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Development of a new device for artificial insemination in cynomolgus macaques

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Abstract. In cynomolgus macaques, an important animal species for biomedical research, efficient reproduction has been hampered partly due to the difficulties of artificial insemination (AI) using straw tubes developed for humans or farm animals, because cynomolgus macaques have a complex cervical canal structure. In this study, taking into consideration the unique structure of the macaque cervical canal, we developed a novel device for AI, comprised of a syringe and an outer cylinder. At 24 and 48 h after using this device to inject semen into one female, viable sperm were observed in the oviduct where the sperm meets the oocytes. We then attempted AI using this new device on 10 females that were at pre-ovulation, and pregnancy was successful in three animals (30% pregnancy rate). These results show that the newly developed device can be used for AI in cynomolgus macaques.

Key words: Artificial insemination, Cynomolgus monkey, Device, Semen

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Cynomolgus macaques are an important animal species in biomedical research due to their evolutionary proximity to humans. Although assisted reproductive techniques have been developed in cynomolgus macaques, including *in vitro* fertilization, *in vitro* maturation, and embryo transfer [1, 2], artificial insemination (AI) has been somewhat difficult. Unlike humans and farm animals, macaques, including cynomolgus and rhesus macaques, have a complex female reproductive tract [3, 4]. The macaque's cervix has five to six folds that do not allow easy passage of an insemination pipette for the deposition of semen into the uterus, making it difficult to utilize the tools and protocols developed for humans and farm animals. In a preliminary study, we attempted to utilize insemination pipettes developed for humans and cattle, but the insemination pipettes failed to get inserted into the cynomolgus macaque's cervix. In rhesus macaques, tools for AI (e.g., insemination pipettes and guides) have been developed [5], but they cannot be used in cynomolgus macaques due to their narrower cervix. New AI methods for cynomolgus macaques are therefore needed.

Macaque semen forms a coagulum after ejaculation [6, 7], possibly to prevent invasion from sperm of other males. Alternatively, seminal coagulation might help sperm move into the cervical canal and the body of uterus by pooling the sperm in the vagina; otherwise, liquefied semen could flow out when the animal sits in a relaxed position. If this hypothesis is true, sperm could swim into the uterus when retained in the vagina long enough during AI. In our preliminary study, we confirmed that motile sperm were released from the coagulated-liquefied semen collected from cynomolgus macaques, suggesting that the semen, even coagulated, can be useful for AI after liquefaction. To test our hypothesis, a new device for AI was developed (Fig. 1A–C) to retain liquefied semen in the vagina and to introduce liquefied semen into the uterus.

In Experiment 1, a naturally menstruating female at pre-ovulation was subjected to AI using the newly developed device and sperm from a male. It should be noted that the same concentration and volume of sperm suspension (50×10^6 cells/ml fresh sperm in 1 ml of BWW medium) was used for all AI procedures performed in this study. At 24 h after AI, we observed at least three viable sperm, and at 48 h, at least two viable sperm in the media flushed from the oviduct of the female (including one motile sperm at each time point), indicating that viable sperm reached the oviduct using this device.

In Experiment 2, to assess the pregnancy rate, 10 females were subjected to AI using the same device. At 20 days after AI, 30% of

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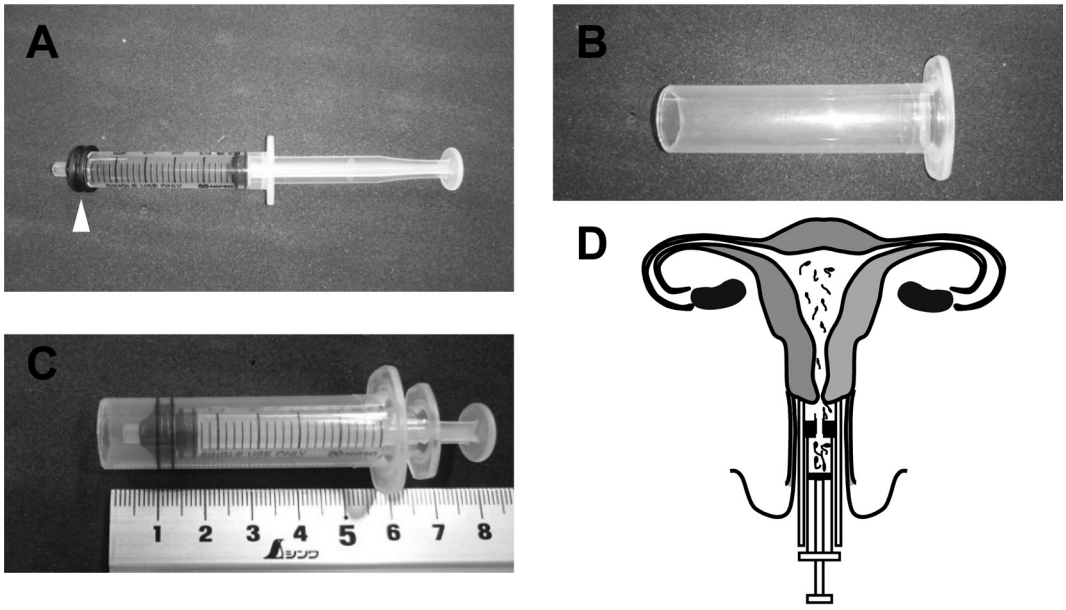

Fig. 1. A newly developed AI device for cynomolgus macaques. The device is composed of a 1-ml syringe (A) attached with silicone rubber (arrow head), and a conventional outer cylinder (B) that is fitted to the size of the vagina. The 1-ml syringe was filled with diluted sperm prepared as described in Methods, and was inserted into the conventional outer cylinder (C). The outer cylinder tightly attached to the cervix and covering the external os (D). The diluted sperm passes through the cervix and is introduced into the uterus when the plunger is depressed.

these females (3/10) were pregnant and delivered healthy offspring. The pregnancy rate was similar to or better than that reported for macaques after intravaginal AI (12.1–13.3%) [8] and intrauterine AI (4–21%) [8–10] with fresh sperm, and after natural mating at our facility (23.3%, 87/373). The good pregnancy rate obtained in the present study is most likely due to an increased number of sperm being introduced into the uterus by retention of liquefied sperm and by covering the external os of the uterus with the new device (Fig. 1D). The new device can be easily prepared, and AI using this device can be performed without special skills or tools, unlike the system described in a previous study [5], suggesting that this new AI method is useful and practical for reproduction in cynomolgus macaques.

In a previous study on rhesus macaques [5], a better pregnancy rate of 57% was achieved after AI with fresh sperm. The authors attributed the high rate principally to depositing sperm into the uterus (not into the vagina or cervix) and to preparing a sperm suspension containing a known sperm concentration (26.7×10^6 fresh sperm) [5]. Smaller numbers of fresh sperm are required for fertilization by unilateral intrauterine insemination as compared with intravaginal insemination in other species such as cats [11, 12]. In humans, smaller numbers of fresh sperm are needed for intravaginal insemination (35.9×10^6 fresh sperm) than intrauterine insemination (90.1×10^6 fresh sperm) [13]. In our study, fewer fresh sperm (50×10^6 cells) than that used in intravaginal insemination in humans resulted in successful fertilization for cynomolgus macaques, suggesting that fewer fresh sperm might be required for AI using the new device. The higher concentration of the fresh sperm used (approximately two-fold) might have partly accounted for the successful pregnancies in our study.

In our study, attempts to retain liquefied sperm near the external os of the uterus using the new AI device successfully led to pregnancy in three of the 10 females. The reasons for unsuccessful pregnancy in 7 females are currently unknown. It is possible that the number of sperm introduced into the uterus was not sufficient to achieve pregnancy. To improve our method, it will be important to investigate the quantity and motility of the sperm in the sperm suspensions and the volume of the sperm suspensions used with this AI devices to achieve a better fertilization rate. In addition, the time period required for a sufficient number of fresh sperm to reach the oviduct needs to be assessed in order to alleviate the potential stress put on the recipient; in this study the device was kept at the right position for 30 min. Nevertheless, the present study provides evidence that the newly developed device is useful for AI in cynomolgus macaques. In the present study, we did not examine the duration from AI to ovulation. To improve the pregnancy rate after AI using this device, we should investigate the relationship between the timing of AI and ovulation, and also develop a procedure for the control of ovulation in female cynomolgus macaques.

Methods

Animals

A total of 14 cynomolgus macaques housed in individual cages were used for the experiments, including three males (from China, 9 years of age, weighing 6–7 kg) and 11 females (from China, 6–8 years of age, weighing 3–4 kg). The present study was reviewed and approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd.

Semen collection and preparation of sperm

Semen was collected from the three males by electric penile stimulation with direct current pulses as described previously [2]. All male monkeys had a history of their semen producing pregnancy by natural mating, and the motility of the sperm in their semen was confirmed. Briefly, ejaculates were collected without anesthesia in the morning, with at least a week intervening between collections. All the male monkeys were chair-trained for semen collection. One pre-sized defibrillator gel electrode was wrapped around the base of the penis and connected to the negative lead. A second gel electrode was positioned immediately behind the glans and connected to the positive lead. A slow and steady stimulation by increasing the output-adjust dial induced a slight erection, engorgement of the glans, and/or elevation of the testicles into the inguinal region. Typically, engorgement and ejaculation occurred at 10–20 V. The ejaculates were collected in clean tubes (15 ml high clarity polypropylene centrifuge tube, BD Falcon, Bedford, MA). The seminal coagulum of three cynomolgus macaques were separately liquefied, and sperm motility in the liquid portion was evaluated under a light microscope. To liquefy the sperm, an equal volume of BWW medium prepared from TALP-HEPES medium supplemented with 4 mg/ml bovine serum albumin [14, 15] was added to the semen, and it was kept at 37°C (5% O_2, 5% CO_2) for 30 min in an incubator (Juji Field, Tokyo, Japan). The liquid portion of the semen was transferred into a 15 ml conical tube and washed with BWW medium, followed by centrifugation (112 × *g*) at room temperature for 7 min. The sperm were counted using a Makler TM counting chamber (Sefi-Medical Instruments, Haifa, Israel), and diluted to 50×10^6 cells/ml with BWW medium. Progressive motility of the sperm samples (sperm moving in a forward direction), assessed subjectively, was > 90%. Each sperm suspension made from an ejaculate was used for three or four females in Experiment 2.

Artificial insemination

The newly developed device is composed of a 1-ml syringe (Nipro, Osaka, Japan) and a conventional outer cylinder that is fitted to the size of the vagina (Fig. 1) so that liquefied sperm cannot flow out of the device and the vagina. All the females exhibited stable menstrual cycles. Before AI, females were anesthetized with 7 mg/kg of ketamine hydrochloride (Kamud Drugs Pvt, India) and 1.25 mg/kg of xylazine (Nippon Zenyaku Kogyo, Tokyo, Japan). After aseptic preparation of the perivaginal area, the outer cylinder was introduced into the vagina and placed around the cervical os. The 1-ml syringe was then placed into the cylinder. In all experiments, the sperm suspension (50×10^6 cells/ml fresh sperm in 1 ml of BWW medium) was placed into the syringe, and slowly injected to the female. This position was maintained for approximately 30 min until the female recovered from anesthesia. In Experiment 1, to assess whether sperm can reach the oviduct, a female showing a normal menstrual cycle, and with at least two births by means of vaginal delivery, was inseminated with 1 ml of sperm suspension diluted to 50×10^6 cells/ml with BWW medium. At 24 and 48 h after AI, the female was anesthetized with isofluran and the oviduct was surgically removed. The lumen of the oviduct was flushed with BWW medium by using a 23-gauge needle (Nipro) attached to a 1-ml syringe and the washed medium was subjected to microscopic examination for sperm. In Experiment 2, to determine whether this AI method can lead to pregnancy, AI was performed once on each of 10 females that were at pre-ovulation as determined by observing the dominant follicle using laparoscopy. Females treated with 50 µg/head/day levonorgestrel (D(-)-Norgestrel, Sigma-Aldrich, St. Louis, MO, USA) for 14 days to synchronize their menstrual cycles, as described previously [16], were subjected to AI using the newly developed device. Levonorgestrel inhibits follicular maturation and ovulation in cynomolgus macaques at different phases of the menstrual cycle, and the menstrual cycle restarts upon withdrawing levonorgestrel administration. AI was performed as described earlier, and pregnancies were assessed at day 20 after insemination by real-time ultrasonography (SonoSite TiTAN, Hitachi Aloka Medical, Tokyo, Japan) under anesthesia using ketamine hydrochloride as described earlier.

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References

1. **Torii R, Nigi H.** Successful artificial insemination for indoor breeding in the Japanese monkey (*Macaca fuscata*) and the cynomolgus monkey (*Macaca fascicularis*). *Primates* 1998; **39**: 399–406. [CrossRef]
2. **Torii R, Hosoi Y, Masuda Y, Iritani A, Nigi H.** Birth of the Japanese monkey (*Macaca fuscata*) infant following in-vitro fertilization and embryo transfer. *Primates* 2000; **41**: 39–47. [CrossRef]
3. **Cuadros A.** New findings relating to the gross and microscopic morphology of the uterine cervix in the rhesus monkey. *Fertil Steril* 1971; **22**: 138–143. [Medline] [CrossRef]
4. **Hafez ESE, Jaszczak S.** Comparative anatomy and histology of the cervix uteri in non-human primates. *Primates* 1972; **13**: 297–314. [CrossRef]
5. **Gabriel Sánchez-Partida L, Maginnis G, Dominko T, Martinovich C, McVay B, Fanton J, Schatten G.** Live rhesus offspring by artificial insemination using fresh sperm and cryopreserved sperm. *Biol Reprod* 2000; **63**: 1092–1097. [Medline] [CrossRef]
6. **VandeVoort CA.** High quality sperm for nonhuman primate ART: production and assessment. *Reprod Biol Endocrinol* 2004; **2**: 33. [Medline] [CrossRef]
7. **Dixson AL, Anderson MJ.** Sexual selection, seminal coagulation and copulatory plug formation in primates. *Folia Primatol (Basel)* 2002; **73**: 63–69. [Medline] [CrossRef]
8. **Dede JA, Plentl AA.** Induced ovulation and artificial insemination in Rhesus colony. *Fertil Steril* 1966; **17**: 757–764. [Medline] [CrossRef]
9. **Czaja JA, Eisele SG, Goy RW.** Cyclical changes in the sexual skin of female rhesus: relationships to mating behavior and sucessful artificial insemination. *Fed Proc* 1975; **34**: 1680–1684. [Medline]
10. **Valerio D, Leverage W, Bensenhaver J, Thornett H.** The analysis of male fertility, artificial insemination and natural matings in the laboratory breeding of macaques. *In:* Goldsmith E, Moor-Jankowski J (eds.), Medical Primatology. Basel: Krager; 1971: 515–525.
11. **Tanaka A, Takagi Y, Nakagawa K, Fujimoto Y, Hori T, Tsutsui T.** Artificial intravaginal insemination using fresh semen in cats. *J Vet Med Sci* 2000; **62**: 1163–1167. [Medline] [CrossRef]
12. **Tsutsui T, Tanaka A, Takagi Y, Nakagawa K, Fujimoto Y, Murai M, Anzai M, Hori T.** Unilateral intrauterine horn insemination of fresh semen in cats. *J Vet Med Sci* 2000; **62**: 1241–1245. [Medline] [CrossRef]
13. **Kathiresan AS, Ibrahim E, Aballa TC, Attia GR, Lynne CM, Brackett NL.** Pregnancy outcomes by intravaginal and intrauterine insemination in 82 couples with male factor infertility due to spinal cord injuries. *Fertil Steril* 2011; **96**: 328–331. [Medline] [CrossRef]
14. **Biggers JD, Whitten WK, Whittingham DG.** The culture of mouse embryos in vitro. *In:* Daniel JC (ed.), Methods in Mammalian Embryology. San Francisco: Freeman; 1971: 86–116.
15. **Jaiswal BS, Cohen-Dayag A, Tur-Kaspa I, Eisenbach M.** Sperm capacitation is, after all, a prerequisite for both partial and complete acrosome reaction. *FEBS Lett* 1998; **427**: 309–313. [Medline] [CrossRef]
16. **Nakama K, Akune A, Kawate N, Takahashi M, Inaba T, Sameshima H, Tamada H.** Delay of ovulation due to diets containing levonorgestrel in cynomolgus monkeys (*Macaca fascicularis*). *J Vet Med Sci* 2012; **74**: 1453–1460. [Medline] [CrossRef]