A controlled ovarian stimulation procedure suitable for cynomolgus macaques

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Abstract: This study aimed to develop a more suitable ovarian stimulation procedure for cynomolgus macaques (Macaca fascicularis). Macaques were divided into 4 groups, 7AG, 8AG, 7AN, and 8AN, according to the ovarian stimulation procedure administered (i.e., administration of either a gonadotropin-releasing hormone agonist [GnRH-a] or GnRH antagonist [GnRH-ant]) and the number of menstruations (≤ 7 times or ≥ 8 times) in the previous year. In both procedures, oocyte growth and maturation were induced by administration of human follicle-stimulating hormone and human chorionic gonadotropin. The mean numbers of metaphase II mature and metaphase I premature oocytes collected from the 7AG, 8AG, 7AN, and 8AN groups were 12.1 and 10.4, 12.0 and 13.8, 9.1 and 8.3, and 15.5 and 8.8, respectively (P>0.05). The fertilization rates of the 7AN and 8AN groups (85.3% and 74.7%) tended to be higher compared with those in the 7AG and 8AG groups (59.1% and 47.3%; P>0.05). The 8AN group yielded 19.9 zygotes, which was the largest number per macaque, compared with the other three groups. Furthermore, regarding the decreases in body weight between the start of the procedures and the time of oocyte collection, those of the 7AN and 8AN groups were significantly smaller than those of the 7AG and 8AG groups (P<0.05), suggesting that the procedure involving GnRH-ant reduced the burden on the macaques. Thus, controlled ovarian stimulation using a GnRH-ant has some advantages for cynomolgus macaques compared with that using a GnRH-a.

Key words: body weight, cynomolgus macaque, GnRH analogue, oocyte, ovarian stimulation

Introduction

Assisted reproductive techniques, such as artificial insemination and in vitro fertilization, are available to artificially avoid some problems involved in the in vivo fertilization steps for producing offspring and zygotes in humans and animals. In cynomolgus macaques (Macaca fascicularis), artificial insemination is difficult because of their meandering cervical canals [1, 2]. However, recent methodological advances have enabled efficient offspring production [3, 4]. Meanwhile, in vitro fertilization, including intracytoplasmic sperm injection (ICSI) followed by embryo transfer, also enables offspring production but requires several steps [5–8]. These techniques have been applied to the production of genetically modified macaques [9–14]. Although the first step is stimulation to induce the growth of multiple ovarian follicles, an efficient procedure to do so in cynomolgus macaques has yet to be developed [15–18].

In cynomolgus macaques, controlled ovarian stimulation procedures for the collection of oocytes involve the administration of follicle-stimulating hormone (FSH) or equine chorionic gonadotropin and gonadotropin-releasing hormone antagonist (GnRH-ant) or GnRH agonist

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(GnRH-a). Administration of a GnRH-ant that blocks GnRH receptors induces a rapid and reversible suppression of gonadotropin secretion and consequently prevents the endogenous luteinizing hormone (LH) surge [19]. Meanwhile, administration of a GnRH-a that regulates pituitary function stimulates ovarian follicle growth in combination with exogenous gonadotropin hormones and prevents the endogenous LH surge [20]. Oocyte maturation is subsequently induced by the administration of human chorionic gonadotropin (hCG) or LH.

There is room to improve the oocyte collection procedure in cynomolgus macaques because the ovarian stimulation procedures vary with respect to the hormone combinations, dosage, and timing of administration [5–8, 15–18]. In addition, although ovarian stimulation procedures have been applied to macaques with regular menstruation [6, 16, 21–23], the detailed information about this has not been reported. In macaques, menstrual cycles generally have an interval of approximately 28 to 32 days [24, 25]. Therefore, while examining the effects of GnRH-ant administration, we also compared the effects of two ovarian stimulation procedures between macaques with ≤ 7 (i.e., irregular) and ≥ 8 (i.e., regular) annual menstruations. This study aimed to develop a more suitable ovarian stimulation procedure for cynomolgus macaques.

Materials and Methods

Animals

Forty-three female cynomolgus macaques aged 4–13 years old were used in this study, which was the first to examine ovarian stimulation with exogenous hormones in cynomolgus macaques. They were divided into 4 groups, 7AG, 8AG, 7AN, and 8AN, according to whether they received administration of GnRH-a or GnRH-ant as well as the number of menstruations (≤ 7 times or ≥ 8 times) in the previous year the procedure was performed (Table 1). In addition, 10 male macaques aged 6–21 years old were used to collect sperm for ICSI. In 6 of the 10 males, fertility had been confirmed; the fertility of the others was not confirmed. They were randomly assigned to the 7AG, 8AG, 7AN, or 8AN groups (Table 1). All macaques were bred and maintained at the Tsukuba Primate Research Center. The cynomolgus macaques were housed in stainless steel cages (0.5 × 0.8 × 0.9 m, width × height × depth, respectively) at 25 ± 3°C and 50–70% humidity with 12 air changes per h and a 12/12-h light/dark cycle. They were given 70 g of commercial food (CMK-2, CLEA Japan, Inc., Tokyo, Japan) and 100 g of apples daily. Tap water was supplied ad libitum. Health status was monitored every morning.

Menstruation was recorded by visual observation of menstrual blood. This study was conducted in accordance with the Rules for Animal Care and Management of the Tsukuba Primate Research Center [26], the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [27], and the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals [28]. The study protocol was approved by the Animal Care and Use Committee of the National Institutes of Biomedical Innovation, Health and Nutrition.

Ovarian stimulation with exogenous hormones

Controlled ovarian stimulation was induced in cynomolgus macaques by two procedures with the administration of GnRH-a or GnRH-ant (Fig. 1); the details of the former procedure have been reported previously [15]. In brief, 25 IU/kg recombinant human FSH (rFSH; follitropin alfa, Gonal-f, Merck Biopharma Co., Ltd., Tokyo, Japan) dissolved in a glycerol/physiological saline (1:1) solution was administered once daily for 9 days starting 2–3 weeks after administration of 1.88 mg leuprolin acetate, a GnRH-a (Leuplin, Takeda Pharmaceutical Co., Ltd., Osaka, Japan). Meanwhile, for the GnRH-ant procedure, the macaques were subcutaneously administered 25 IU/kg rFSH once daily for 8 days starting at 3–4 days of menstruation. Then, 0.125 mg cetorelix acetate, a GnRH-ant (Cetrodite, Merck Biopharma Co., Ltd., Tokyo, Japan) dissolved in a glycerol/physiological saline solution was administered once daily for 4 days starting from day 6 of rFSH administrations. In both procedures, 1,200 IU hCG (Gonatropin, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) was administered once daily for 4 days starting from day 7 of rFSH administrations.

### Table 1. Female and male cynomolgus macaques used in this study

| Group | GnRH analog administered | No. of female macaques used | Average age (range) | No. of menstruations during the past year | Average no. of menstruations during the past year ± SD | Fertility of males that donated sperm assigned to ICSI |
|-------|--------------------------|-----------------------------|---------------------|----------------------------------------|---------------------------------------------|-----------------------------------------------|
|       |                          |                             |                     |                                         |                                             | No. of confirmed | No. of unconfirmed |
| 7AG   | Agonist                  | 9                           | 6.1 ± 0.9 (4–10)    | 1–7                                    | 4.1 ± 0.8                                   | 3                | 2                  |
| 8AG   | Agonist                  | 10                          | 9.3 ± 0.8 (5–12)    | 8–13                                   | 10.2 ± 0.5                                  | 4                | 0                  |
| 7AN   | Antagonist               | 7                           | 7.9 ± 0.8 (5–11)    | 2–7                                    | 5.0 ± 0.6                                   | 2                | 0                  |
| 8AN   | Antagonist               | 17                          | 7.9 ± 0.7 (4–13)    | 8–14                                   | 10.9 ± 0.6                                  | 3                | 3                  |

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kyo, Japan) was administered intravenously 34–36 h after the final rFSH administration.

**Oocyte collection**

To collect oocytes from ovarian follicles, the female macaques were anaesthetized with 10 mg/kg ketamine hydrochloride (Ketalar, Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and isoflurane (Pfizer Inc., New York, NY, USA) 36–38 h after hCG administration. The contents of the ovarian follicles were aspirated with a 25-gauge needle connected to a 2.5-ml syringe containing Special Cleavage Modified Medium (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 2.5 IU/ml heparin (Novo Nordisk Pharma Ltd., Tokyo, Japan) via an abdominal incision. The suspension was then treated with 0.1% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) to release the oocytes from cumulus cells. The oocytes were subsequently washed with CMRL-1066 medium (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum, GlutaMAX (×100, Life Technologies), and penicillin-streptomycin solution (×100, FUJIFILM WAKO Pure Chemical Corp., Osaka, Japan; hereafter, CMRL). The washed oocytes were classified into 4 groups as follows: (a) metaphase II (MII; mature) stage with a polar body, (b) metaphase I (MI; pre-mature) stage without a polar body and a large nucleus, (c) germinal vesicle (GV) stage with a large nucleus, and (d) degenerative.

**Intracytoplasmic sperm injection**

To verify the fertilization ability of the mature oocytes collected, ICSI was conducted to produce zygotes derived from the denuded oocytes using a micromanipulation system equipped with a piezo drive unit (PMM-150FU, Prime Tech Ltd., Ibaraki, Japan) under an inverted microscope (IX-70, Olympus, Tokyo, Japan). Male macaques were anaesthetized with ketamine hydrochloride, and fresh semen was collected into TYH medium \(^{[29]}\) by transrectal probe electrostimulation. The semen suspension was layered onto 90% Percoll (GE Healthcare, Chicago, IL, USA) and centrifuged at 1,200 × g for 10 min. The precipitate was resuspended in TYH medium and then centrifuged again at 1,200 × g for 10 min. Collected sperm were suspended in 10% polyvinylpyrrolidone medium (Kitazato Corporation, Shizuoka, Japan) and then used for ICSI. Using piezo pulses, individual MII oocytes were injected with a spermatozoon drawn into an injection pipette (7–8 µm in diameter). Following spermatozoon injection, the oocytes were transferred into CMRL and cultured at 37°C in a humidified 5% CO\(_2\) atmosphere. Fertilization rates for evaluating the fertilization ability of MII oocytes were defined as the proportions of the oocytes with two pronuclei and two polar bodies (normal fertilization) to the total number of the surviving oocytes showing no pronuclei, one pronucleus, and multiple pronuclei. Note that there is a possibility that some of the oocytes showing no pronuclei or one pronucleus failed to retain the injected sperm in the cytoplasm after ICSI.
Statistical analysis

Statistical comparisons were made by one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparison test using the Statcel 4 software (OMS Publishing Inc., Saitama, Japan). Note that fertilization rates and maturation rates (i.e., MI vs. MII oocytes) were subjected to arcsine conversion prior to one-way ANOVA. The level of significance was set at $P<0.05$.

Results

Oocytes collected from stimulated ovarian follicles

The numbers (mean ± SD) of oocytes collected from follicles in the 7AG (n=9), 8AG (n=10), 7AN (n=7), and 8AN (n=17) groups were 34.9 ± 6.3 (range: 12–71), 35.8 ± 3.9 (range: 18–62), 29.4 ± 5.3 (range: 9–50), and 32.2 ± 5.1 (range: 6–71), respectively. The numbers (mean ± SD) of MII, MI, GV, and degenerative oocytes collected were as follows: 12.1 ± 5.2, 10.4 ± 3.0, 9.6 ± 3.0, and 2.8 ± 1.3 in the 7AG group; 12.0 ± 3.7, 13.8 ± 2.3, 8.5 ± 2.3, and 1.5 ± 0.8 in the 8AG group; 9.1 ± 2.1, 8.3 ± 2.4, 7.6 ± 3.4, and 4.4 ± 1.9 in the 7AN group; and 15.5 ± 3.8, 8.8 ± 1.7, 6.4 ± 1.4, and 1.5 ± 0.4 in the 8AN group, respectively ($P>0.05$, Fig. 2; Supplementary Table 1). The rates of maturation from the MI to the MII stage within 6 h of in vitro culture were 48.9% (46/94), 39.9% (55/138), 50.0% (29/58), and 42.0% (63/150) in the 7AG, 8AG, 7AN, and 8AN groups, respectively ($P>0.05$).

Fertilization of mature oocytes by intracytoplasmic sperm injection

To examine fertilization ability, MII oocytes were subjected to ICSI. The fertilization rates in the 7AG, 8AG, 7AN, and 8AN groups were 59.1% (55/93, n=5), 47.3% (69/146, n=8), 85.3% (58/68, n=5), and 74.7% (198/265, n=10), respectively (Table 2, Fig. 3A). The fertilization rates in the 7AN and 8AN groups tended to be higher compared with those in the 7AG and 8AG groups, although there were no significant differences in the 4 groups ($P>0.05$). The mean numbers of zygotes per macaque in the 7AG, 8AG, 7AN, and 8AN groups were 11.0 ± 3.4 (55/5), 8.6 ± 2.8 (69/8), 11.0 ± 2.8 (44/4), and 19.9 ± 4.6 (179/9), respectively (Fig. 3B). The number of zygotes per macaque in the 8AN group was the largest compared with in the other groups, although there were no significant differences in the 4 groups ($P>0.05$).

Body weight changes

To determine the physical influence of the ovarian stimulation procedures on cynomolgus macaques, the difference in body weight between the start of the procedures and the time of oocyte collection was investigated. The body weights at the time of the first rFSH administration in the GnRH antagonist groups and at GnRH agonist administration in the GnRH agonist groups were used as the baseline values. In the 7AN and 8AN groups (n=24), the mean body weight decrease at the time of oocyte collection was 75.0 ± 19.4 g. For the 7AG and 8AG groups (n=14), the difference in body weight was 11.0 ± 3.4 g.

Table 2. Fertilization ability of MII oocytes microinseminated in cynomolgus macaques

| Group | No. of oocytes injected | No. (%) of surviving oocytes | No. (%) of normally fertilized oocytes* | No. (%) of abnormally fertilized oocytes with | No pronucleus | One pronucleus | Multiple pronuclei |
|-------|-------------------------|-------------------------------|----------------------------------------|---------------------------------------------|---------------|---------------|-------------------|
| 7AG   | 99                      | 93 (93.9)                     | 55 (59.1)                              | 36 (38.7)                                   | 1 (1.1)       | 1 (1.1)       |
| 8AG   | 153                     | 146 (95.4)                    | 69 (47.3)                              | 74 (50.7)                                   | 1 (0.7)       | 2 (1.4)       |
| 7AN   | 70                      | 68 (97.1)                     | 58 (85.3)                              | 8 (11.8)                                    | 2 (2.9)       |               |
| 8AN   | 282                     | 265 (94.0)                    | 198 (74.7)                             | 64 (24.2)                                   | 2 (0.8)       | 1 (0.4)       |

There were no significant differences in the 4 groups ($P>0.05$). *Fertilized oocytes with two pronuclei and two polar bodies (normal fertilization).
weight at the times of rFSH administration and oocyte collection was also investigated, and the mean body weight decreases at the times of rFSH administration and oocyte collection were 366.4 ± 39.8 and 444.3 ± 53.2 g, respectively. The body weight decreases in the GnRH-a groups (the 7AG and 8AG groups) were significantly greater than those in the GnRH-ant groups (the 7AN and 8AN groups; \( P < 0.05 \), Fig. 4).

**Discussion**

This study aimed to develop a more effective ovarian stimulation procedure suitable for collecting oocytes as well as reducing the burden in cynomolgus macaques. Although there are various procedures to induce ovarian stimulation in nonhuman primates, such as cynomolgus and rhesus macaques [5–8, 15–18], they cannot be directly compared. One reason for this is that the genetic background of macaques is not controlled, unlike that of experimental mice. We previously collected oocytes from cynomolgus macaques individually maintained in a uniform indoor environment by using a GnRH-a that is also a GnRH analogue [15]. As an alternative, the present study investigated the use of another GnRH analogue that is a GnRH-ant. The results show that the ovarian stimulation procedure using the GnRH-ant had some advantages compared with that using the GnRH-a.

Our previous study showed that rFSH administration was more effective for collecting mature oocytes than equine chorionic gonadotropin administration for controlled ovarian stimulation in cynomolgus macaques [15]. The present study also investigated the effect of the number of menstrual cycles prior to the ovarian stimulation cycle on the number of oocytes collected and fertilization rate. However, there was no significant difference in the number of oocytes collected between the GnRH-ant and GnRH-a administration groups. One reason why there were no significant differences between the two groups may be due to the similar effect of the same exogenous hormones (rFSH and hCG) administered to all the macaques. On the other hand, regardless of the past frequency of the menstrual cycle, fertilization rates tended to be lower in the GnRH-a administration groups compared with those in the GnRH-ant administration groups. Furthermore, the results indicate that the number of fertilized oocytes produced by a given macaque tended to be highest in the 8AN group. These findings indicate that for oocyte collection and zygote preparation in cynomolgus macaques, the ovarian stimulation procedure using GnRH-ant is more effective than that using GnRH-a.

In addition, body weight decreased significantly more in the GnRH-a administration groups than in the GnRH-ant administration groups. Although ovarian stimulation
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procedures have been reported for oocyte collection [5–8, 15–18], to our knowledge, the present study is the first report of the effects of these procedures on the body weight of macaques. Unlike mice, cynomolgus macaques are never euthanized at the time of oocyte collection [17]; this enables cynomolgus macaques from which oocytes have been collected to be available for subsequent breeding, embryo transfer, and other experiments. Therefore, it is important to establish an appropriate ovarian stimulation procedure that reduces the burden on cynomolgus macaques. The GnRH-ant and GnRH-a used in this study are also used to treat human infertility [30, 31]. The medical package insert for Leuplin, the GnRH-a used in this study, states that it has side effects such as loss of appetite and weight loss. However, the GnRH-ant used in this study, Cetrodite, does not incur such side effects. Accordingly, the results of examining the effects of these GnRH analogues on body weight change in the present study suggests that even in cynomolgus macaques, the burden of the ovarian stimulation procedure using the GnRH-ant on individuals is extremely low or nonexistent.

There were no significant differences in the number of oocytes collected between macaques with irregular menstruation and those with regular menstruation. In previous studies, although ovarian stimulation for oocyte collection was conducted in macaques with regular menstruation [6, 16, 21–23], the definition of regular menstruation was not specified. Macaques have a menstrual cycle length (approximately 28–32 day intervals) similar to humans [24, 25]. Therefore, we compared the effects of ovarian stimulation between macaques with ≤ 7 (i.e., irregular) and ≥ 8 (i.e., regular) annual menstruations. The results indicated that the procedures for the 8AN group with regular menstruation were relatively effective for maximizing the numbers of MII oocytes and zygotes and the fertilization rates compared with the other three groups. The ovarian stimulation procedure using the GnRH-ant in cynomolgus macaques with regular menstruation may have had some positive effects on oocyte growth and maturation by reducing the burden of body weight changes.

There are two reports of oocyte collection using ovarian stimulation procedures similar to our procedures [16, 17]. However, the numbers of oocytes collected in them varied. This may be due to differences such as in genetic background, details of the ovarian stimulation procedures, foods and their quantity, housing environment, and age.

In conclusion, this study shows that for cynomolgus macaques, an ovarian stimulation procedure using a GnRH-ant has some advantages in terms of oocyte collection, production of zygotes, and reduction of burden compared with that using a GnRH-a. Adopting this procedure using a GnRH-ant may also contribute to artificial reproduction in macaques with hereditary disorders [32, 33] compared with that using the conventional procedure with a GnRH-a.

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