Birth of Healthy Offspring following ICSI in In Vitro- Matured Common Marmoset (Callithrix jacchus) Oocytes

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Abstract

Intracytoplasmic sperm injection (ICSI), an important method used to treat male subfertility, is applied in the transgenic technology of sperm-mediated gene transfer. However, no study has described successful generation of offspring using ICSI in the common marmoset, a small non-human primate used as a model for biomedical translational research. In this study, we investigated blastocyst development and the subsequent live offspring stages of marmoset oocytes matured in vitro and fertilized by ICSI. To investigate the optimal timing of performing ICSI, corrected immature oocytes were matured in vitro and ICSI was performed at various time points (1–2 h, 2–4 h, 4–6 h, 6–8 h, and 8–10 h after extrusion of the first polar body (PB)). Matured oocytes were then divided randomly into two groups: one was used for in vitro fertilization (IVF) and the other for ICSI. To investigate in vivo development of embryos followed by ICSI, 6-cell- to 8-cell-stage embryos and blastocystcs were nonsurgically transferred into recipient marmosets. Although no significant differences were observed in the fertilization rate of blastocysts among ICSI timing after the first PB extrusion, the blastocyst rate at 1–2 h was lowest among groups at 2–4 h, 4–6 h, 6–8 h, and 8–10 h. Comparing ICSI to IVF, the fertilization rates obtained in ICSI were higher than in IVF (p<0.05). No significant difference was noted in the cleaved blastocyst rate between ICSI and IVF. Following the transfer of 37 ICSI blastocysts, 4 of 20 recipients became pregnant, while with the transfer of 21 6-cell- to 8-cell-stage ICSI embryos, 3 of 8 recipients became pregnant. Four healthy offspring were produced and grew normally. These are the first marmoset offspring produced by ICSI, making it an effective fertilization method for marmosets.

Introduction

The common marmoset (Callithrix jacchus) is a small New World primate that has been used in biomedical research because of its physiological similarity to humans, its small body size, and its prolificacy. Marmosets show specific reproductive characteristics including a relatively short gestation period (about 144 days), reaching sexual maturity at 12–18 months, and female marmosets ovulate two or three oocytes in each ovarian cycle [1]. Utilization of this reproductive efficiency and the successful production of transgenic marmosets with germine transmission has been reported [2] and can be applied to genetically modified non-human primate models in life sciences [3].

In vitro production techniques of preimplantation embryos increase our understanding of the physiology of early embryonic development and improve animal production. In marmosets, in vitro fertilization (IVF) with fresh ejaculated sperm has achieved over 50% fertilization rates [4–6]. However, IVF requires high-quality and abundant sperm, which are difficult to obtain from fertile males, and it often gives rise to polyspermic embryos. To avoid these problems, intracytoplasmic sperm injection (ICSI) could be applied as an inseminating technique in marmosets. The ICSI procedure has improved assisted reproduction technologies in rabbits [7], cattle [8,9], mice [10], rhesus monkeys [11], and humans [12,13], providing opportunities to investigate fundamental aspects of fertilization, such as the mechanisms of gamete interaction and sperm-induced oocyte

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activation. Furthermore, ICSI is a useful technique for efficient animal production of genetically modified animals or infertile male animals. This technology could be applied to transgenic animals via intracytoplasmic sperm injection-mediated transgenesis (ICSI-Tr) [14]. ICSI-Tr can be used to insert very large DNA fragments into the host genome. Currently, in non-human primates, the generation of transgenic animals has been reported using only lentiviral systems, which have limited insertional DNA sizes. ICSI-Tr can be applied to create various transgenic animal models, such as marmosets.

In marmosets, blastocyst stage embryos have been produced by ICSI and IVF using in vivo-matured oocytes [5]. However, since the production of offspring from the ICSI embryos has not been reported, the developmental competence to neonate of ICSI embryos remains unknown. Furthermore, ICSI embryos from in vivo-matured oocytes have not yet been described.

Oocyte maturation depends on nuclear meiotic progression and is influenced by a quality and maturity of ooplasm [15,16]. Previous studies observed frequent chromatin aberrations and decreasing developmental rates of the blastocyst stage when sperm insemination to oocytes was performed immediately after MII arrest [17,18]. Therefore, essential cytoplasmic changes may occur during the MII arrest period, and successful embryo development depends on the proper timing of oocyte maturation, as well as oocyte fertilization.

This study aimed to determine the suitable timing of sperm injection into oocytes after in vitro maturation (IVM) and to investigate developmental competence of blastocysts of ICSI embryos using in vivo-matured oocytes in marmosets. Finally, we performed a quality evaluation of ICSI embryos using embryo transfer, reporting the first birth of a normal infant from ICSI embryos.

Table 1. List of primers and their sequences in multiplex PCR.

| Name   | Sequence of the labeled primer (5’-3’) | Dye     | Name   | Sequence of the non-labeled primer (5’-3’) |
|--------|--------------------------------------|---------|--------|------------------------------------------|
| 2463P-TH | GCACAGGCAGATTTCAAGACAACCTC           | FAM     | 2463P-TH-NL | CCAAGACCTCAAGGAGGTAGTACC |
| CJ060  | TGCTAGGTGCCTCCACTGT                 | PET     | CJ060-NL | GGCATGTCTAGCTAACCCTCTG |
| CJ077  | ATTCATTCTGAGGCAAGAAG                | PET     | CJ077-NL | CTCCTCATACAGATGAGGA |
| CJ081  | TTCCCCCTCCTTCTCAGACACA              | VIC     | CJ081-NL | CACCTCTCTTCAGTAACACCC |
| CJ103  | CCCCCTCTCGTAACTCAACAGAAG            | VIC     | CJ103-NL | CTGGGTAACCAGATTGAACTC |
| CJ187  | TGGAGAAGAATCTTGTGCCAACCC           | PET     | CJ187-NL | GCTTGTCAGCGACGATGAG |
| CJ003  | AGATTGGGAGATGTCTTG                   | PET     | CJ003-NL | TCTCTGCAATAGTGAACCTC |
| CJ083  | TTTGCCCTTTGGCTGAC                    | VIC     | CJ083-NL | TTCTCTTTTTGGGAGTGT |
| CJ091  | CCTGCACCCGTAAAATAGGTTCC            | PET     | CJ091-NL | CATCCTGGCGCAAAGAGTG |
| CJ146  | CTTAAATCTCCAGTTAGACAC           | PET     | CJ146-NL | GAGAGTCCAATATGCAAGGA |

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Figure 1. ICSI of marmoset oocytes after in vitro maturation. (A) The zona pellucida was drilled using piezo pulses, and the pipette was inserted deep into the oocyte and a single piezo pulse was applied. Sperm were inserted into the oocyte (bar = 100 μm). (B) Marmoset blastocysts produced by ICSI. (bar = 100 μm).
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Materials and Methods

Animals
A total of 66 adult marmosets, purchased from a marmoset breeding company for experimental animals (CLEA Japan Inc., Tokyo, Japan), were used in this study. The body weights and ages of the marmosets ranged from 284–534 g and 2–8 years, respectively. The animals were not sacrificed for the current experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Central Institution for Experimental Animals (CIEA) (CIEA approval no: 11028A) and were performed in accordance with the CIEA guidelines that agree with the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan (2006). Animal care was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). The marmosets were housed in pairs in stainless steel living cages (39x65x70 cm) with wire mesh floors maintained at 25–26°C with 45–55% humidity and illumination for 12 h per day. Wood perches for locomotion and gouging and a platform for a bed were placed in each cage for environmental enrichment. Marmosets were kept healthy and well-nourished with a balanced diet (CMS-1M; CLEA Japan Inc.), including mixed L (+)-ascorbic acid (Nacalai Tesque, Tokyo, Japan), vitamins A, D3, and E (Duphasol AE 3D; Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan), and honey (Nihonhatimitsu Co., Ltd., Gifu, Japan). In addition, chicken liver boiled in water (DBF Pet Co., Ltd., Niigata, Japan) was given as a supporting meal once a week. The animals were supplied with tap water ad libitum from feed valves.

Anesthesia and Postoperative Care
Animals were pre-anesthetized with an intramuscular injection of 0.04 mg/kg of medetomidine (Domitor; Nippon Zenyaku Kogyo, Koriyama, Japan), 0.40 mg/kg of midazolam (Dormicam 10 mg; Astellas Pharma, Tokyo, Japan), and 0.40 mg/kg of butorphanol (Vetorphale; Meiji Seika Pharma, Tokyo, Japan). They were also administered 15 mg/kg of ampicillin (Viccillin; Meiji Seika Pharma, Co., Ltd.) and hydrated subcutaneously with 2 mL/head of fluid (KN No.1 injection; Otsuka Pharmaceutical, Tokyo, Japan). Thereafter, animals were anesthetized by inhalation with 1.0–3.0% isoflurane (Forane; Abbott Japan, Tokyo, Japan) via a ventilation mask. Anesthetization management was performed by spontaneous respiration during the operation, monitoring the heart rate and the arterial oxygen saturation. After oocyte collection or embryo transfer, 0.20 mg/kg of atipamezole (Antisedan; Nippon Zenyaku Kogyo) was administered intramuscularly into the animals. For postoperative analgesia and infection control, 1.2 mg/kg ketoprofen and 15 mg/kg ampicillin were administered once daily for 3 consecutive days following the operations.

Ovarian Stimulation and Collection Oocytes
Thirty-three donor female adult marmosets were used in the present study. For oocyte collection from ovaries, donor female marmoset ovarian stimulation was performed using the follicular stimulation protocol, as described previously [2]. Briefly, ovarian cycles were monitored based on plasma progesterone levels using an enzyme immunoassay (EIA) kit (TOSO Progesterone Kit; TOSO, Tokyo, Japan). Luteolysis was induced with 0.8 mg of cloprostenol, an analog of prostaglandin F2α (Estrumate; Schering-Plough Animal Health, Union, NJ), which was administered intramuscularly into the animals. For postoperative analgesia and infection control, 1.2 mg/kg ketoprofen and 15 mg/kg ampicillin were administered once daily for 3 consecutive days following the operations.

Table 2. Fertilization and developmental rates after ICSI of in vitro-matured marmoset oocytes.

| ICSI time post-first PB extrusion | No. of oocytes | No. (%)*1 of fertilized oocytes | No. (%)*2 of embryos developed to |
|---------------------------------|----------------|---------------------------------|---------------------------------|
|                                 |                | No. (%) | 2-cell | 8-cell | Blastocyst |
| 1–2 h                           | 23             | 20 (87) | 18 (90) | 14 (70) | 1 (5) |
| 2–4 h                           | 20             | 19 (95) | 18 (94.7) | 16 (84.2) | 6 (31.6) |
| 4–6 h                           | 25             | 25 (100) | 25 (100) | 20 (80) | 4 (16.0) |
| 6–8 h                           | 16             | 15 (93.8) | 15 (93.8) | 13 (86.7) | 4 (26.7) |
| 8–10 h                          | 20             | 20 (100) | 20 (100) | 16 (80) | 4 (20) |

*1Numbers in parentheses were calculated from total oocytes.
*2Numbers in parentheses were calculated from fertilized oocytes.

Table 3. Effect of fertilization method on in vitro development of marmoset oocytes.

| No. of matured oocytes | No. (%)*1 of Fertilized oocytes | No. (%)*2 of embryos developed to |
|------------------------|---------------------------------|---------------------------------|
|                        | No. (%) | 2-cell | 8-cell | 16-cell | Morula | Blastocyst |
| ICSI                   | 88      | 82(93.2)* | 80(97.6) | 63(76.8) | 43(52.4) | 32(39.0) | 29(35.4) |
| IVF                    | 90      | 74(82.2)* | 69(93.2) | 62(83.8) | 48(64.9) | 36(48.6) | 29(39.2) |

*1Numbers in parentheses were calculated from total oocytes.
*2Numbers in parentheses were calculated from fertilized oocytes.

Values within the same column with different letters (a, b) differ significantly (p<0.05), χ²-test.
the day after PGE₂ injection. Marmoset follicles were stimulated by intramuscular injection of recombinant human follicle-stimulating hormone (rhFSH; 50 international units [IU]; FOLYRMON – P injection; Fuji pharma Co., Ltd, Tokyo, Japan) for 9 days at 10:00, and human chorionic gonadotropin (hCG; 75IU; Gona-tropin; ASKA Pharmaceutical. Co. Ltd., Tokyo, Japan) was administered on the day of the ninth FSH administration at 17:30. Thirty hours after hCG injection, animals were anesthetized as described above and follicular aspiration was performed. Oocytes were collected in porcine oocyte medium (POM; Research Institute for the Functional Peptides, Yamagata, Japan) [19].

### In Vitro Maturation (IVM)

Collected oocytes were washed three times with mPOM (POM supplemented with 5% heat-inactivated calf serum (FBS; Gibco, Carlsbad, CA) and 100 IU/mL FSH (Folyrmon-P; FujiPharma, Tokyo, Japan). In vitro oocyte maturation was performed by incubation in an 80 μL mPOM drop covered with mineral oil (Nacalai Tesque, Tokyo, Japan) under a gas phase of 5% CO₂, 5% O₂, and 90% N₂ at 37.5°C. To measure the suitable ICSI timing after the first PB extrusion, cumulus cells were degraded with 1 mg/mL hyaluronidase (Sigma) for 1 min after 19 h of IVM, oocytes were changed to independent cultures and the first PB extrusion was observed. The oocytes from each animal were divided into two groups, IVF and ICSI. For ICSI, the cumulus cells were completely degraded with hyaluronidase. Twenty-seven hours after the IVM, ICSI and IVF were performed.

### Sperm Collection and Preparation

Eight male adult marmosets were used in the present study. Of the eight male animals, three were selected based on their physical conditions for each experiment. Marmoset semen was collected as described previously [20]. The animal was restrained in an upright position, and penile vibratory stimulation was performed using a FeriCare personal vibrator (Fertility Healthcare and Supplies, Inc., Silverado, CA, USA) in three sexually mature donor animals. Pre-equilibrated 700 μL TYH medium (Mitsubishi Chemical Medience Corp., Tokyo, Japan) was immediately added to the ejaculate and the suspension was incubated at 37.5°C for approximately 30 min to disperse sperm from the coagulum. The semen was washed twice with 700 μL of TYH medium by aspiration of the supernatant after centrifugation for 5 min at 400×g, after which 200 μL of fresh TYH medium was added. The resuspended sample was then supplied to the bottom of a conical tube containing 500 μL fresh TYH. A total of 700 μL TYH with sperm suspension was incubated for 30 min at 37.5°C under a gas phase of 5% CO₂, 5% O₂, and 90% N₂ to allow the sperm to swim upwards. After incubation, 400 μL of supernatant was collected and the quality of sperm samples was measured using the Sperm Motility Analysis System (SMAS; Ditect, Tokyo, Japan) based on viability, motility, and concentration. Samples of the highest quality were chosen for IVF and ICSI, and the sperm suspension was divided into two samples. For the IVF study, the sperm suspension was adjusted to a final sperm concentration of 5.6x10⁶ sperms/mL and then into 30 μL drops. For the ICSI study, the sperm suspension was washed with TYH and centrifuged for 5 min at 400×g. After washing, the sperm pellet was resuspended in M2 medium (Sigma, St. Louis, MO) and adjusted to a final sperm concentration of 1x10⁵ sperms/mL.

### Intracytoplasmic Sperm Injection (ICSI)

An oil-covered micromanipulation chamber containing a 20 μL drop of M2 for oocytes and a 10 μL drop of M2 medium containing 10% polyvinyl pyrrolidone (PVP; Sigma) for spermatozoa was prepared. Approximately 1 μL of sperm suspension was transferred into M2 medium containing 10% (w/v) PVP drops and mixed thoroughly. One to three oocytes were placed in the M2 medium drop. Using an inverted microscope with micromanipulators (Narishige, Tokyo, Japan), only motile sperm with normal morphology were selected and immobilized by pressing the tail with the injection needle tip against the dish bottom prior to injection. A single sperm was aspirated from the sperm drop and moved to a droplet containing oocytes. An oocyte was captured by the holding pipette and immobilized with its PB at either the 6 or 12 o’clock position, and the zona pellucida was drilled using piezo pulses. The pipette was inserted deeply into the oocyte and a single piezo pulse was applied. A spermatozoon was then injected into the cytoplasm. After ICSI, oocytes were placed in ISM1 medium drops (Medicult; Nosan Corp., Kanagawa, Japan) and washed three times. The day of performing ICSI was designated as day 0.

### In Vitro Fertilization (IVF)

After sperm preparation, the matured oocytes were washed three times with 50 μL of TYH medium drops. The oocytes were transferred into 30 μL drops of TYH medium containing 3.6x10⁶ sperms/mL and incubated for 18 h under a gas phase of 5% CO₂, 5% O₂, and 90% N₂ at 37.5°C. After 18 h, embryos were washed three times with 70 μL ISM1 drops. The day of performing IVF was designated as day 0.

### In Vitro Culture (IVC)

Human embryo culture medium ISM 1 and 2 (ORIGIO, Målov Denmark) were used in this study to culture marmoset embryos. Pronuclear formations of embryos followed by IVF or ICSI were confirmed under microscopic observation. Embryos...
Figure 2. Devices for nonsurgical embryo transfer. Newly developed devices for nonsurgical embryo transfer. (A) I: 23-G, 120-mm-long blunt-end stainless steel stylet II: polyethylene 160-mm-long cannula (inner diameter 0.28 mm, outer diameter 0.61 mm) for embryo transfer, III: Fluon ETFE 20-G, 108-mm-long cannula (A blunt/tapered cannula; inner diameter 0.8 mm, outer diameter 1.10 mm), IV: endoscope for small animals, V: tapered to 4.2 mm, 6.0 mm in diameter and tapered to 5.0 mm, 7.0 mm in diameter and tapered to 5.3 mm at one end of the glass tubes for vaginal dilation and manipulation of the cannulae. (B) Scheme for nonsurgical embryo transfer.

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were transferred in 70 μL fresh drops of ISM1 and cultured under mineral oil cover at 37.5°C in 5% CO₂, 5% O₂, and 90% N₂ for 3 days. On day 3, embryos were transferred to 70 μL of ISM2 drops covered with mineral oil and were placed in the incubator at 37.5°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ until day 12. The media exchange and checking of embryo development were performed every 2 days. The embryos that developed to blastocysts were examined at days 9, 10, and 11.

Non-surgical Embryo Transfer

Twenty-eight recipient female adult marmosets were used in the present study. The ovarian cycles of donor and recipient animals were synchronized using PGF₂α, and ovarian cycles were monitored based on plasma progesterone levels. The embryos produced by ICSI were transferred to surrogate mothers using nonsurgical embryo-transfer techniques, as described previously with modified instruments [21]. The three different diameter-sized, 7.5-cm-long glass tubes were newly developed for the noninvasive embryo transfer technique. The thinnest glass tube size was tapered to 4.0 mm with a 4.2 mm diameter, the middle size glass tube was tapered to 5.0 mm with a 6.0 mm diameter, and the widest size glass tube was tapered to 5.3 mm with a 7.0 mm diameter at one end. The Fluon ETFE 20-gauge (G) was comprised of a cannula 108 mm in length (a blunt/tapered cannula; 0.8-mm inner diameter and 1.10-mm outer diameter; Oviraptor; Altair Corp., Yokohama, Japan). The 23-G had 120-mm-long blunt-end stainless steel stylets and a polyethylene 160-mm-long cannula (inner diameter 0.28 mm and outer diameter 0.61 mm; Oviraptor; Altair Corp.). These devices were developed to reduce invasiveness to the animals and improve the insertion of the cannula into the uterus. All instruments were sterilized before use with a hydrogen peroxide gas plasma sterilizer (Sterrad 50; Advanced Sterilization Products, Irvine, CA), and the surgeon and surgical assistants wore sterile surgical gloves.

Vaginal dilation of the anesthetized recipients was performed gradually by serial introduction and removal of three sizes of 75-mm-long glass tubes (from thinnest to widest). The widest glass tube was placed in the vagina to manipulate the endoscope and cannula to prevent vaginal injury. An endoscope (1.6 mm in

Figure 3. First offspring produced by ICSI in marmosets. Four healthy neonates were generated using ICSI. A female (A) 635 produced by blastocyst transfer and a male neonate (B) 732, male (C) 737 and female neonate (D) 640 from 6-cell- to 8-cell stage embryo transfer. doi:10.1371/journal.pone.0095560.g003
diameter; TESALA AE-C1; AVS Co., Ltd., Tokyo, Japan) was
inserted into the glass tube to observe the ostium uteri externum. A
blunt/tapered Fluon ETFE 20-G outer cannula combined with a
23-G, 120-mm-long blunt-end stainless steel stylet were inserted
into the cervix via the glass tube. After inserting the Fluon ETFE
20-G outer cannula/23-G blunt-end stainless steel stylet into the
uterus, the blunt inner stainless steel stylet was removed. At this
time, the polyethylene cannula was inserted into the Fluon ETFE
20-G outer cannula as the dummy inner cannula. The uterus was
observed using linear ultrasound probe (Prosound 7: Hitachi
Aloka Medical, Ltd., Tokyo, Japan) by longitudinally placing it
onto the abdomen to confirm insertion of the dummy inner
cannula via the outer cannula into the uterus. Approximately 2 µL
of medium containing one to three embryos was loaded into a new
polyethylene cannula that attached to a 50-µL glass syringe
(Hamilton Co., Reno, NV). After removing the dummy inner
cannula, the inner catheter containing the embryos was inserted
into the outer cannula. When the inner catheter was approxi-
mately 3 mm from the distal end of the uterus, the outer cannula
was pulled back until the cannula tip reached the proximal end
of the uterus from the cervix. The embryos were then delivered into
the uterine lumen by gentle pressure on the glass syringe. The
cannula and catheter were withdrawn after embryo transfer and
washed with medium to confirm that no embryos remained in
the cannula and catheter.

The recipients were tested for pregnancy based on plasma
progesterone measurements once a week until the time at which
pregnancy could be monitored using ultrasonography in the
uterus.

The blastocyst stage embryos underwent embryo transfer
12 days after ICSI and the 6-cell- to 8-cell-stage embryos were
transferred into a surrogate mother uterus at 5 days after ICSI.

Genotyping of Neonates Using Microsatellite Markers

Parental testing based on microsatellite polymorphisms for the
delivered offspring was performed using 10 microsatellite markers
(Table 1). Genomic DNA was extracted from hair root for live
animals or frozen skin for dead animals using the DNA Micro Kit
(Qiagen KK, Tokyo, Japan). To detect microsatellite polymor-
phisms, polymerase chain reaction (PCR) amplification was
performed using the extracted genomic DNA as template. The
PCR product was loaded directly on an ABI 3130 Genetic
Analyzer (Life Technologies, NY, USA) along with the GS500 LIZ
dye Size Standard (Life Technologies, NY, USA). The electro-
phoresis data was processed using GeneMapper 4.0 software (Life
Technologies), and alleles were assigned according to the PCR
product size.

Statistical Analysis

To evaluate differences between experimental groups, a χ² - test
was performed. Differences at p<0.05 were considered significant.

Results

Fertilization Competence after PB Extrusion

PB extrusions were observed approximately 20–24 h after IVM.
To determine the optimal timing of performing ICSI, 104 in vitro-
matured oocytes derived from 12 female marmosets were divided
into five groups and subjected to ICSI at various time points: 1–
2 h, 2–4 h, 4–6 h, 6–8 h, and 8–10 h after extrusion of the first PB
(Figure 1A, Table 2). Although no significant differences were
observed in the fertilization and blastocyst rate, the blastocyst rate
tended to be low among the groups when ICSI was performed at

Table 5. Pregnancy and birth rates following nonsurgical embryo transfer to recipients of common marmoset embryos produced by ICSI.

| Embryo stage at transfer | No. of Embryos transferred | No. of recipients | No. (%)*1 of pregnant recipients | No. (%)*2 of offspring born | No. (%)*2 of dead offspring after birth |
|--------------------------|---------------------------|------------------|----------------------------------|---------------------------|----------------------------------------|
| 6-cell–8-cell (day 5)    | 21                        | 8                | 3 (37.5)                         | 3 (37.5)                  | 0 (0)                                   |
| Blastocyst (day 12)      | 37                        | 20               | 4 (20)                           | 1 (5)                     | 3 (15)                                 |

*1Numbers in parentheses were calculated from total recipients.
*2Numbers in parentheses were calculated from total embryos.

Values within the same column with different letters (a, b) differ significantly (p<0.05, χ²-test).

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Table 6. Details of offspring produced by ICSI.

| Transferred embryo (stage/number) | The neonates ID | Body weight (g) | Status after birth |
|-----------------------------------|----------------|-----------------|-------------------|
| Blastocyst/2                      | 635            | 36              | alive             |
| 8-cell/3                          | 731            | 27.7            | dead on day 5     |
|                                   | 732            | 25.6            | alive             |
|                                   | 733            | 24.6            | dead on day 16    |
|                                   | 734            | ND              | dead on day 1     |
| 8-cell/2                          | 737            | 32              | alive             |
| 8-cell/2, 6-cell/1                | 640            | 32              | alive             |

1–2 h after PB extraction (5.0%, 31.6%, 16.0%, 26.7%, and 20.0%, respectively).

Developmental Competence of Oocytes Fertilized by ICSI or IVF

To compare embryo developmental competencies, ICSI and IVF were performed (Table 3). Since relatively high embryo development was shown beyond 2 h after PB extrusion, ICSI and IVF were performed after 27 h following IVM that fit to 3–7 h after PB extrusions. In total, 178 in vitro-matured oocytes derived from 21 female marmosets were divided into two groups for ICSI or IVF. The fertilization and developmental rates during the blastocyst stage of ICSI and IVF embryos are shown in Figure 1B and Table 3. The fertilization rate of the ICSI embryos (93.2%) was significantly higher than that of the IVF embryos (82.2%, p<0.05). No significant differences in developmental rate were observed between ICSI and IVF embryos (35.4% and 39.2%, respectively) in the blastocyst stage.

The length to reach the blastocyst stage of embryos using ICSI and IVF are shown in Table 4.

Although the developmental speed to blastocyst was not significantly different, ICSI embryos tended to develop later than IVF embryos.

Developmental Abilities of the Embryos to Neonates

To assess the in vivo developmental potential of ICSI-derived blastocysts (Figure 1B), 37 blastocysts after 12 days of culture were transferred into 20 recipient surrogate mothers by nonsurgical embryo transfer (Figure 2). Four of 20 recipients receiving blastocysts produced by ICSI were pregnant. Although one normal and healthy offspring (Figure 3, Table 5, Table 6) was delivered, other recipients interrupted pregnancy by spontaneous abortion at days 83, 104, and 105. Therefore, to investigate whether the in vivo developmental potential of relatively early ICSI embryos was higher, 21 embryos from the 6-cell- to 8-cell stage after 5 days of culture were transferred into eight recipients (Table 5). We found that three of eight recipients were pregnant and six offspring were delivered. Although three offspring were dead a few days after delivery by caesarean section, the other three offspring grew to be healthy. The birth rate of the 6-cell- to 8-cell-stage embryo transfer was 28.6%, which was significantly higher than that of the blastocyst stage embryo transfer (2.7%, p<0.05).

Parentage Evaluation Tests Using Microsatellite Markers

The results of genotyping of neonates using microsatellite markers clearly demonstrated that all offspring were derived from donor embryos (Table 7). For offspring 635, the genotype with CJ060-PET, CJ077-VIC, CJ081-VIC, CJ103-NED, CJ003-NED, and CJ083-VIC microsatellite markers demonstrated that this neonatal animal was derived from donors 3464 and 691. The genotype with CJ060-PET, CJ081-VIC, and CJ103-NED microsatellite markers indicated that offspring 731, 732, 733 and 734 was not derived from the recipients, but rather from donors 3525 and 666. Similarly, the paternity testing indicated that offspring 737 and 640 were from the donor animals.

Discussion

The present study is the first to report the birth of common marmoset offspring using ICSI with oocytes matured in vitro. Based on the results of genotyping tests using microsatellite markers, all neonates were derived from the ICSI embryos.

Since ICSI can control the timing of fertilization, the optimal fertilization timing through oocyte maturation was determined. Oocyte nuclear maturation implies re-initiation and completion of the first meiotic division from the GV stage to MII stage. Besides these nuclear aspects of oocyte maturation, cytoplasmic aspects are also important for fertilization and development of the oocyte [22]. These two processes are completely independent events [22–25]. The study of human, mouse, and bovine oocytes indicated that cytoplasmic maturation may occur during MI arrest [17,18]. In humans, IVM oocytes require at least 1 h incubation for cytoplasmic maturation after the first PB extrusion to fertilize by ICSI [26,27]. In this study, the fertilization and embryonic developmental rates of the marmoset ICSI embryos after IVM oocytes at various time intervals following extrusion of the first PB showed no significant differences. Although significant differences were not observed, the blastocyst rate in 1–2 h groups was lowest among the groups, suggesting that the optimal timing of fertilization was more than 2 h after PB extrusion in marmosets. In this study, both ICSI and IVF were performed after 27 h following IVM, which were adjusted to 3–7 h after PB extrusion, and both embryos showed comparable developmental rates. Furthermore, the observation of marmoset oocyte PB extrusions approximately 20–24 h after IVM (data not shown) was consistent with previous reports [4]. The ICSI procedure bypasses the normal fertilization process through IVF of zona penetration and fusion of sperm and oocyte membranes. Thus, the initial steps involved in oocyte activation may also be bypassed. In several species such as mice, humans, and rabbits, ICSI can activate oocytes for further embryonic development, comparable to IVF [10,28,29]. In bovine and porcine embryos, the developmental competencies of embryos followed by ICSI were low. To improve the development of embryos, ICSI has been combined with artificial stimuli, such as exposure to ethanol, ionomycin, 6-
Table 7. Analysis of microsatellite genotypes of donors, recipients, and offspring in embryo transfer of marmoset embryo produced by ICSI.

| Pedigree | The neonates ID | Sex | 2463-TH-FAM | CJ060-PET | CJ077-VIC | CJ081-VIC | CJ103-NED | CJ187-FAM | CJ003-NED | CJ083-VIC | CJ091-FAM | CJ146-PET |
|----------|-----------------|-----|--------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| #1       | Recipient 2370  | Female | 109/109 | 135/146 | 205/205 | 170/184 | 119/121 | 201/201 | 90/94 | 119/125 | 139/145 | 134/134 |
|          | Recipient 3298  | Male | 109/109 | 146/151 | 205/213 | 184/186 | 119/121 | 201/207 | 94/94 | 119/121 | 139/139 | 132/134 |
|          | Donor 3464      | Female | 109/109 | 135/135 | 205/205 | 160/168 | 111/121 | 201/201 | 94/96 | 121/121 | 139/139 | 132/134 |
|          | Donor 691       | Male | 109/109 | 135/135 | 205/209 | 168/186 | 107/117 | 201/207 | 94/94 | 115/121 | 139/139 | 132/134 |
|          | Offspring 635    | Female | 109/109 | 135/135 | 205/209 | 168/186 | 107/117 | 201/207 | 94/94 | 121/121 | 139/139 | 134/134 |
| #2       | Recipient 2343  | Female | 109/109 | 135/135 | 205/211 | 160/186 | 117/117 | 201/201 | 94/98 | 121/121 | 139/139 | 132/132 |
|          | Recipient 659   | Male | 109/109 | 149/151 | 213/213 | 170/170 | 111/121 | 201/209 | 94/96 | 121/125 | 139/147 | 134/134 |
|          | Donor 3525      | Female | 109/109 | 135/137 | 205/213 | 160/186 | 119/125 | 201/209 | 94/94 | 121/121 | 139/139 | 132/134 |
|          | Donor 666       | Male | 109/109 | 135/135 | 205/213 | 168/186 | 119/121 | 201/207 | 94/98 | 121/121 | 139/139 | 134/134 |
|          | Offspring 731    | Male | 109/109 | 135/137 | 213/213 | 186/186 | 121/125 | 201/201 | 94/98 | 121/121 | 139/139 | 132/134 |
|          | Offspring 732    | Male | 109/109 | 135/137 | 213/213 | 186/186 | 121/125 | 201/201 | 94/98 | 121/121 | 139/139 | 132/134 |
|          | Offspring 733    | Male | 109/109 | 135/137 | 213/213 | 186/186 | 121/125 | 201/201 | 94/98 | 121/121 | 139/139 | 132/134 |
| #3       | Recipient 2706  | Female | 109/109 | 135/146 | 205/205 | 170/184 | 117/125 | 201/201 | 90/94 | 119/125 | 139/145 | 134/134 |
|          | Recipient 3346  | Female | 109/109 | 135/139 | 205/205 | 184/186 | 117/117 | 201/201 | 94/98 | 119/121 | 139/139 | 134/134 |
|          | Donor 2703      | Female | 109/109 | 135/146 | 205/205 | 168/186 | 107/115 | 201/207 | 94/98 | 121/121 | 139/139 | 132/134 |
|          | Donor 666       | Male | 109/109 | 135/135 | 205/213 | 168/186 | 117/119 | 201/201 | 94/98 | 121/121 | 139/139 | 134/134 |
|          | Offspring 737    | Male | 109/109 | 135/135 | 205/205 | 168/168 | 115/117 | 201/201 | 94/94 | 121/121 | 139/139 | 134/134 |
| #4       | Recipient 3196  | Female | 109/109 | 135/137 | 205/205 | 160/170 | 107/121 | 201/201 | 90/94 | 121/123 | 139/139 | 132/134 |
|          | Recipient 3343  | Female | 109/109 | 135/135 | 203/205 | 168/168 | 111/121 | 201/201 | 94/98 | 121/123 | 139/139 | 132/134 |
|          | Donor 2703      | Female | 109/109 | 135/146 | 205/205 | 168/168 | 107/115 | 201/207 | 94/98 | 121/121 | 139/139 | 132/134 |
|          | Donor 666       | Male | 109/109 | 135/135 | 205/213 | 168/186 | 117/119 | 201/201 | 94/98 | 121/121 | 139/139 | 134/134 |
|          | Offspring 640    | Female | 109/109 | 135/146 | 205/205 | 168/168 | 107/117 | 201/201 | 94/94 | 121/121 | 139/139 | 134/134 |

*1 Size in bp.
*2 Underlined numbers indicate unique alleles inherited from the donor, and bold numbers indicate the genotypes used to determine whether the offspring were derived from donor animals.
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in vitro embryo transfer may have been the long-term embryo culture recipient uterus. One reason for the low birth rate of blastocyst identical triplets, indicating that one ICSI embryo split in the in vivo reported six days, whereas it takes 10 days in marmosets. A previous study human IVC, embryos reach the blastocyst stage in approximately # approximately eight days after ovulation [32]. The prolonged development period suggested this culture condition was not optimized for marmosets. Consequently, the application of other animal embryo culture conditions would not be adequate for marmosets. Therefore feasible long-term culture conditions for marmoset embryos need further investigation. Additionally, the long incubation period of the embryos after reaching the blastocyst stage on days 9–11 probably affected the birth rate of blastocysts because all embryos were transferred on day 12 and, as described above, the embryo culture condition was not optimized, thus potentially affecting the embryo quality.

The marmoset is the only nonhuman primate that has been used to generate transgenic animals with the lentiviral system and a exogenous gene, which encoded a green fluorescent protein (GFP) and were germine-transmitted [2]. This lentiviral system is the most successful transgenic method to efficiently obtain offspring in several species [33–35]. However, the major drawback of this technique is the limited size of the transgene (up to 8 kb) [36]. To overcome this limitation, ICSI-Tr combined with recombinases or transposases could be a powerful technique to introduce very large DNA transgenes with relatively highly efficient integration into the host genomes [37,38]. This ICSI-Tr technique would be applicable to the marmoset and facilitate transgenesis.

In conclusion, in the marmoset, embryos produced by ICSI using in vitro-matured oocytes could develop to blastocysts and neonates. Several offspring were successfully derived from embryo transfer after ICSI, which is a suitable fertilization method in marmosets.

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Author Contributions

Conceived and designed the experiments: TT KH HS ES. Performed the experiments: TT CY MY ES. Analyzed the data: TT CY MY ES. Contributed reagents/materials/analysis tools: TT TI KS AS JO HS CY. Wrote the paper: TT TI HS HO MS ES.

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