

—Review—

Practical reproductive techniques for the common marmoset

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Abstract: The common marmoset (*Callithrix jacchus*) is commonly used as a subject model animal in experimental research. The species has several advantages compared with other laboratory primates and we succeeded in creating a transgenic (Tg) marmoset with germline transmission of the transgene, the first time in a nonhuman primate. We have been attempting to further improve marmoset reproductive technology, which is more similar to that of humans than rodent experimental animals, such as mice. We have produced many genetically modified marmosets as human disease models and have also improved marmoset reproductive techniques to obtain many fertilized embryos and neonates. For ethical reasons, it is difficult to perform human reproductive studies; thus, we must rely on nonhuman primate models in basic research. For this reason, reproductive studies of marmosets may help the development of assisted reproduction technologies (ART) for humans and may also be useful in human preclinical studies. In this mini-symposium, we describe practical marmoset reproductive technologies performed at the Central Institute for Experimental Animals (CIEA) and discuss our planned future research using marmosets in reproductive studies.

Key words: Common marmoset, Reproduction, Ovum pick-up, *in vitro* fertilization, Intracytoplasmic sperm injection

Introduction

The common marmoset (*Callithrix jacchus*) is a New World monkey that is commonly used in the laboratory (Fig. 1, Table 1). Marmosets are extensively used in many areas of biomedical research and have several advantages over other nonhuman primate laboratory animals. Specifically, marmoset reproductive traits are suit-

able for breeding, the production of genetically modified models, and the study of reproductive biology, because they deliver 2–3 pups every half year, have a gestation period of 145–148 days, and are sexually mature at approximately one and a half years of age.

We have succeeded in creating the world's first transgenic (Tg) nonhuman primate (marmoset) with germ line transmission of the transgene [1]. Currently, we are attempting to further improve marmoset reproductive technologies in order to efficiently create genetically modified marmosets. To obtain marmoset early embryos as much as possible, we continuously monitor ovarian cycles via serum progesterone levels in order to determine the timing of ovarian stimulation and ovum pick-up (OPU) which is followed by *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). In particular, these procedures need improving. In previous studies, human reproductive technologies were adopted to obtain or manipulate marmoset early embryos, because of the similarities in reproductive physiological characteristics and properties of pre-implantation embryos between marmosets and humans. For example, since ovarian hormone sensitivity varies between individual marmosets, ovarian stimulation protocols are occasionally ineffective; and this is similar to infertility in humans, but not in mice [2]. Despite the properties of murine pre-implantation embryos differing from those of humans, they are still used in quality control of culture media for human embryos because ethical considerations make it difficult to use human pre-implantation embryos for quality tests. In this respect, marmoset embryos might be a good alternative model, and may be helpful in the development of assisted reproductive technologies (ARTs) for humans, as well as help to improve our understanding of human embryonic molecular mechanisms and developmental metabolisms [3–5]. In this mini-symposium, we describe the results and future direction of reproductive technologies practised at the Central Institute for Experimental Animals (CIEA) and discuss the results and future direc-

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Fig. 1. Common marmoset (*Callithrix jacchus*). Common marmosets have white ear tufts, and the tail is striped with dark and pale bands. Common marmosets live in family groups, each of which consists of one or two breeding females and a breeding male. The infants are cared for not only by their mother but also by their father and siblings.

tion of our at marmoset reproductive studies.

Ovarian Cycle Control and Ovarian Stimulation

The ovarian cycle of adult female marmosets is the same as that of humans, i.e., 28 days (Fig. 2). The cycle consists of 8–11 days of follicular phases and 17–20 days of the luteal phase; and ovulation occurs around 9–13 days after the follicular phase. Marmoset ovarian cycles can be curtailed to 21 days by artificial method because the follicular phase is promoted from the luteal phase by prostaglandin F₂ alpha (PGF₂α, Estrumate; Schering-Plough Animal Health, Union, NJ, USA), the uterine factor responsible for luteolysis [1, 6]. This procedure is performed using a single intramuscular injection with 0.1 ml of 8 μg/ml PGF₂α per animal. After administration, the shift of the follicular phase can be confirmed by a low plasma progesterone concentration, which is monitored by an enzyme immunoassay (EIA) kit (TOSOH Progesterone Kit; TOSO, Tokyo, Japan). Ovulation can

Table 1. Physiology of the common marmoset

Binomial name	<i>Callithrix jacchus</i>
Geographical distribution	East-central Brazil
Body weight	250–500 g (adult)
Body length	14–22 cm
Chromosome number	2n = 46
Lifespan	12–15 years
Sexual maturation	1.5 years
Gestational period	145–148 days
Litter size	1–3
Research uses	Neuroscience, reproductive biology, infection disease, behavior, etc.

be detected by elevation of the plasma progesterone concentration above 10 ng/ml [7, 8]. After confirmation of reset in the ovarian cycle by PGF₂α, the marmosets are administered human follicle-stimulating hormone (hFSH, 25 IU; Folyrmon-P; Fuji Pharma Co., Ltd, Tokyo, Japan) on alternate days at 10:00 AM, for 9 days. Then human chorionic gonadotropin (hCG, 75 IU; Gonatropin; ASKA Pharmaceutical. Co., Ltd, Tokyo, Japan) is administered on day 10 at 5:00 PM.

Anesthesia and Pre- and Postoperative Care

For marmoset oocyte collection by OPU, ovarian stimulated marmosets are pre-anesthetized with an intramuscular injection of 0.15 ml MMB cocktail solution containing 0.04 mg/kg medetomidine (Domitor; Nippon Zenyaku Kogyo, Koriyama, Japan), 0.4 mg/kg midazolam (Dormicam; Astellas Pharma, Tokyo, Japan), 0.4 mg/kg butorphanol (Vetorphale; Meiji Seika Pharma, Tokyo, Japan), and saline solution at a ratio of 1:2:2:5 per head. In order to prevent infection and dehydration, marmosets are administered 15 mg/kg ampicillin (Viccillin; Meiji Seika Pharma, Co., Ltd.) and electrolyte solution (KN No. 1 injection; Otsuka Pharmaceutical, Tokyo, Japan) of 2 ml per head. Subsequently, they are anesthetized with 1.0–3.0% isoflurane (Forane; Abbott Japan, Tokyo, Japan), using inhalation masks. During anesthetization maintenance, heart rate and arterial oxygen saturation are monitored. After OPU, an intramuscular dose of 0.20 mg/kg atipamezole (Antisedan; Nippon Zenyaku Kogyo, Japan) is administered for recovery from sedation status. For the purposes of post-operative analgesic care and infection management, 1.2 mg/kg ketoprofen and 15 mg/kg ampicillin are administered once a day for 3 days, beginning the day after the procedure.

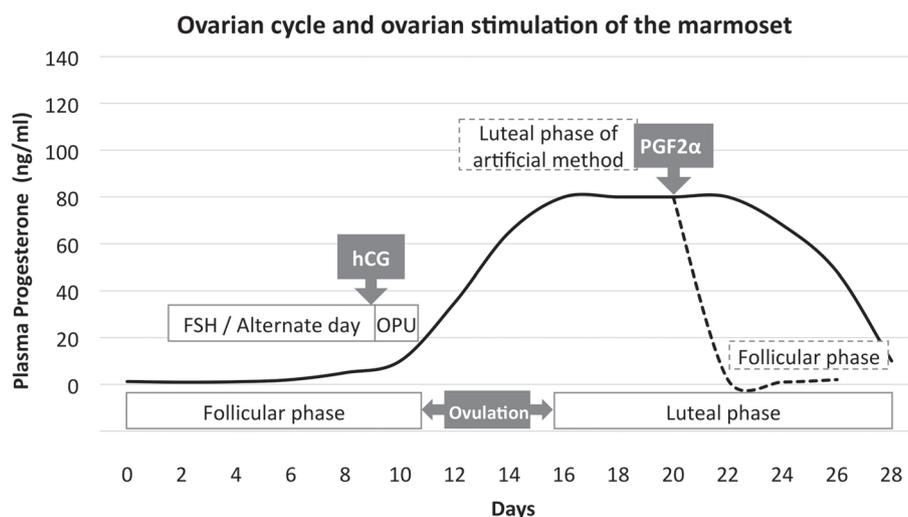


Fig. 2. Ovarian cycle and ovarian stimulation of the marmoset. The marmoset ovarian cycle is 28 days long. It consists of 8–11 days of follicular phases and 17–20 days of luteal phases. The ovarian cycle in marmosets can be curtailed to 21 days (broken line) by artificial methods using prostaglandin F2 alpha (PGF2 α) for luteolysis, and is shown here in comparison with the typical ovarian cycle (continuous line). After confirmation of the reset in the marmoset ovarian cycle, ovarian stimulation is carried out using hFSH for 9 days at 10:00 AM, on alternate days. Then, hCG is administered on the tenth day at 5:00 PM. OPU is performed on the marmosets the next morning.

OPU and IVM

Unlike in mice, marmoset superovulation has not been achieved. Therefore, marmoset oocytes require OPU followed by IVM and IVF. In order to obtain Tg marmosets, we routinely perform OPU every week. Ovarian stimulation methods for marmoset and IVM techniques have previously been reported [9–11]. This accumulated knowledge has contributed to the development of routine reproductive techniques for marmosets at our laboratory. Below, we describe our protocols for marmoset OPU and IVM.

Eighteen hours after hCG administration, OPU is performed on ovarian stimulated marmosets to collect oocytes by laparotomy. The ovaries and uterus of the marmoset are exposed by midline laparotomy and follicular oocytes are aspirated with a 25-G needle attached to a syringe containing heparin and porcine oocyte medium (POM; Research Institute for the Functional Peptides Co., Ltd., Japan). Then, the aspirates containing oocytes are collected into a Petri dish [12, 13]. After all the follicular oocytes have aspirated, the marmoset's abdomen is sutured and the animal is rested for at least 2 months before the next OPU. As described above, postoperative care protocol is followed after OPU. The oocytes collected in the Petri dish are classification, according to oocyte

stage and quality, then transferred to separate droplets (Fig. 3). For the visual, the cytoplasm of metaphase II (MII) and metaphase I (MI) oocytes are smooth, whereas the cytoplasm of germinal vesicle (GV) stage oocytes are rough. For the classification of the GV stage oocytes, the GVA oocytes are attached to multiple layers of cumulus cells surrounding the oocytes, the GVB oocytes are attached to incomplete or few layers of cumulus cells, and the GVC oocytes have no cumulus cells. Marmosets undergo multiple OPU procedures. However, the number of oocytes collected reduces with successive OPU repetitions (Fig. 4); this problem has not yet been resolved.

POM has been developed as an IVM medium for porcine oocytes [12, 13]. Tomioka et al. reported that POM is also effective in marmoset oocyte IVM and IVF [14]. Recently, we have been using POM for IVM supplemented with 5% fetal bovine serum (FBS), 0.15 IU/ml hFSH, and 10 IU/ml hCG. A previous study indicated that hCG induces marmoset oocyte maturation *in vivo*, and we recently found that hCG promotes marmoset oocyte maturation *in vitro* as well (data not shown) [10]. The immature oocytes of the GVA, GVB and GVC stages are cultured overnight in modified POM in a Petri dish at 38 °C, with a gas phase of 5% CO₂, 5% O₂, and 90% N₂. During the overnight IVM culture, the multiple layers of cumulus cells attached to the GVA and GVB oocytes

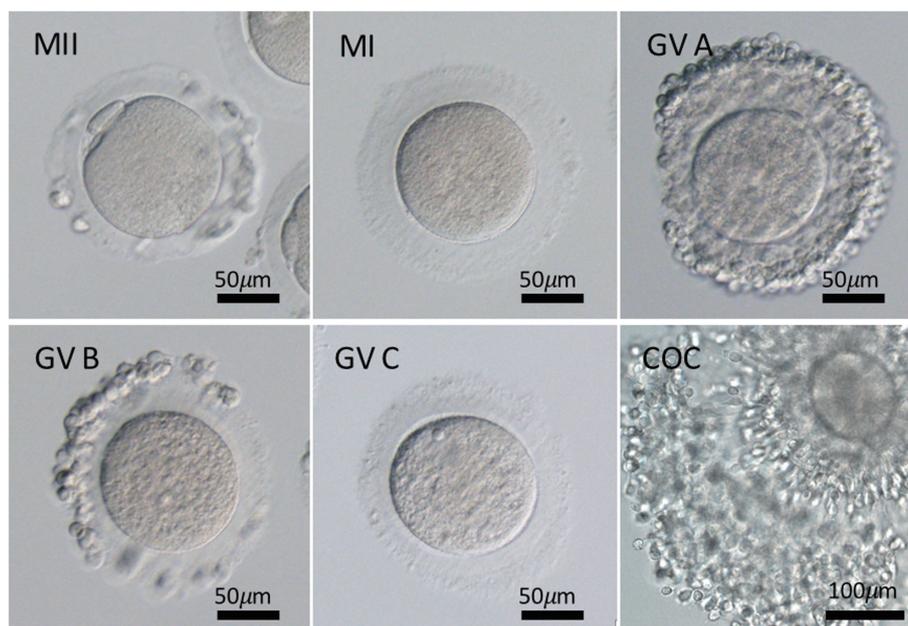


Fig. 3. Oocytes on the days of OPU (day 0) and IVM (day 1). Oocytes are classified as belonging to stages MII, MI, GVA, GVB, or GVC on the day of OPU. The cytoplasm of MII and MI oocytes is smooth, whereas the cytoplasm of GVA, GVB, and GVC oocytes is rough. Multiple layers of cumulus cells attach to the GVA and GVB oocytes after IVM (day 1), and the cumulus cells expand to surround the oocyte, forming a cumulus-oocyte complex (COC).

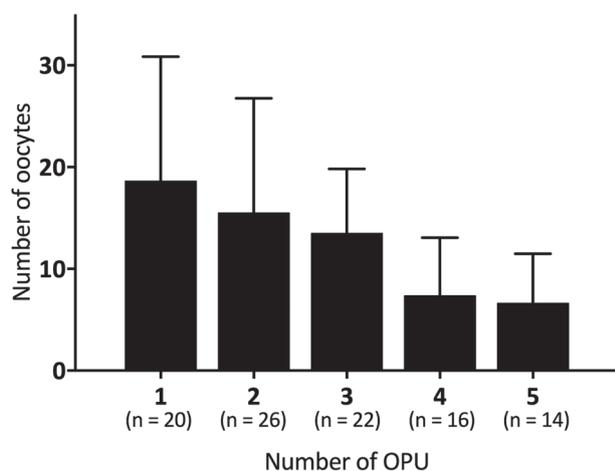


Fig. 4. Effects of repeated OPU. The number of oocytes obtained decreases as OPU is repeated. The mean oocyte counts (\pm SEs) were as follows: 18.7 (12.2) for the first collection (n=20); 15.5 (11.3) for the second collection (n=26); 13.5 (6.3) for the third collection (n=22); 7.4 (6.0) for the fourth collection (n=16); and 6.6 (4.8) for the fifth collection (n=14).

expand around the oocyte, forming a cumulus-oocyte complex (COC; Fig. 3). Subsequently, not only MII oo-

cytes but also MI oocytes are subjected to IVF on the day following IVM culture.

Preparation of Spermatozoa

Marmoset ejaculates can be collected from unsedated marmosets using noninvasive penile vibrostimulation with a vibrator [15, 16]. The unsedated marmoset is placed in a restrainer and the genitals are sterilized with alcohol-impregnated cotton; a vibrator is then placed at the base of the penis for stimulation. The ejaculated semen is collected in TYH medium (LSI Medience Corporation, Tokyo, Japan) which is the same medium used for mouse IVF [17]. Since marmoset semen is produced in small quantities, density gradient centrifugation is not used as in human semen preparation. To wash the marmoset semen, the samples are centrifuged at $500\times g$ for 5 min at room temperature. Then, the supernatant is discarded, and the pellet is resuspended in 0.5 ml TYH. To liquefy the semen, the suspension is incubated for 30 min at 38°C , with a gas phase of 5% CO_2 , and after incubation, the samples are gently mixed and centrifuged at $500\times g$ for 5 min at room temperature. To prepare motile sperm for IVF swim-up, the most common technique used for sperm preparation is as follows. The sperm pel-

let is gently resuspended in 0.1 ml TYH. This suspension is carefully applied to the bottom of a 5-ml polystyrene conical tube containing 0.7 ml TYH. The conical tube containing the semen sample is tilted to an angle of 30° and incubated for 30 min at 38 °C, with a gas phase of 5% CO₂, 5% O₂, and 90% N₂. After the swim-up step, the motile sperm are extracted from the supernatant in the tube and measured using a sperm motility analysis system (SMAS).

IVF and IVC

Several studies have reported the establishment of marmoset IVF techniques. Not all of these studies performed embryo transfer of IVF-derived embryos; however, a few studies have succeeded in obtaining offspring [2, 9–11, 18–22]. The following protocols are used at our laboratory. TYH medium droplets 40 µl are prepared in an IVF dish, covered with paraffin liquid (a specially prepared reagent; Nacalai Tesque, Inc., Japan), and equilibrated in a CO₂ incubator for 1 h before use. Motile sperm is added to the equilibrated TYH medium droplets and adjusted to 3.6 × 10⁶ sperm/ml /droplet. Mature (MII) or premature (MI) oocytes are placed in each droplet in the IVF dish. The IVF dishes containing the oocytes and sperm are then incubated for 18 h at 38 °C, with a gas phase of 5% CO₂, 5% O₂, and 90% N₂, for fertilization. After insemination, the eggs are recovered, washed with TYH medium, and transferred to fresh medium droplets of Cleav (Origio, Denmark). Fertilization of the washed oocytes is then confirmed the presence of two pronuclei and two polar bodies (2PN-2PB; Fig. 5A). A comparison of the fertilization rates between the first and third versus fourth and eighth OPU repetitions showed that fertilization is significantly lower in the repetitions (Fig. 6).

In order to confirm the developmental abilities of the fertilized embryos and to check transgene expression in genetically modified marmoset production, the zygotes are usually cultured *in vitro* for 3–8 days before embryo transfer to surrogate mothers. Human pre-implantation embryo culture systems provide appropriate environments for the changes occurring in embryo metabolic and physiological status. Media for human pre-implantation embryos, the Origio Sequential series, which consists of Cleav and Blast (Origio, Denmark), are used for *in vitro* culture of the fertilized marmosets embryos. Cleav nourishes cleavage stage embryos using pyruvate, whereas Blast supports glycolysis to promote blastocyst development. In order to promote the rate of blastocyst development in the marmoset embryos, Blast is supplemented with 10% FBS and 2 mM L-glutamine. Fertilized eggs are

cultured in Cleav medium up to the 8- to 10-cell stage (Fig. 5B–D). The 8- to 10-cell stage pre-implantation embryos are then replaced in Blast medium and cultured for 3–4 days for development to the blastocyst stage (Fig. 5E–G).

Intracytoplasmic Sperm Injection (ICSI)

Some genetically modified marmosets sometimes show conditions such as asthenozoospermia and idiopathic infertility. ICSI can be adapted for these cases [22]. The shape of the marmoset sperm is similar to that of the human sperm (Fig. 7C). Commercial injection needles (PINU06-20FT, Prime Tech Ltd., Japan) for ART and 7% polyvinylpyrrolidone (PVP; Origio, Denmark) can therefore be used for marmoset ICSI (Fig. 6A, B).

To perform marmoset ICSI, an MII oocyte is held using a holding pipette and rotated to the 12 o'clock position of the polar body. A selected sperm is immobilized by pressing down on the tail with an injection needle and then aspirated into the injection needle. In the next step, the zona pellucida is punctured with a piezo pulse to allow insertion of the injection needle using a piezo driver system (Prime Tech Ltd., Japan). This minimizes damage to the oocyte. The injection needle is inserted deeply into the oocyte (Fig. 7D) and the oolemma is punctured by activating a single piezo pulse to inject sperm into the cytoplasm. These ICSI procedures are performed on a plate stage maintained at 38 °C.

Embryo Transfer (ET)

ET is the critical final procedure used to obtain genetically modified marmosets. One of the differences between marmoset and human ART is that in marmosets, adoptive parents are used for embryo transplantation. Adoptive marmoset parents cannot conceive because the adoptive father has previously been subjected to a vasectomy. ET female recipients are selected 2–6 days after an ovulation day which is identified by a plasma progesterone concentration elevated above 10 ng/ml.

Marmosets are anesthetized for nonsurgical ET, as described in previous studies [22, 23]. First, to avoid injury to the recipient marmoset's vagina and to ensure ease of access to the uterus, a glass tube (Fig. 8A: V, VI, or VII) is inserted into the vagina and fitted to the uterine cervix. Second, a long cannula (Fig. 8A: IV; Fluon ETFE 20-G, 108 mm), combined with a stainless steel stylet (Fig. 8A: I; 23-G, 120 mm long blunt-end), is inserted into the uterus via the glass tube. Then, the inner stainless steel stylet is removed and replaced with a dummy catheter (Fig.

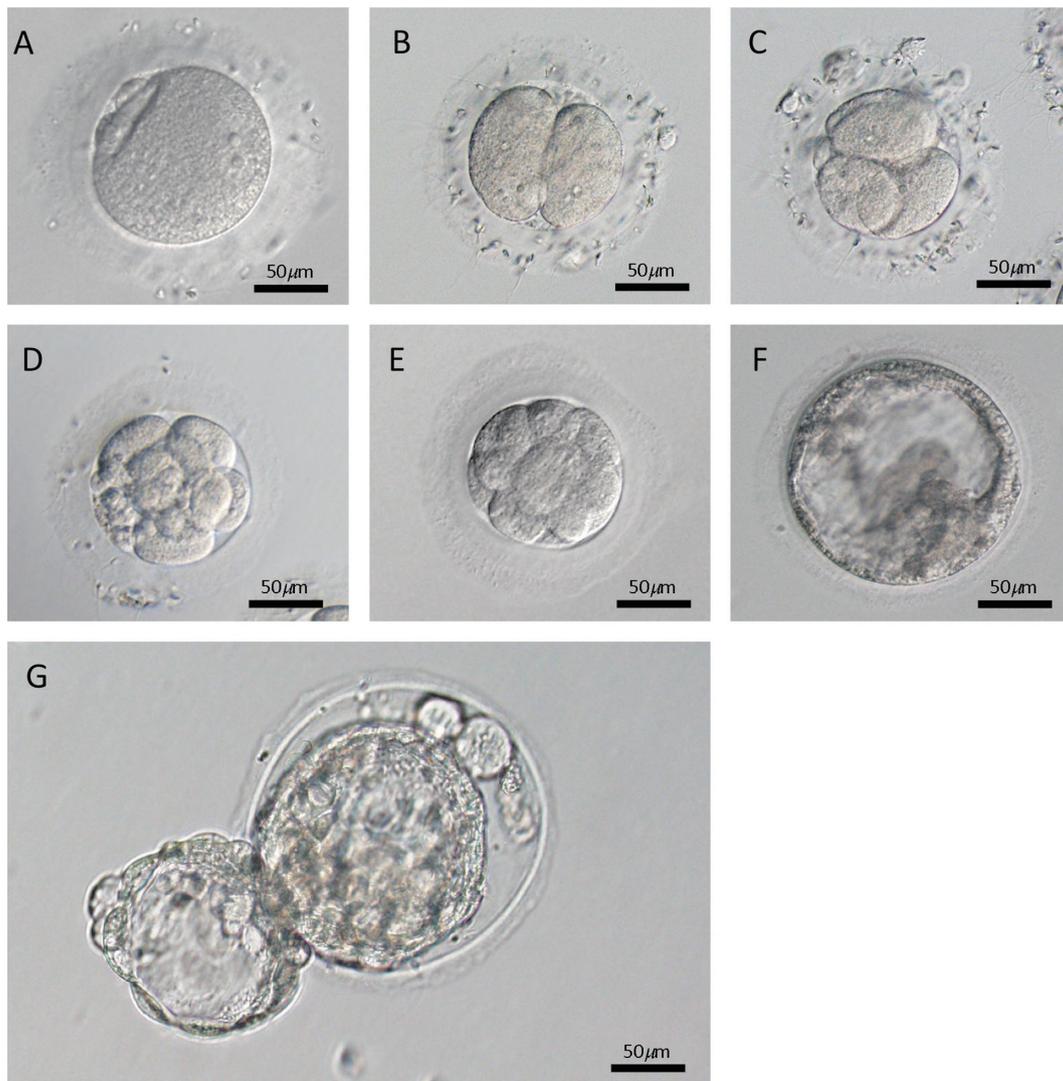


Fig. 5. Fertilized eggs and pre-implantation embryos in marmosets. (A) Pronuclear stage (1 day after IVF). The light area in the cytoplasm contains male and female pronuclei and two polar bodies. (B) Two-cell stage (evening of the first day after IVF). (C) Four-cell stage (2 days after IVF). (D) Eight-cell stage (3 days after IVF). At this stage, the medium must be changed from Cleav medium to Blast medium. (E) Compact morula stage. The borders between blastmeres are not as clearly defined in this image as in D. (F) Blastocyst stage showing some lineage, i.e., the trophectoderm (TE), and inner cell mass (ICM). (G) Hatching blastocyst stage. The blastocyst is hatching from the zona pellucida and preparing for implantation in the uterus.

8A: II; inner diameter: 0.28 mm, outer diameter: 0.61 mm) (Fig. 8B). Ultrasound via the abdomen is used to confirm that the dummy catheter has entered the uterus. The dummy catheter is then removed from the long cannula and replaced by the embryo-loaded (1–3 embryos) ET-catheter (Fig. 8A: III) with a Hamilton syringe. Embryos of all stages are simultaneously injected into the uterus, and ultrasound is used to confirm the location of the embryo by the air accompanying the pre-implantation embryos. Even if cleavage-stage embryos, such as those

at the 8-cell stage, are transfused into the uterus of primates, including humans, pregnancy can be established, unlike in mice.

Discussion and Conclusion

This paper described our approach to reproductive technology in marmosets. While basic techniques for reproductive technologies have been established in the marmoset, various issues need to be resolved. Improve-

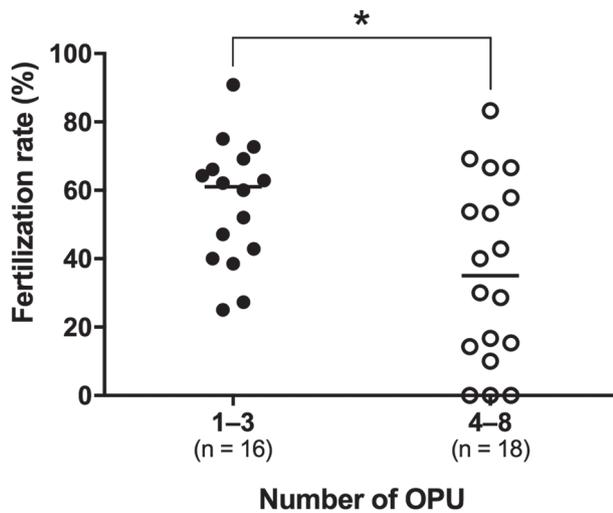


Fig. 6. Effects of OPU repetition on the fertilization success rate. A significant difference was found between the first and third collections (n=16) and the fourth and eighth collections (n=18; $p < 0.05$). It is clear from this analysis that repeated OPU from marmosets significantly decreases the fertilization rate.

ments in ovarian stimulation and IVF protocols will be needed to achieve greater efficiency in genetically modified marmoset production. Figs. 4 and 6 show reduced numbers of collected oocytes and rates of IVF fertilization repetitive OPU, indicating that OPU may cause damage to the physical properties and quality characteristics of oocytes. Therefore, further optimization of ovarian stimulation in repetitive OPU in marmosets is needed to improve follicle growth and oocyte quality, in order to improve the efficiency of marmoset reproductive technologies.

Recent advances in mouse ovarian stimulation methods have been exciting. Since the discovery that anti-inhibin serum (AIS) promotes follicular development, AIS has been used in high-yielding superovulation protocols [24–26]. Several papers have reported that AIS also increases the numbers of oocytes in other animals, e.g., golden hamsters, cows, guinea pigs, and rats [27–30]. These studies have shown that FSH-inhibin interactions occur during the follicular phase of the ovarian cycle. Inhibins are gonadal proteins that act to suppress exces-

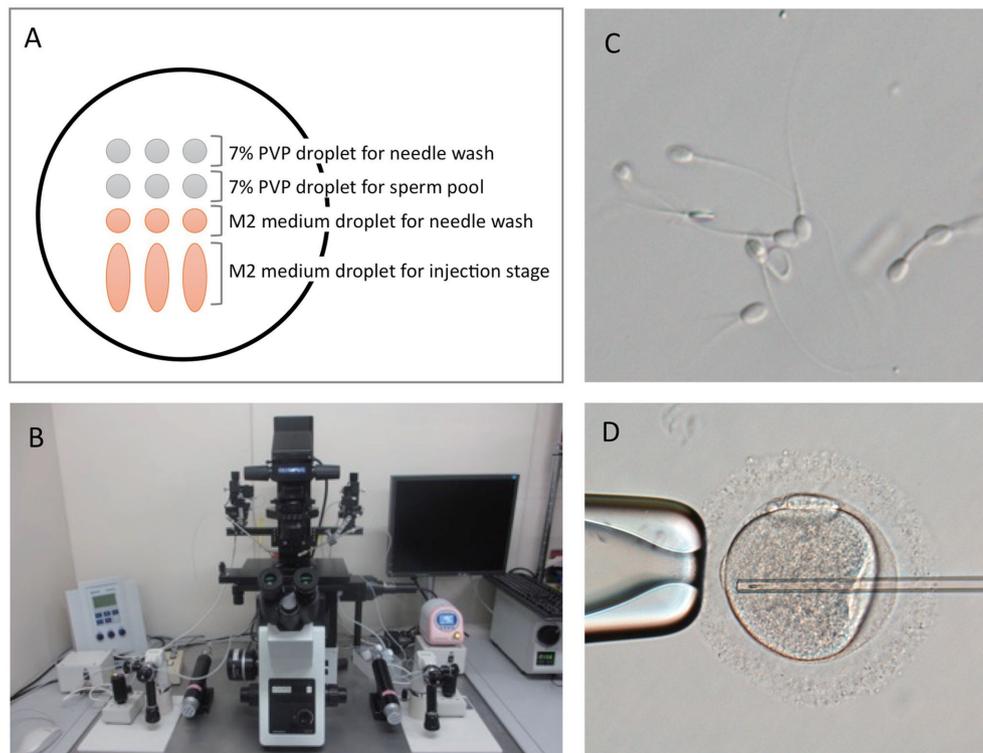


Fig. 7. Intracytoplasmic sperm injection (ICSI). (A) Arrangement of the ICSI dish. Commercial 7% polyvinylpyrrolidone and M2 medium can be used for marmoset ICSI. (B) Piezo-manipulation system. (C) Marmoset sperm. Marmoset and human sperm have similar shapes. (D) The injection needle is inserted deep into the oocyte. The oolemma can be punctured by activating a single piezo pulse to inject sperm into the cytoplasm. These ICSI procedures are performed on a plate maintained at 38 °C.

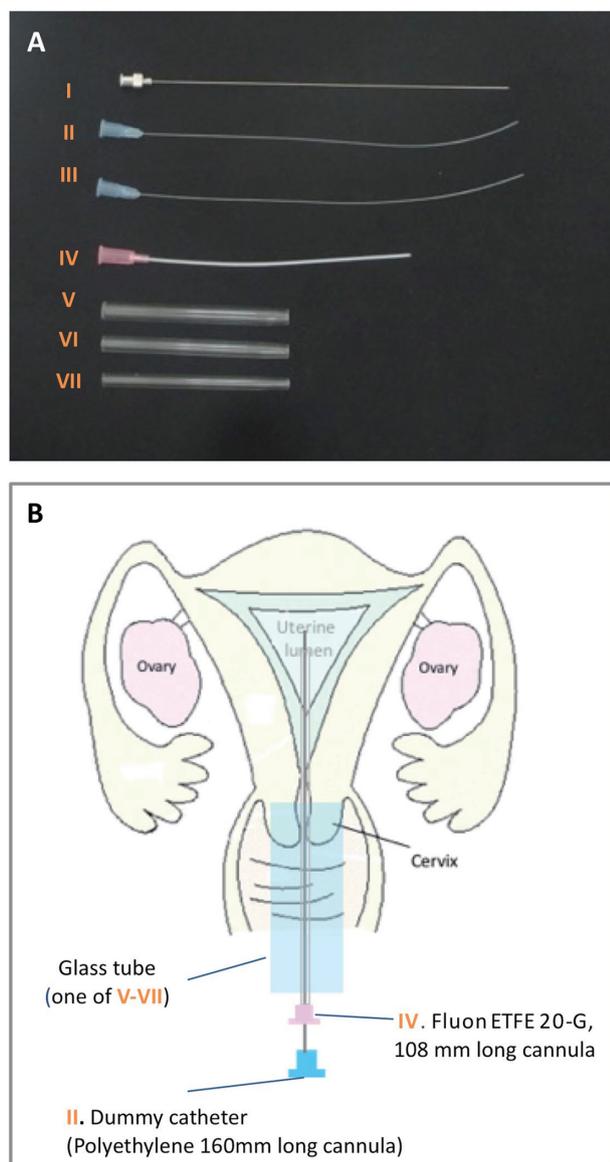


Fig. 8. Embryo transfer (ET). (A) First, a glass tube (one of V–VII) is inserted into the vagina and fitted to the uterine cervix. Second, a long cannula (IV), with a stainless steel stylet (I), is inserted into the uterus via the glass tube. Then, the inner stylet is removed and replaced with a dummy catheter (II). Abdominal ultrasonography is used to confirm that the dummy catheter has reached the uterus. The dummy catheter is then removed from the long cannula and replaced with the embryo-loaded (1–3 embryos) ET-catheter (III) which, using a Hamilton syringe, is inserted into the uterus. (B) This procedure is shown schematically.

sive FSH secretion. As AIS has the ability to suppress inhibins, while endogenous FSH secretion is not suppressed, it is highly effective for ovarian stimulation. This

may also be the case in marmosets, because inhibin is secreted from ovarian follicles during the ovarian cycle in the marmoset [31]. This secreted inhibin may suppress endogenous FSH during ovarian stimulation in marmosets.

Recently, Kawamura et al. reported a new growth follicle treatment for patients with primary ovarian insufficiency (POI) [32, 33]. They showed that disruption of the Hippo signaling pathway, which is essential for maintaining optimal organ size, via fragmented murine ovaries led to the increased expression of the CCN growth factors, CCN2, CCN3, CCN5 and CCN6, consequent promotion of follicle growth, and generation of mature oocytes. In addition, follicle growth was induced when drugs were used to stimulate Akt (protein kinase B) signaling in the ovaries of infertile patients. In our experience with marmosets, physical damage to the ovaries may occur when the follicular oocytes are aspirated with a 25-G needle during OPU. This damage may be repaired by Akt treatment.

The current fertilization rate in marmoset IVF is around 50%. In order to improve this, we are attempting to perform IVF using sperm treated with the antimicrobial protein, defensin, a major family of antimicrobial peptides. Diao et al. found that human recombinant defensin improves sperm motility in patients with asthenozoospermia and leukocytospermia [34]. Sperm motility does not increase when human recombinant defensin is applied to marmoset sperm; however, the fertilization rate improves (data not shown).

Improvements to the efficiency of marmoset reproductive techniques will advance compliance with the “3Rs” for laboratory animals. These are the guiding principles for the ethical use of animals in animal experimentation. They consist of replacement (methods which avoid or replace the use of experimental animals), reduction (methods which minimize the number of animals used per experiment), and refinement (methods which minimize suffering and improve animal welfare). The optimization of repetitive OPU and IVF protocols for marmosets will facilitate a reduction in the number of animals used and achieves refinement of the 3Rs in animal experimentation. Therefore, success in achieving our objectives will both improve reproductive techniques and help advance the aims of the 3Rs.

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