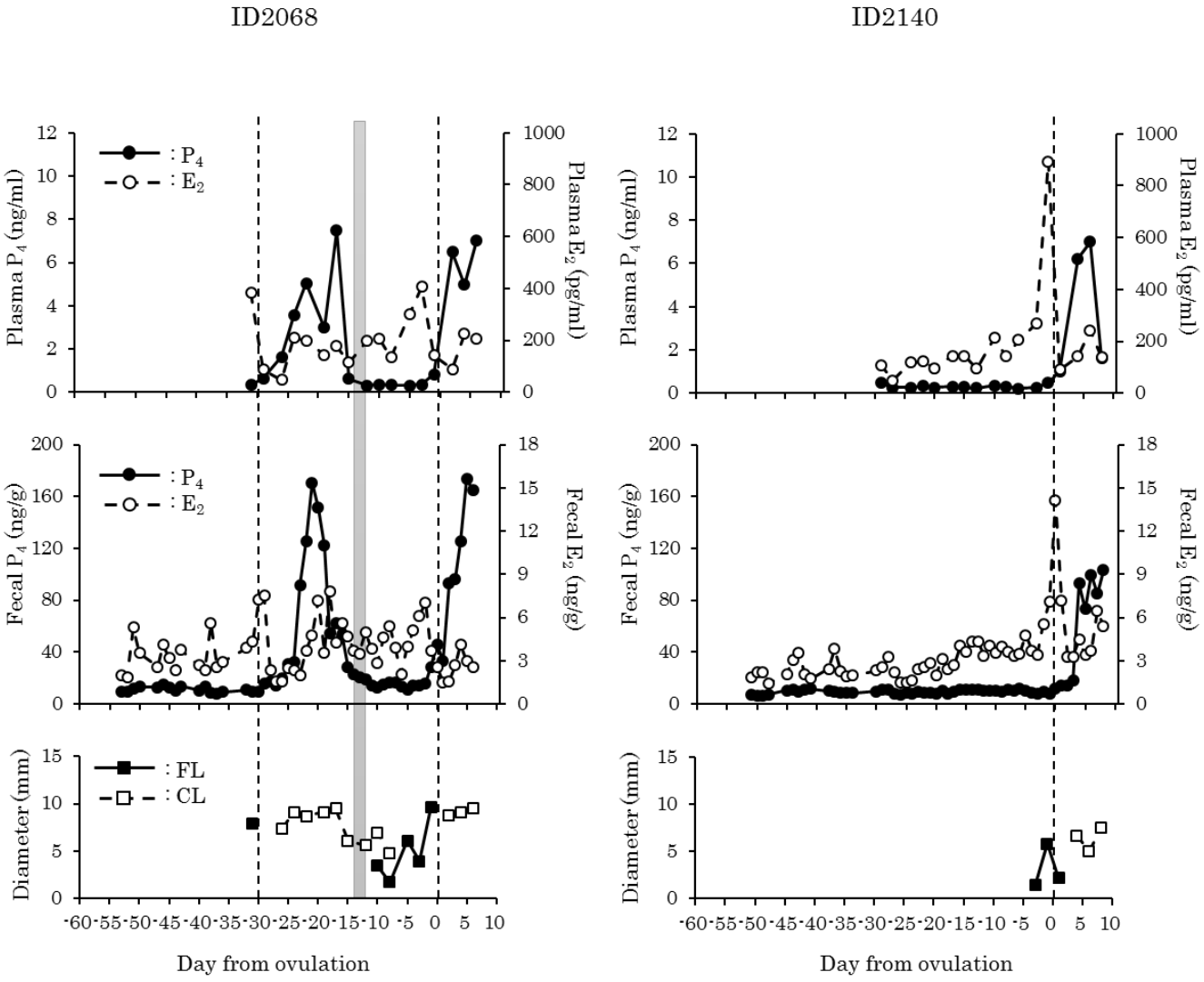


Fig. II-2. Hormonal and ovarian dynamics of female monkeys with menstrual cycle monitoring.

Broken line indicates ovulation day. Shaded box indicates menses.

P_4: Progesterone, E_2: Estradiol-17β, FL: Follicle, CL: Corpus luteum

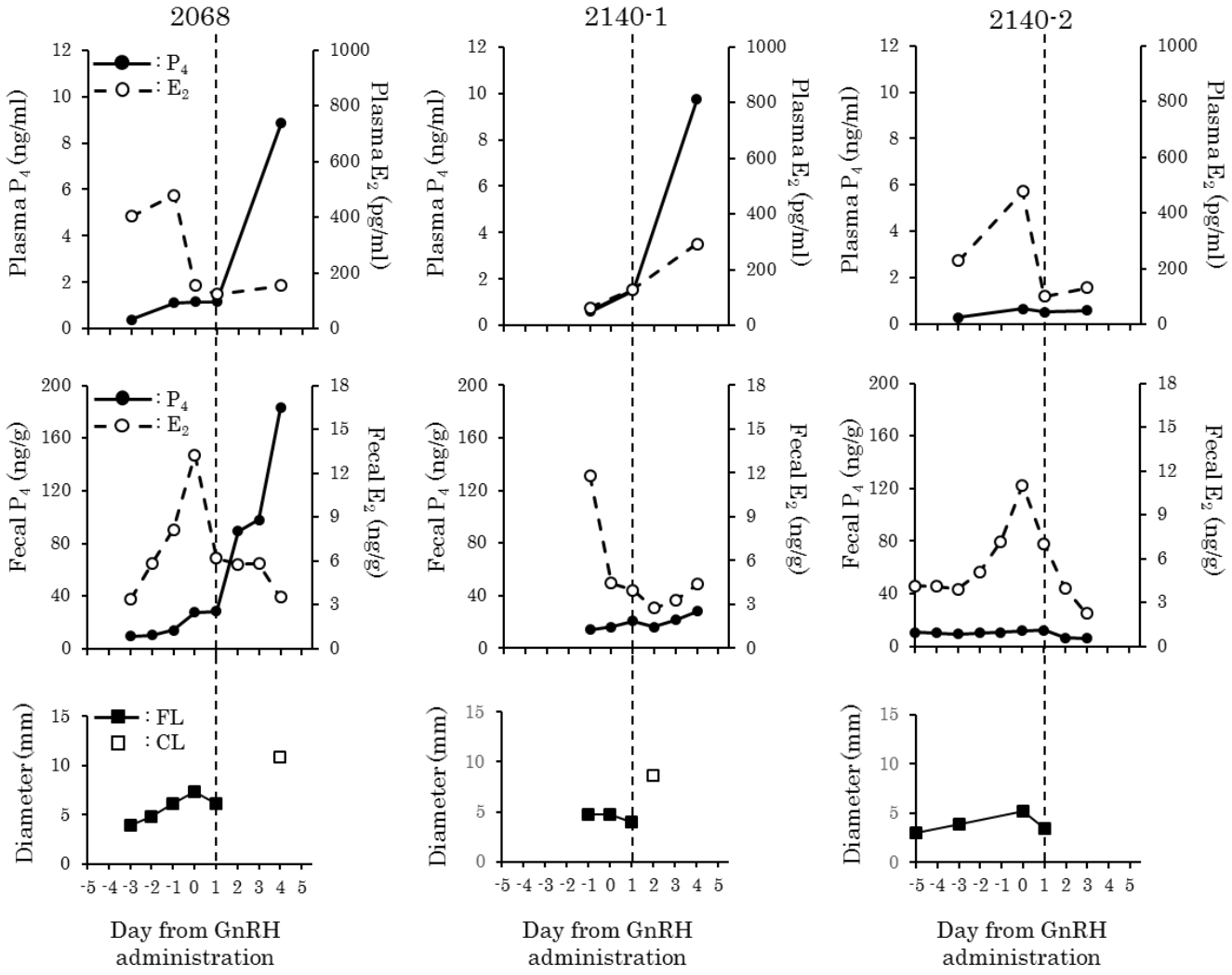


Fig. II-3. Hormonal and ovarian dynamics of female monkeys subjected to ovulation induction by gonadotropin releasing hormone (GnRH). Broken line indicates ovulation day.

P_4: Progesterone, E_2: Estradiol-17β, FL: Follicle, CL: Corpus luteum

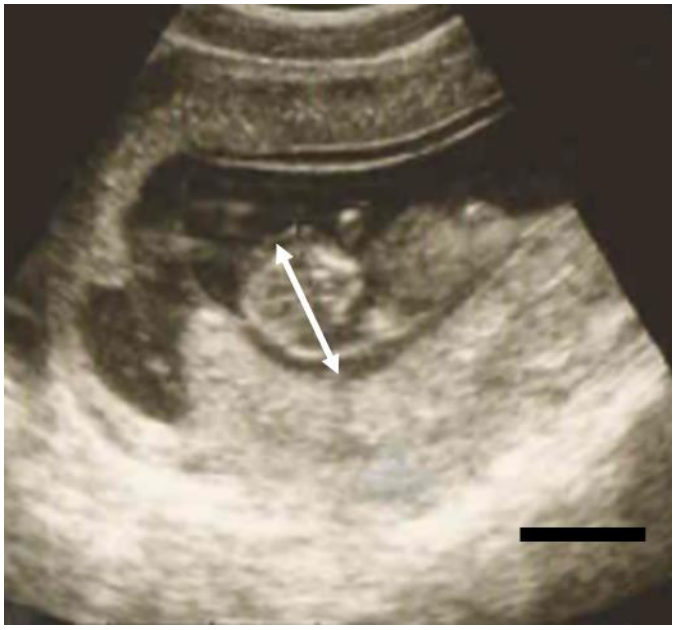


Fig. II-4. Ultrasonographic image of the fetus of ID2068 at 71 days after artificial insemination (bar = 10 mm)

Fetal age was estimated to be 50 days based on the biparietal diameter (white arrow: 12 mm).

SUMMARY AND CONCLUSION

The Japanese macaque is endemic species in Japan, and its physiology and society have been the subject of scientific research over 70 years. The Japanese macaque is popular in zoos, and usually exhibited as troop consisting of both sexes to replicate the behavior in the wild. However, overpopulation and decreasing genetic diversity often become problems due to uncontrolled reproduction in closed troop and the clannish nature. For reproductive management of the Japanese macaque in zoos, artificial insemination (AI) with preserved semen is required as a solution of the two major problems involving reproduction, although success of AI using cryopreserved semen in the Japanese macaque has not been reported. In this thesis, to establish the AI protocol for practical use in the Japanese macaque, improvement of procedures involving males and females was aimed.

In chapter 1, to improve semen collection method and semen cryopreservation, efficacy of semen collection applying urethral catheterization (UC), appropriate temperature for temporary holding, and faster cooling rate of semen before freezing were investigated in the Japanese macaque. In semen collection of the Japanese macaque, electro-ejaculation (EE) has been used with high voltage of stimulation, which could cause invasiveness. However, lower invasive method is preferred in terms of animal welfare. Though, in carnivores, semen can be collected with UC under sedation using α_2-adrenergic agonist, the efficacy of UC had been unclear in primates. UC enables to reduce the invasiveness and the cost involving semen collection. Therefore, semen collection by UC and a combination of EE and UC (EE-UC) for the Japanese macaque were evaluated. Semen collection rate by EE with high voltage (5–10–15 V) and low voltage (3–4–5 or 4–5–6 V) stimuli was compared to investigate the efficacy of practical use of low voltage stimuli for semen collection. As a result, UC with sedative dose of medetomidine (25–50 μg/kg) did not work for semen collection, but EE-UC increased the total sperm number retrieving semen remaining in urethra. Semen collection rate reach maximum with series of 5–10–15 V of stimuli, but 5–10 V of stimulation series did not decrease semen collection rate. Up to 10 V of stimulation can reduce the invasiveness for semen collection. Furthermore, appropriate temperature for temporary holding time in

the Japanese macaque was evaluated since different appropriate temperatures among species were reported. Progressive motile sperm rates after 60 min of holding time at four different temperatures (4°C, 15°C, 27°C and 37°C) were evaluated. Also, cooling rate can affect post thawed sperm motility, then semen characteristics processed with two cooling procedure (slow cooling before second dilution and fast cooling after second dilution) were compared. It was suggested that holding semen at 25°C and fast cooling after second dilution had advantage to improve sperm motility but post thawed sperm motility was not sufficient for AI.

In chapter 2, subjects of the female side were investigated. In previous AI protocol in the Japanese macaque, laparoscopy and insemination were repeated until ovulation, but repeated anesthesia and surgical procedure is invasive for monkeys. Also, the size of ovulatory follicle is important for decision of AI timing, but it had not been clarified in the Japanese macaque. Furthermore, efficacy of ovulation induction with gonadotropin releasing hormone (GnRH), which can facilitate the AI procedure, has not been studied in the Japanese macaque. Thus, monitoring the follicle size with ultrasonography was evaluated around spontaneous ovulation and induced ovulation. Six spontaneous ovulations were observed, and it was clarified that the diameter of ovulatory follicle immediately before ovulation was 7.4 ± 1.5 (5.1–9.6) mm and ovulation occurred during Day 12 to 14 of menstrual cycle. GnRH induced ovulation in three monkeys, and the diameter of the follicle at GnRH injection was 5.8 ± 1.2 mm (4.7–7.4 mm). Single AI with ovulation induction with GnRH, which was expected to reduce the invasiveness and duration of isolation, was conducted seven times for four monkeys. Semen were inseminated in uterus in four cases and in cervix in other three cases between Day 8 to 14 of menstrual cycle. Only in one case a female monkey inseminated in uterus on Day 12 got pregnant. However, the fetus death was confirmed at pregnancy examination with ultrasonography 71 days after AI, and estimated fetal age was about 50 days old.

In this thesis, in the male side, EE-UC improved semen collection increasing the total number of sperm and up to 10 V of stimulation was effective to reduce the invasiveness. Further semen preservation at 25˚C and faster cooling after second dilution could increase preserved sperm motility. In the female side, the size of ovulatory

follicle and efficacy of GnRH to induce ovulation were clarified. And single AI, which was suitable for practical use, could make monkeys pregnant. These results will contribute to reproductive management and animal welfare in captive Japanese macaque. Moreover, it is thought that these attempts also important for wildlife conservation. In fact, the population of the Japanese macaque is not endangered and then, occasion of applying AI technique for conservation is limited currently. However, risk of extinction often comes obscurely and recovery of the population is difficult after the serious situation has been recognized. Accumulation of information about reproduction and refinement of artificial reproductive techniques while the species still have a certain population is important for wildlife conservation in future. And more, some species of genus *Macaca*, relative species of the Japanese macaque, are endangered to extinction. Improvement of artificial reproductive techniques in this study also contributes to *ex situ* conservation of those endangered species in zoos.

ACKNOWLEDGEMENTS

I would like to acknowledge Professor Toshio Tsubota, Laboratory of Wildlife, Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido University for valuable advice to this thesis and review of the present manuscript.

I greatly thank Assistant Professor Yojiro Yanagawa, Laboratory of Theriogenology, Faculty of Veterinary Medicine, Hokkaido University, for great support through all the process of this research, from data collection to writing articles. I could not accomplish this thesis without his considerable supports and advices.

I really appreciate Professor Seiji Katagiri, Laboratory of Theriogenology, Faculty of Veterinary Medicine, Hokkaido University, for valuable advice to this manuscript and review of the present manuscript.

I would like to thanks to Michito Shimozuru, Laboratory of Wildlife, Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido University for review of the present manuscript, providing encouragement and valuable advices.

I truly appreciate Professor Masashi, Nagano, Laboratory of Animal Reproduction, School of Veterinary Medicine, Kitasato University and Assistant Professor Chihiro Kannno, Laboratory of Clinical Veterinary Medicine for Large Animal, School of Veterinary Medicine, Kitasato University for their all supports.

I really thank to Kosuke Sugimoto and Masako Minami, graduates of Laboratory of Theriogenology, Undergraduate School of Veterinary Medicine, Hokkaido University for their daily effort for sample collection and data analysis.

I would like to express my gratitude to former Professor Hirohisa Hirai, Professor Hiroo Imai, Professor Munehiro Okamoto, Yoriko Indo and Akihisa Kaneko, Primate Research Institute, Kyoto University, for their coordinate and support of experiments through the Cooperative Research Program. All the experiments except for post thawed semen evaluation were conducted in Primate Research Institute, Kyoto University, in which use of Japanese macaques and facilities for experiments are widely opened for researchers outside the institute with financial support through Cooperate Research Program. Since progress of

research only by one zoo is often difficult due to the limitation of the budget, staffs and use of animals, this program assists strongly to tackle the problems involving research in zoos and gratefully connects the research institute and research of wildlife and *ex situ* conservation in zoos.

At last, I would announce accomplishment of this manuscript and express grateful thanks to my father and mother who passed away without seeing this manuscript completed.

REFERENCES

1) Amato, F., Warnes, G. M., Kirby, C. A. and Norman, R. J. 2002. Infertility caused by hCG autoantibody. The Journal of Clinical Endocrinology and Metabolism, 87: 993-997.

2) Andrabi, S. M. H. and Maxwell, W. M. C. 2007. A review on reproductive biotechnologies for conservation of endangered mammalian species. Animal Reproduction Science, 99: 223-243.

3) Aoki, K. 2017. Actual state and subjects in zoo, In: Monkey of Japan: mammalogical study of Japanese macaques. (Tsuji, Y. and Nakagawa, N. eds.) pp. 245-264, University of Tokyo Press, Tokyo. (In Japanese)

4) Arakaki, P. R., de Carvalho, F. M., de Castro, P. H. G., Muniz, J. and do Valle, R. D. 2017. Collection, evaluation, and coagulum dissolution of semen from Goeldi's monkey, *Callimico goeldii*. Folia Primatologica, 88: 334-343.

5) Araujo, G. R., Paula, T. A. R., Deco-Souza, T., Morato, R. G., Bergo, L. C. F., Silva, L. C., Costa, D. S. and Braud, C. 2018. Comparison of semen samples collected from wild and captive jaguars (*Panthera onca*) by urethral catheterization after pharmacological induction. Animal Reproduction Science, 195: 1-7.

6) Beekman, N. J. C. M., Schaaper, W. M. M., Turkstra, J. A. and Meloen, R. H. 1999. Highly immunogenic and fully synthetic peptide-carrier constructs targetting GnRH. Vaccine, 17: 2043-2050.

7) Bishop, C. V., Sparman, M. L., Stanley, J. E., Bahar, A., Zelinski, M. B. and Stouffer, R. L. 2009. Evaluation of antral follicle growth in the macaque ovary during the menstrual cycle and controlled ovarian stimulation by high-resolution ultrasonography. American Journal of Primatology, 71: 384-392.

8) Braunstein, G. D., Bloch, S. K., Rasor, J. L. and Winikoff, J. 1983. Characterization of antihuman chorionic-gonadotropin serum antibody appearing after ovulation induction. Journal of Clinical Endocrinology and Metabolism, 57: 1164-1172.

9) Carvalho, F. M., Arakaki, P. R., Nichi, M., Muniz, J., Duarte, J. M. B. and Valle, R. R. 2014. Evaluation of sperm quality in successive regular collections from

captive black-and-gold howler monkeys (*Alouatta caraya*). Animal Reproduction, 11: 11-18.

10) Cavalero, T. M. S., Segabinazzi, L., Scheeren, V. F. C., Canuto, L. E. F., Gobato, M. L. M. and Papa, F. F. 2020. Alternative protocol using imipramine, detomidine, and oxytocin for semen collection in stallion with ejaculatory dysfunction. Journal of Equine Veterinary Science, 93: 103205.

11) Chenault, J. R., Kratzer D. D., Rzepkowski, R. A. and Goodwin, M. C. 1990. LH and FSH response of Holstein heifers to fertirelin acetate, gonadorelin and buserelin. Theriogenology, 34: 81-98.

12) Colazo, M. G. and Mapletoft, R. J. 2014. A review of current timed-AI (TAI) programs for beef and dairy cattle. Canadian Veterinary Journal, 55: 772-780.

13) Cunto, M., Küster, D., Bini, C., Cartolano, C., Pietra, M. and Zambelli, D. 2015. Influence of different protocols of urethral catheterization after pharmacological induction (Ur.Ca.P.I.) on semen quality in the domestic cat. Reproduction in Domestic Animals, 50: 999-1002.

14) Enari, H. and Suzuki, T. 2010. Risk of agricultural and property damage associated with the recovery of Japanese monkey populations. Landscape and Urban Planning, 97: 83-91.

15) Fickel, J., Wagener, A. and Ludwig, A. 2007. Semen cryopreservation and the conservation of endangered species. European Journal of Wildlife Research, 53: 81-89.

16) Fujita, S., Mitsunaga, F., Sugiura, H. and Shimizu, K. 2001. Measurement of urinary and fecal steroid metabolites during the ovarian cycle in captive and wild Japanese macaques, *Macaca fuscata*. American Journal of Primatology, 53: 167-176.

17) Fukui, D., Nagano, M., Nakamura, R., Bando, G., Nakata, S., Kosuge, M., Sakamoto, H., Matsui, M., Yanagawa, Y. and Takahashi, Y. 2013. The effects of frequent electroejaculation on the semen characteristics of a captive siberian tiger (*Panthera tigris altaica*). Journal of Reproduction and Development, 59: 491-495.

18) Fumagalli, F., Villagrán, M., Damián, J. and Ungerfeld, R. 2012. Physiological and biochemical parameters in response to electroejaculation in adult and yearling anesthetized pampas deer (*Ozotoceros bezoarticus*) males. Reproduction in Domestic Animals, 47: 308-312.

19) Gibb, Z. and Aitken, R. J. 2016. Recent developments in stallion semen preservation. Journal of Equine Veterinary Science, 43: S29-S36.

20) Granados, M. D. G., Lopez, L. E. H., Aguilar, A. C., Molina, A. L. C., Perez-Ramirez, O. and Mondragon-Ceballos, R. 2014. Effect of photoperiod on characteristics of semen obtained by electroejaculation in stump-tailed macaques *(Macaca arctoides)*. Primates, 55: 393-401.

21) Hafez, E. S. E. and Jaszczak, S. 1972. Comparative anatomy and histology of the cervix uteri in non-human primates. Primates, 13: 297-314.

22) Imanishi, K. 1957. Social behavior in Japanese monkey, *Macaca fuscata*. Psychologia, 1: 47-54.

23) The International Union for Conservation of Nature (IUCN). Red list of threatened species.
(https://www.iucnredlist.org/search?taxonomies=130129&searchType=species)

24) Ishikawa, A., Matsui, M., Tsuruga, H., Sakamoto, H., Takahashi, Y. and Kanagawa, H. 1998. Electroejaculation and semen characteristics of the captive Hokkaido brown bear (*Ursus arctos yesoensis*). Journal of Veterinary Medical Science, 60: 965-968.

25) Itani, J. 1963. Vocal communication of the wild Japanese monkey. Primates, 4: 11-66.

26) Jacobowitz, D. and Koelle, G. B. 1965. Histochemical correlations of acetylcholinesterase and catecholamines in postganglionic autonomic nerves of cat, rabbit and guinea pig. Journal of Pharmacology and Experimental Therapeutics, 148: 225-237.

27) Japanese Association of Zoos and Aquariums. Breeding animal search.
(https://www.jaza.jp/animal/search?_keyword=macaca&_method=true#paragraph-2, In Japanese)

28) Kamischke, A. and Nieschlag, E. 2002. Update on medical treatment of ejaculatory disorders. International Journal of Andrology, 25: 333-344.

29) Kaneko, R. and Miyazaki, K. 1993. Study on semen collection from patients with anejaculation by ellectorostimulation. The Japanese Journal of Urology, 84: 1479-1488.

30) Kanno, C., Sakamoto, K. Q., Yanagawa, Y., Takahashi, Y., Katagiri, S. and Nagano, M. 2017. Comparison of sperm subpopulation structures in first and second ejaculated semen from Japanese black bulls by a cluster analysis of sperm motility evaluated by a CASA system. Journal of Veterinary Medical Science, 79: 1359-1365.

31) Kanno, C., Yanagawa, Y., Takahashi, Y., Katagiri, S. and Nagano, M. 2017. Effects of semen preservation procedure in egg yolk-tris based extender on bull spermatozoa characteristics. Japanese Journal of Veterinary Research, 65: 151-157.

32) Kappelhof, J. and Windig, J. J. 2021. Controlling inbreeding rate in the European zoo population of hamadryas baboons *Papio hamadryas* with a breeding circle. Journal of Zoo and Aquarium Research, 9: 26-34.

33) Kinoshita, K., Inada, S., Seki, K., Sasaki, A., Hama, N. and Kusunoki, H. 2011. Long-term monitoring of fecal steroid hormones in female snow leopards (*Panthera uncia*) during pregnancy or pseudopregnancy. PloS One, 6: e19314

34) Koyama, S., Fukuda, K., Watanabe, S., Kohara, S., Tsuchiya, H., Fukuzaki, K., Nagano, M., Uno, Y. and Hosoi, Y. 2016. Development of a new device for artificial insemination in cynomolgus macaques. Journal of Reproduction and Development, 62: 527-529.

35) Kuczmarski, A. H., Alves De Barros, M., Souza De Lima, L. F., Motheo, T. F., Bento, H. J., Iglesias, G. A., Sônego, D. A. and Rodrigues Da Paz, R. C. 2020. Urethral catheterization after pharmacological induction for semen collection in dog. Theriogenology, 153: 34-38.

36) Leong, S. K. and Singh, G. 1990. Innervation of the monkey vas-deferens. Journal of Anatomy, 171: 93-104.

37) Li, Y. H., Cai, K. J., Kovacs, A. and Ji, W. Z. 2005. Effects of various extenders and permeating cryoprotectants on cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa. Journal of Andrology, 26: 387-395.

38) Lone, F. A., Islam, R., Khan, M. Z. and Sofi, K. A. 2011. Effect of transportation temperature on the quality of cauda epididymal spermatozoa of ram. Animal Reproduction Science, 123: 54-59.

39) Lueders, I. and Allen, W. R. T. 2020. Managed wildlife breeding-an undervalued conservation tool? Theriogenology, 150: 48-54.

40) Lueders, I., Luther, I., Scheepers, G. and van der Horst, G. 2012. Improved semen collection method for wild felids: Urethral catheterization yields high sperm quality in African lions (*Panthera leo*). Theriogenology, 78: 696-701.

41) Martorana, K., Klooster, K. and Meyers, S. 2014. Suprazero cooling rate, rather than freezing rate, determines post thaw quality of rhesus macaque sperm. Theriogenology, 81: 381-388.

42) Matsubayashi, K. 1982. Comparison of the two methods of electroejaculation in the Japanese monkey (*Macaca fuscata*). Experimental Animals, 31: 1-6.

43) Matsubayashi, K. and Mochizuki, K. 1982. Growth of male reproductive organs with observation of their seasonal morphologic changes in the Japanese monkey (*Macaca fuscata*). The Japanese Journal of Veterinary Science, 44: 891-902.

44) Millar, R. P. 2005. GnRHs and GnRH receptors. Animal Reproduction Science, 88: 5-28.

45) Moore, S. G. and Hasler, J. F. 2017. A 100-Year Review: Reproductive technologies in dairy science. Journal of Dairy Science, 100: 10314-10331.

46) Morgan, P. M., Hutz, R. J., Kraus, E. M., Cormie, J. A., Dierschke, D. J. and Bavister, B. D. 1987. Evaluation of ultrasonography for monitoring follicular-growth in rhesus-monkeys. Theriogenology, 27: 769-780.

47) Morishita, S. 1986. Prompt effect of progesterone on the adrenergic response of smooth muscles. Japanese Journal of Pharmacology, 42: 289-296.

48) Muiño, R., Tamargo, C., Hidalgo, C. O. and Peña, A. I. 2008. Identification of sperm subpopulations with defined motility characteristics in ejaculates from

Holstein bulls: Effects of cryopreservation and between-bull variation. Animal Reproduction Science, 109: 27-39.

49) Munro, C. J., Laughlin, L. S., Illera, J. C., Dieter, J., Hendrickx, A. G. and Lasley, B. L. 1997. ELISA for the measurement of serum and urinary chorionic gonadotropin concentrations in the laboratory macaque. American Journal of Primatology, 41: 307-322.

50) Murphy, E. M., O' Meara, C., Eivers, B., Lonergan, P. and Fair, S. 2018. Optimizing storage temperature of liquid bovine semen diluted in INRA96. Journal of Dairy Science, 101: 5549-5558.

51) Nagai, Y., Miyakawa, N., Takuwa, H., Hori, Y., Oyama, K., Ji, B., Takahashi, M., Huang, X.-P., Slocum, S. T., Diberto, J. F., Xiong, Y., Urushihata, T., Hirabayashi, T., Fujimoto, A., Mimura, K., English, J. G., Liu, J., Inoue, K.-I., Kumata, K., Seki, C., Ono, M., Shimojo, M., Zhang, M.-R., Tomita, Y., Nakahara, J., Suhara, T., Takada, M., Higuchi, M., Jin, J., Roth, B. L. and Minamimoto, T. 2020. Deschloroclozapine, a potent and selective chemogenetic actuator enables rapid neuronal and behavioral modulations in mice and monkeys. Nature Neuroscience, 23: 1157-1167.

52) Nakayama, T., Hori, T., Nagasaka, T., Tokura, H. and Tadaki, E. 1971. Thermal and metabolic responses in Japanese monkey at temperatures of 5-38 degrees C. Journal of Applied Physiology, 31: 332-337.

53) Nicholson, H. D., Parkinson, T. J. and Lapwood, K. R. 1999. Effects of oxytocin and vasopressin on sperm transport from the cauda epididymis in sheep. Reproduction, 117: 299-305.

54) Nigi, H. 1977. Laparoscopic observations of ovaries before and after ovulation in the Japanese monkey (*Macaca fuscata*). Primates, 18: 243-259

55) Nigi, H. 1983. Periovulatory time courses of plasma estradiol and progesterone in the Japanese monkey (*Macaca fuscata*). Primates, 24: 410-418

56) Nigi H. 1983. Japanese macaque, physiology of reproduction. pp. 146-219, Doubutsu-sha, Tokyo. (In Japanese)

57) Nigi, H., Hayama, S., Sawada, T. and Sakamoto, M. 1991. Estimation of delivery

date using biparietal diameter of fetus in Japanese macaque. The 1991 annual meeting of the mammal society of Japan. (In Japanese)

58) Nigi, H. and Torii, R. 1991. Periovulatory time courses of serum LH in the Japanese monkey (*Macaca fuscata*). Experimental Animals, 40: 401-405.

59) Nomura, T., Nigi, H., Ohsawa, N., Ando, A., Tanaka, T., Tajima, Y. and Kotera, S. 1972. Reproduction of Japanese monkeys. Acta Endocrinologica, 71: 473-482.

60) Oishi, T., Imai, H., Go, Y., Imamura, M., Hirai, H. and Takada, M. 2014. Sporadic premature aging in a Japanese monkey: A primate model for progeria. PloS One, 9: e111867.

61) Paulenz, H., Soderquist, L., Perez-Pe, R. and Berg, K. A. 2002. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. Theriogenology, 57: 823-836.

62) Perry, G. A., Smith, M. F., Lucy, M. C., Green, J. A., Parks, T. E., MacNeil, M. D., Roberts, A. J. and Geary, T. W. 2005. Relationship between follicle size at insemination and pregnancy success. Proceedings of the National Academy of Sciences of the United States of America, 102: 5268-5273.

63) Pezo, F., Romero, F., Zambrano, F. and Sanchez, R. S. 2019. Preservation of boar semen: An update. Reproduction in Domestic Animals, 54: 423-434.

64) Pierson, R. A. and Ginther, O. J. 1988. Ultrasonic-imaging of the ovaries and uterus in cattle. Theriogenology, 29: 21-37.

65) Prochowska, S., Dzimira, S., Tomaszek, A. and Niżański, W. 2021. Immunolocalization of adrenoceptors in the reproductive tract of male domestic cats in comparison to rats. Animals, 11: 1049.

66) Ravindra, J. P., Rawlings, N. C., Evans, A. C. O. and Adams, G. P. 1994. Ultrasonographic study of ovarian follicular dynamics in ewes during the oestrous cycle. Reproduction, 101: 501-509.

67) Roughley, M. and Lyall, M. 2019. Retrograde ejaculation associated with quetiapine and treatment with low-dose imipramine. Bmj Case Reports, 12: e228539.

68) Sanchez-Partida, L. G., Maginnis, G., Dominko, T., Martinovich, C., McVay, B.,

Fanton, J. and Schatten, G. 2000. Live rhesus offspring by artificial insemination using fresh sperm and cryopreserved sperm. Biology of Reproduction, 63: 1092-1097.

69) Sankai, T., Terao, K., Yanagimachi, R., Cho, F. and Yoshikawa, Y. 1994. Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). Journal of Reproduction and Fertility, 101: 273-278.

70) Schaffer, N., Jeyendran, R. S. and Beehler, B. 1991. Improved sperm collection from the lowland gorilla - recovery of sperm from bladder and urethra following electroejaculation. American Journal of Primatology, 24: 265-271.

71) Schramm, R. D. and Bavister, B. D. 1994. Follicle-stimulating hormone priming of rhesus monkeys enhances meiotic and developmental competence of oocytes matured in vitro. Biology of Reproduction, 51: 904-912.

72) Schramm, R. D. and Bavister, B. D. 1999. A macaque model for studying mechanisms controlling oocyte development and maturation in human and non-human primates. Human Reproduction, 14: 2544-2555.

73) Sherwood, N. 1987. The GnRH family of peptides. Trends in Neurosciences, 10: 129-132.

74) Shimozawa, N., Ageyama, N., Nakayama, S., Koie, H. and Yasutomi, Y. 2021. Ultrasound-guided, transabdominal, intrauterine artificial insemination for cynomolgus macaques (*Macaca fascicularis*) based on estimated timing of ovulation. Journal of the American Association for Laboratory Animal Science, 60: 125-132.

75) Si, W., Zheng, P., Tang, X. H., He, X. C., Wang, H., Bavister, B. D. and Ji, W. Z. 2000. Cryopreservation of rhesus macaque (*Macaca mulatta*) spermatozoa and their functional assessment by in vitro fertilization. Cryobiology, 41: 232-240.

76) Sokol, R. Z., McClure, R. D., Peterson, M. and Swerdloff, R. S. 1981. Gonadotropin therapy failure secondary to human chorionic gonadotropin-induced antibodies. Journal of Clinical Endocrinology and Metabolism, 52: 929-933.

77) Sukesh, B., Puttabyatappa, M., Peter, A. T., Medhamurthy, R. and Seshagiri, P.

B. 2017. Assessment of ovarian follicular dynamics and folliculogenesis associated endocrine profiles following gonadotropin stimulation in the bonnet monkey. General and Comparative Endocrinology, 253: 25-32.

78) Swanson, W., Bateman, H. and Vansandt, L. 2017. Urethral catheterization and sperm vitrification for simplified semen banking in felids. Reproduction in Domestic Animals, 52: 255-260.

79) Thau, R. B., Goldstein, M., Yamamoto, Y., Burrow, G. N., Phillips, D. and Bardin, C. W. 1988. Failure of gonadotropin therapy secondary to chorionic gonadotropin-induced antibodies. The Journal of Clinical Endocrinology and Metabolism, 66: 862-867.

80) Thijssen, A., Klerkx, E., Huyser, C., Bosmans, E., Campo, R. and Ombelet, W. 2014. Influence of temperature and sperm preparation on the quality of spermatozoa. Reproductive Biomedicine Online, 28: 436-442.

81) Thomsen, R. 2014. Non-invasive collection and analysis of semen in wild macaques. Primates, 55: 231-237.

82) Tokuda, K. 1961. A study on the sexual behavior in the Japanese monkey troop. Primates, 3: 1-40.

83) Tollner, T. L., Vandevoort, C. A., Overstreet, J. W. and Drobnis, E. Z. 1990. Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). Journal of Reproduction and Fertility, 90: 347-352.

84) Torii, R., Hosoi, Y., Iritani, A., Masuda, Y. and Nigi, H. 1998. Establishment of routine cryopreservation of spermatozoa in the Japanese monkey (*Macaca fuscata*). Japanese Journal of Fertility and Sterility, 43: 125-131.

85) Torii, R. and Nigi, H. 1992. The artificial fecundation in Japanese monkeys (*Macaca fuscata*) by induced ovulation and artificial-insemination in nonmating season. Experimental Animals, 41: 243-246.

86) Torii, R. and Nigi, H. 1998. Successful artificial insemination for indoor breeding in the Japanese monkey (*Macaca fuscata*) and the cynomolgus monkey (*Macaca fascicularis*). Primates, 39: 399-406.

87) Tsuji, Y. and Nakagawa, N. 2017. Monkeys of Japan: A mammalogical studies

of Japanese macaques, University of Tokyo Press. (In Japanese)

88) Turner, R. M. O., McDonnell, S. M. and Hawkins, J. F. 1995. Use of pharmacologically induced ejaculation to obtain semen from a stallion with a fractured radius. Journal of the American Veterinary Medical Association, 206: 1906-1908.

89) Valle, R. R., Guimaraes, M., Muniz, J., Barnabe, R. C. and Vale, W. G. 2004. Collection and evaluation of semen from captive howler monkeys (*Alouatta caraya*). Theriogenology, 62: 131-138.

90) van Diepen, H. A., Pansier, J., Wesselink, P. O., van Drie, A., van Duin, M. and Mulders, S. 2012. Non-invasive translational cynomolgus model for studying folliculogenesis and ovulation using color doppler ultrasonography. Journal of Medical Primatology, 41: 18-23.

91) Warner, H., Martin, D. E. and Keeling, M. E. 1974. Electroejaculation of the great apes. Annals of Biomedical Engineering, 2: 419-432.

92) Yanagawa, Y., Matsuura, Y., Suzuki, M., Saga, S. I., Okuyama, H., Fukui, D., Bando, G., Nagano, M., Katagiri, S., Takahashi, Y. and Tsubota, T. 2015. Accessory corpora lutea formation in pregnant Hokkaido sika deer (*Cervus nippon yesoensis*) investigated by examination of ovarian dynamics and steroid hormone concentrations. Journal of Reproduction and Development, 61: 61-66.

93) Yang, S., He, X., Niu, Y., Wang, X., Lu, B., Hildebrandt, T. B., Goeritz, F., Jewgenow, K., Zhou, Q. and Ji, W. 2009. Dynamic changes in ovarian follicles measured by ultrasonography during gonadotropin stimulation in rhesus monkeys. Theriogenology, 72: 560-565.

94) Zambelli, D., Cunto, M., Prati, F. and Merlo, B. 2007. Effects of ketamine or medetomidine administration on quality of electroejaculated sperm and on sperm flow in the domestic cat. Theriogenology, 68: 796-803.

SUMMARY IN JAPANESE

ニホンザル(*Macaca fuscata*)は日本の固有種であり、動物園で広く飼育・展示され、野生での生息状況を模すため雌雄混合の群れで飼育されることが多い。しかし、ニホンザルの繁殖力は強く、飼育環境下においても容易に繁殖するため、多くの動物園で飼育個体数過剰が問題となる。繁殖制限のため、オスについては精管切除術、メスについてはプロジェステロン製剤のインプラントが処置されるが、意図しない精管再疎通やサル自身によるインプラント剤の抜去により失敗することが多い。また、ニホンザルの群れは排他的で群れ内の結びつきが強く、外部から個体を導入すると攻撃されて死に至ることがある。結果として、外部からの遺伝子導入がないまま閉鎖的な群れで繁殖を繰り返すことで、遺伝的多様性の低下も問題となる。保存精液を使用した人工授精（AI）が家畜および一部の動物園動物で繁殖管理に利用されており、ニホンザルにおいても精液を提供するオスおよび AI の対象となるメスの選別と厳密な避妊処置により、血統管理と繁殖数管理を同時に実施可能であると考えられる。しかし、ニホンザルの AI についての報告は少なく、凍結保存精液を使用した産子獲得については報告がない。

AI を成功させるには、オスおよびメス両方へのアプローチが必要となる。また動物福祉の改善および実用性の向上のため、侵襲性が低く簡便な方法が求められる。オスについては、精液の採取と保存が課題として挙げられる。ニホンザルの精液採取には直腸プローブによる電気刺激法（EE 法）が一般的に用いられるが、同属であるマカク属を含む他の動物種よりも高い電圧が使用されている。また、精液が副生殖腺液の作用により射精後すぐに凝固し、採取精子数や精子運動性を減少させることが知られている。食肉目動物では、α_2 アドレナリン受容体作動薬の精管収縮作用によって尿管内に分泌された精液を尿管カテーテルの挿入により回収する方法（UC 法）が報告されている。霊長目動物における UC 法の有効性は不明であるが、電気刺激による侵襲なしで精液を回収すること、ならびに通常の射精を伴わないため精液凝固による精子数および運動性に対する負の影響を軽減することが期待できる。

一方、ニホンザルの精液凍結処理において、精液の濃縮と検査のため 30 分から 60 分間精液を一時的に保管する時間が必要であるが、精液の短時間保存に適切な温度は動物種によって異なり、ニホンザルの適正保存温度は不明である。また同属のアカゲザルにおいて、精液の冷却速度を速めることで凍結融解後の精子の活性が上昇したという事例が報告されており、ニホンザルの精液凍結処理においても、冷却速度を検討する価値が

あると考えられる。

第 1 章では、精液採取技術の改善および侵襲性の低減のため。ニホンザルの精液採取における UC 法および電気刺激後に UC 法を実施する方法（EE-UC 法）の有用性について検討した。併せて低電圧刺激での精子採取の有用性を検討するため、低電圧群（3–4–5、4–5–6 V）と高電圧群（5–10–15 V）の、精液採取率を比較した。また、凍結保存処理中の精液性状改善のため、採取した精液を 4˚C、15˚C、25˚C および 37˚C で 60 分保存した後の精子運動性を比較し、適切な保存温度について検討した。さらに、2 次希釈のタイミングと冷却スピードを変えた 2 種類の冷却方法で精液を凍結保存し、各凍結処理段階および凍結融解後の精子性状を比較した。

その結果、ニホンザルにおいて鎮静量の α_2 アドレナリン受容体作動薬投与（メデトミジン、25–50 μg/kg）では UC 法によって精液は採取できないことが明らかとなった。しかし、EE-UC 法によって EE 法のみでは採取不可能な尿道内に貯留する液状精液を回収し、総採取精子数を増加させられることが明らかとなった。高電圧群では低電圧群より精子採取率が有意に上昇した。精液採取率を最大にするためには 5 V から 15 V までの電圧上昇が必要であるが、個体への侵襲を抑えたい場合は 10 V までの上昇が有効であることが示唆された。精液の保存については、60 分間保存した後の前進運動精子率が 25˚C の保存温度下で高い傾向にあり、一時保存温度として 25˚C が適正であることが示唆された。また、二次希釈を冷却前に行い 20 分で素早く 4˚C 付近まで冷却することで、60 分から 90 分かけて冷却した後に二次希釈を行う方法に比べ、凍結前の精子運動性が改善した。

一方、AI 実施のメス側の課題として、精液注入適期の検査および調整が挙げられる。ニホンザルの AI では、排卵確認のため排卵前後に複数回の腹腔鏡検査が実施され、精液注入も数日に渡って実施されていた。しかし、検査に伴う頻回の麻酔および外科的処置は侵襲性が高く、動物福祉の観点から改善が必要である。さらに動物園での実施を考慮すると、AI のためにサルを群れから隔離することは、群れ内の順位が混乱し闘争の原因となるため、隔離期間の短縮が求められる。超音波画像診断装置による卵巣動態観察は家畜およびマカク属のサルを含む野生動物において広く実施されているが、ニホンザルにおける報告はない。また AI 適期のコントロールに誘起排卵が利用されるが、ニホンザルにおいては性腺刺激ホルモン放出ホルモン（GnRH）の使用例はない。

第 2 章では超音波画像診断装置によるニホンザルの卵巣動態観察、GnRH による誘起

排卵およびそれらの知見を応用した単回精液注入による AI の有効性を評価した。超音波画像診断装置によって 6 回の自然排卵を観察した結果、ニホンザルの自然排卵は生理周期の 12–14 日目に主席卵胞が 7.4 ± 1.5(5.1–9.6) mm の時に起こることが明らかとなった。また主席卵胞が 5.8 ± 1.2(4.7–7.4) mm の時、GnRH 投与により投与翌日までに排卵を誘起することが明らかとなった。GnRH による排卵誘起と単回の精液注入を併用した人工授精を生理周期の 8–14 日目に計 7 回実施した結果、生理周期の 12 日目で AI した 1 頭が妊娠したが、胎齢 50 日付近における胎子の発達停止が確認された。

本研究から、ニホンザルの精液採取において、EE-UC 法によって採取精子数を増加させられることが判明した。また、精液採取には高い電圧が必要であるが、最大 10 V の刺激で 15 V と同等な精液採取率が得られることが明らかとなった。精液凍結処理については、一時保存には 25°C が適正であること、二次希釈後に素早く冷却することで精子運動性を改できることが併せて示された。また、ニホンザルの卵巣動態把握および排卵誘起に関して、超音波画像診断装置と GnRH 投与がそれぞれ有効であることが明らかとなった。ニホンザルの AI について、GnRH を使用した排卵誘起後の単回精子注入により妊娠が達成でき、侵襲性の低減および隔離期間の短縮が可能であることが示された。これらの知見は動物園等のニホンザル飼育施設における人工授精プロトコルの低侵襲化および効率化につながるものであり、将来的なニホンザル飼育群の繁殖管理および動物福祉の改善に貢献するものである。また、絶滅が危惧される近縁種の飼育下繁殖に応用できる可能性もあり、生息域外保全への貢献も期待される。