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Original Article

Successful blastocyst production by intracytoplasmic injection of sperm after *in vitro* maturation of follicular oocytes obtained from immature female squirrel monkeys (*Saimiri boliviensis*)

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Abstract. Advanced reproductive technologies are being applied for the propagation of squirrel monkeys, to ensure their preservation as a genetic resource and the effective use of their gametes in the future. In the present study, oocytes and spermatozoa were collected from live squirrel monkeys, following which piezo intracytoplasmic sperm injection (ICSI) was performed using these gametes. Follicular development was induced by administering equine chorionic gonadotropin (eCG) containing inhibin antiserum to an immature squirrel monkey female. The unilateral ovary was excised after the administration of human chorionic gonadotropin (hCG), to induce ovulation, following which the larger developed follicular oocytes were collected. Follicular oocytes were prepared for ICSI using sperm from the epididymal tail of a unilateral testis extracted from a mature male. The embryos were continuously incubated in CMRL 1066 medium supplemented with 10% (v/v) fetal bovine serum. Embryo culture was performed with cumulus cells. Two experiments of ICSI carried out with three females resulted in 14 mature oocytes from the 49 cumulus-oocyte complexes collected and five embryos, three of which developed into blastocysts. These blastocysts were vitrified, thawed, and transferred to recipient monkeys, but no pregnancies resulted. In conclusion, the present study is the first to successfully produce ICSI-derived blastocysts from MII oocytes obtained by means of hormone administration (a combination of eCG+inhibin antiserum and hCG) and *in vitro* maturation in immature squirrel monkeys.

Key words: Blastocyst, *In vitro* maturation of oocytes, Intracytoplasmic sperm injections, Squirrel monkey (*Saimiri boliviensis*), Superovulation

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The Bolivian squirrel monkey (*Saimiri boliviensis*) is a new world monkey native to South America. At the Amami Laboratory of the Institute of Medical Science, University of Tokyo, located on the warm Amami Oshima Island, new world monkeys, owl monkeys (*Aotus lemurinus*), and Bolivian squirrel monkeys are bred annually. Owl monkeys are annual breeders with an oestrus cycle of approximately 17 days [1]. In contrast, squirrel monkeys breed seasonally, from November to March, with an estrus cycle of approximately 10 days [2]. Squirrel monkeys are used as home-breeding monkeys for a variety of experiments at Amami Laboratory. For example, because squirrel monkeys exhibit spontaneous hypercortisolemia, these were used in a study [3] that compared the histocytologic specificity of the adrenal cortex between squirrel monkeys and owl monkeys. Owing to the well-known susceptibility of squirrel monkeys to *Plasmodium*, these have also been used as experimental models in malaria research [4]. Recently, these have been used as a pathogen model for measles virus [5].

Advanced reproductive technologies are being used for the propagation of new world monkeys, owl monkeys, and squirrel monkeys in the laboratory, to ensure their preservation as a genetic resource and the effective use of their gametes in the future. We previously reported the morphology and normal characteristics of oocytes [6] and spermatozoa [7] in new world monkeys, owl monkeys, and squirrel monkeys. Although fresh spermatozoa obtained from the epididymis of owl monkeys exhibit poor motility and low concentration [7], we attempted to produce embryos using intracytoplasmic sperm injection (ICSI) in owl monkeys [8]. In contrast to owl monkeys, fresh spermatozoa of squirrel monkeys exhibited excellent motility and very high concentrations [7]. However, after freezing and warming, the squirrel monkey spermatozoa exhibit very poor motility compared to those of the owl monkey.

Successful *in vitro* fertilization (IVF) of squirrel monkeys has been

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reported [9–12]. If sperm are cryopreserved, it becomes necessary to employ an ICSI method to produce embryos, because of the low motility of the sperm after freezing and warming. However, to date, there have been no reports on the production of blastocysts in the squirrel monkey using ICSI.

Steptoe and Edwards reported the first human birth by IVF in 1978 [13], following which Cohen *et al.* reported the first birth by blastocyst transfer in 1985 [14], and Palermo *et al.* reported the first successful birth of a child resulting from ICSI in 1992 [15]. In non-human primates, Gould *et al.* reported the first successful IVF in a squirrel monkey in 1973 [12]. Hosoi *et al.* successfully produced blastocysts from Japanese monkeys in 2003 [16] and Takahashi *et al.* obtained blastocysts from common marmosets using *in vitro* maturation (IVM)-ICSI and delivered a baby in 2014 [17]. Our group recently grew an embryo to the 10-cell stage using ICSI in the owl monkey, but the embryo failed to develop to the blastocyst-stage [8]. Based on this study, we speculated that selection of culture medium is important for blastocyst development, and thus, in the present study, we used CMRL 1066 medium, which has been used to produce rhesus monkey blastocysts [18], instead of the potassium simplex optimized medium (KSOM) that was used in our previous studies [8]. We hypothesized that if we could repeat ICSI in the owl monkey for embryo production, we could produce blastocysts using CMRL 1066 medium, but unfortunately, the only female at the Amami Laboratory died.

In our previous reports [6–8] related to owl monkeys and squirrel monkeys, the gametes used were obtained from ovaries and testes that were removed during the dissection of monkeys used for other experiments; however, due to time constraints, we could not obtain dissected individuals in the present study. Because of the limited time frame at Amami laboratory and the limited number of animals used in the present study, we selected the methodology of unilateral ovariectomy for this study, in consideration of the safety of the precious squirrel monkeys. Furthermore, because we did not have ultrasound imaging equipment for the aspiration of oocytes, we decided that directly aspirating oocytes from ovaries could be more dangerous for small squirrel monkeys than unilateral ovariectomy. In addition, we wanted to avoid affecting any of the original experiments designed at the Amami laboratory for the present study.

As a pre-experiment to induce follicle development in immature female squirrel monkeys, we employed superovulation, which has been carried out previously in immature female mice by administration of a combination of equine chorionic gonadotropin (eCG) and inhibin antiserum [19, 20]. In this methodology, after reviewing the successful results of superovulation in immature female mice, removal of the unilateral ovary was performed in immature female mice post hormone treatment, followed by successful IVF using oocytes obtained from the ovary. Furthermore, the females gave birth to many babies naturally after sexual maturation (unpublished data).

Based on the results of the pre-experiment using mice, follicular development was induced by administering eCG containing inhibin antiserum to an immature female squirrel monkey. The unilateral ovary was excised after administration of human chorionic gonadotropin (hCG), to induce ovulation, following which the larger developed follicular oocytes were collected. Follicular oocytes were also prepared for ICSI using sperm from the epididymal tail of a unilateral testis extracted from a mature male, because we had failed to use sperm ejaculated by means of electrical stimulation in squirrel monkeys for the coagulation reaction. The successful production of embryos using ICSI in squirrel monkeys could also open future prospects for owl monkeys. In the present study, we report successful *in vitro* production of ICSI embryos and *in vitro* culture of embryos to the blastocyst stage in immature female squirrel monkeys.

Materials and Methods

All animal experiments were approved by the Animal Experiment Committee at the University of Tokyo (approval number: PA14-40) and were performed in accordance with the Regulations for Animal Care and Use of the University of Tokyo.

The present study used three female (one in Experiment 1 and two in Experiment 2) and two male (one male each in experiments 1 and 2) squirrel monkeys raised at the Amami Laboratory of the Institute of Medical Science, University of Tokyo.

The monkeys used in the present study were individually housed in cages (L: 450 mm, H: 550 mm, W: 450 mm) with a bar that the monkey could climb on and exposed to a natural light/dark cycle that tracked local sunrise and sunset. The monkeys could see and hear each other. Food and water were available to the monkeys *ad libitum*, and a pellet diet (CMS-1M, Clea Japan Inc., Tokyo, Japan) was supplemented daily with fresh fruits and sweet potato.

Experiment 1

The squirrel monkey breeding season at the Amami Laboratory spans from October to March. The first experiment was conducted in September 2016. Although it was a non-breeding season and the animal was immature, follicle oocytes were obtained from a female after administering it with hormone. However, the spermatozoa obtained from the male did not exhibit good motility, and the sperm density was similar to that of oligospermia. This was attributed to the fact that the experiment was being carried out outside the breeding season. Administration of a combination of eCG and inhibin antiserum was employed to induce follicle development in the immature female, because it has been used as a highly effective method for inducing superovulation in immature female mice [19, 20].

As a superovulatory stimulus, an immature female squirrel monkey (15 months old, 490 g body weight [BW]) was administered with 1.25 ml (46.88 IU) of a mixture of eCG and inhibin antiserum (CARD HyperOva®; Kyudo Company, Tosu, Japan) by means of a subcutaneous injection twice a day (0800 and 1500 h) for 4 consecutive days, beginning prior to collection of oocytes. Furthermore, hCG (ASKA Animal Health, Tokyo, Japan; 250 IU/body, intramuscular injection) was administered as an ovulation trigger, 18 h before ovariectomy. The unilateral ovary was removed from the female monkey following intramuscular injection of ketamine (Ketalar for intramuscular injection 500 mg; Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) (30 mg/kg)/medetomidine (Domitor; Nippon Zenyaku Kogyo Co., Ltd., Kooriyama, Japan; 0.05 mg/kg) with isoflurane (MSD Animal Health K.K., Tokyo, Japan).

Follicle oocytes, as cumulus-oocyte complexes (COCs), were collected according to a previously reported procedure [8]. COCs were graded by attaching layers of cumulus cells according to a previous report [6] and subjected to IVM for 25 h at 37°C in a 5% CO_2 atmosphere in CMRL 1066 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 U/ml recombinant follicle-stimulating hormone (Follistim Injection 50; MSD Japan, Tokyo, Japan), 50 ng/ml epidermal growth factor (Merck, Darmstadt, Germany), and 10% (v/v) fetal bovine serum (FBS) (lot. no. 057K8414; Merck), previously lot-checked using mouse embryos. After IVM, oocytes with the first polar body in the perivitelline space were defined as mature oocytes at the MII-stage.

On the day MII oocytes were obtained, the right testis with epi-

didymis was excised from one adult male squirrel monkey (8 years old, 1000 g BW) under a similar anesthesia protocol as the female, following which sperm were collected from its tail epididymis. A portion of the solution in which the sperm were suspended was mixed with m-HTF medium (modified HTF medium with gentamicin-HEPES [no. 90126]; Irvine Scientific, Fujifilm, Santa Ana, CA, USA) drops containing 10% (v/v) FBS and used for ICSI.

ICSI was performed according to the piezo method using an inverted microscope (IX-71; Olympus, Tokyo, Japan), a micromanipulator (Narishige, Tokyo, Japan), a piezo impact drive (PMM System; Primetech, Tsuchiura, Japan), and a sperm injection pipette (Cooper Surgical, Trumbull, CT USA) with an external diameter of 7 µm.

The ICSI procedure was initiated by placing gametes in m-HTF drops supplemented with 33% (v/v) FBS. Spermatozoa were immobilized and aspirated from the tail into the pipette. Penetration of the zona pellucida was carried out with a continuous piezo pulse (speed 3, intensity 2), and destruction of the oolemma was carried out with another piezo pulse (speed 1, intensity 1). The pipette aspirating the spermatozoon was inserted deep into the oocyte cytoplasm, and after sperm injection, the pipette was withdrawn and the whole course of ICSI was completed. The squirrel monkey's oolemma was often weak, and if the piezo-ICSI was performed from a narrow-enclosed oocyte cavity, the impact of the piezoelectric pulse could cause oocyte degeneration; therefore, we prioritized the size of the enclosed oocyte cavity over the position of the polar body.

Oocytes, into which a sperm was successfully injected, were cultured and evaluated as fertilized oocytes and embryos, based on the appearance of two pronuclei. The embryos were continuously incubated for 6 days at 37°C (5% CO_2 in air) in CMRL 1066 medium supplemented with 10% (v/v) FBS. Embryo culture was performed with cumulus cells.

Experiment 2

The second experiment was conducted in December 2018, during the breeding season for squirrel monkeys. The regimen used successfully in Experiment 1, of administering a combination of eCG + inhibin antiserum (which effectively induced follicle development) and hCG, was adapted to Experiment 2 as well.

As a superovulatory stimulus, two immature female squirrel monkeys (monkey 1: 2 years old, 500 g BW; monkey 2: 3 years old, 560 g BW) were administered 1.7 ml of a mixture of eCG (63.75 IU) by subcutaneous injection twice a day (0800 and 1500 h), for 6 consecutive days prior to the collection of oocytes. Furthermore, hCG was administered as an ovulation trigger 18 h before ovariectomy. The dose of eCG and inhibin antiserum mixture was increased in order to collect more COCs than in Experiment 1, while administration of hCG was performed in the same manner as in Experiment 1. The dosage was determined to be lower than that provided by the applicable animal welfare law.

Surgical removal of a unilateral ovary from the female monkey was performed under an anesthesia protocol similar to that used in Experiment 1. Pre-ovulation follicle oocytes were collected using the same procedure as that used in Experiment 1. COCs were cultured until ICSI in CMRL 1066 medium supplemented with 10% (v/v) FBS.

Oocyte maturity was confirmed using a microscope. Cumulus cells were removed using 0.1% (w/v) hyaluronidase (H3506-1G, Merck), if necessary, to determine maturity. For germinal vesicle-stage oocytes, IVM culture (37°C, 5% CO_2 in air) was performed using the same protocol as that used in Experiment 1. Oocytes with the first polar body in the perivitelline space were defined as mature (at the MII-stage). When MII-stage oocytes were obtained, the right testis with epididymis was excised from one male squirrel monkey (8 years old, 980 g BW) under a similar anesthesia protocol as the female, and sperm were collected from the tail epididymis for ICSI, which was performed according to the same protocol as that used for Experiment 1.

Fertilization was confirmed by identifying the appearance of two pronuclei, 19 h after ICSI. The resulting embryos were evaluated continuously during culture for 8 days at 37°C (5% CO_2 in air) in CMRL 1066 medium supplemented with 10% (v/v) FBS. For embryos, co-culture with cumulus cells was performed.

Vitrification of embryos that developed to the blastocyst stage was performed using a commercially available kit (Vitrification Kit; Kitazato Corp., Fuji, Japan).

Photographs of gametes, the performance of ICSI, and the oocyte showing two pronuclei were captured with Viewfinder Lite (version 1.0; Pixera Corporation, Santa Clara, CA, USA) and Olympus DP50 (a digital camera for microscopy) connected to an Olympus BX51 microscope. Photographs of the blastocysts were captured using the built-in camera of CCM-iBIS (Astec, Shime, Japan).

Embryo transfer

One year later, in March 2019, the blastocysts were thawed and transferred to females in which pseudopregnancy was induced *via* hormone administration. Recipient monkeys received eCG containing inhibin antiserum (63.75 IU/body, subcutaneous injection) for 6 days, followed by hCG (252 IU/body, intramuscular injection), and underwent embryos transfer 6 days after hCG administration. Although we could not confirm ovulation or check the progesterone level, when the abdomen was opened for embryo transfer, the uterus and fallopian tubes were found to be swollen and reddish in appearance; thus, the pseudopregnancy effect upon hormone administration was considered obvious. The embryo transfer procedure was carried out as follows: anesthetized monkeys were laparotomized, and approximately 5 µl of medium containing an embryo was transferred *via* an incision in the oviduct cavity near the tubouterine junction using a gel-loader tip (20 µl GELoader tips, catalog no. 0030001222, Eppendorf, Hamburg, Germany) that was cut off at the tip end to correspond to the diameter of the embryo. The recipient mother monkeys were kept in a group cage of only females (four, including the mother monkey) before and after the experiment.

Results

Experiment 1

A total of 17 COCs were collected from one ovary surgically removed from a live immature female monkey stimulated with hormones (Fig. 1A). The COCs were graded into three types based on the attached layers of cumulus cells (Table 1, Fig. 1B–C). There were 3 'excellent', 1 'good', and 13 'poor' COCs. IVM of these COCs resulted in two MII oocytes, which were then subjected to ICSI. One of the oocytes was judged to have been fertilized normally, because the second polar body and two pronuclei were observed in its case, at 19 h after ICSI. However, the other oocytes exhibited poor oolemma flexibility when ICSI was performed, and these oocytes were found to have degenerated by 19 h after ICSI. The fertilized oocyte was cleaved to the 2-cell-stage at the time of observation, at 27 h after ICSI, and developed to the blastocyst-stage after 140 h under *in vitro* culture; the blastocyst was found to be vitrified.

Experiment 2

A total of 32 COCs were collected from two ovaries surgically

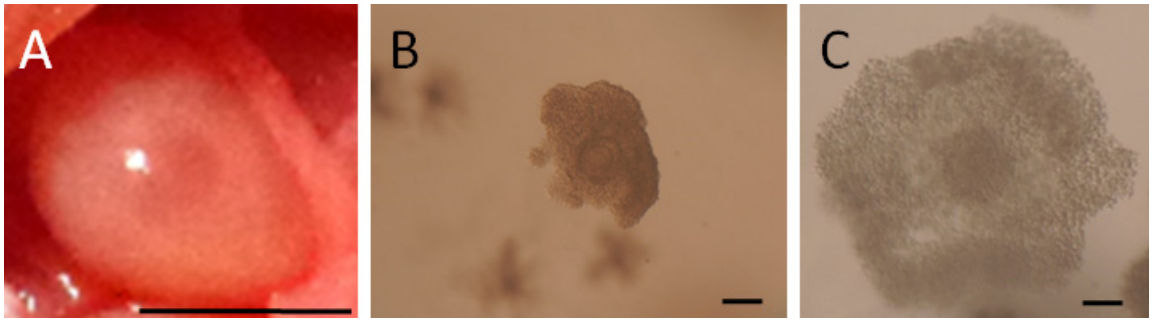

Fig. 1. The ovary and cumulus-oocyte complexes (COCs) before and after *in vitro* maturation in the squirrel monkey. A) The ovary of the immature female squirrel monkey, stimulated with a combination of eCG and inhibin antiserum, followed by administration of hCG. There are large follicles in the ovary. Scale bar: 5 mm. eCG: equine chorionic gonadotropin, hCG: human chorionic gonadotropin. B–C) The follicle oocytes surrounded by cumulus cells in the squirrel monkey. Each 'excellent' COC (B) before and (C) after *in vitro* maturation. Scale bar: 100 µm.

Table 1. Number of cumulus-oocyte complexes, graded by attachment of cumulus cells, and results of *in vitro* maturation of metaphase I oocytes

Experiment 1

Evaluation *	Number of oocytes				
	Total	MII-stage	MI-stage	GV-stage	Degeneration
Excellent	3	0	1	1	1
Good	1	1	0	0	0
Poor	13	1	1	7	4
Total	17	2	2	8	5

Experiment 2

Evaluation *	Number of oocytes				
	Total	MII-stage	MI-stage (MII after IVM)	GV-stage	Degeneration
Excellent	11	0	11 (10)	0	0
Good	3	0	2 (2)	1	0
Poor	18	0	0	17	1
Total	32	0	13 (12)	18	1

* COCs: cumulus-oocyte complexes were graded by attaching layers of cumulus cells, according to a previous report [6]. MI, metaphase I; MII, metaphase II; GV, germinal vesicle; IVM, *in vitro* maturation.

removed from two live female monkeys (no. 1: 15 COCs; no. 2: 17 COCs). The COCs were graded into three types based on the attachment of cumulus cells (Table 1). Of the 13 oocytes in the MI phase, 12 released the first polar body within 2–29 h of IVM after collection. After confirming that the oocytes were mature and at the MII-stage (Fig. 2A), sperm were collected from the epididymal tail of the male monkey. The fresh sperm used in the present study exhibited good motility (Fig. 2B). These sperms were used to perform ICSI on 12 MII oocytes (Fig. 2C). Eighteen of the remaining oocytes were at the GV-stage, and one was degenerated.

Four oocytes were judged to have been fertilized normally, because the second polar body and two pronuclei were observed in the oocytes, at 19 h after ICSI (Fig. 2D). Eight oocytes exhibited poor oolemma flexibility upon ICSI and were found to have degenerated at 19 h after ICSI.

Three of the four fertilized oocytes had started cleavage at the time of observation, at 44 h after ICSI, and the remaining oocytes exhibited no cleavage. Two of the three cleaved embryos developed to the blastocyst-stage after 173 h and 189 h of *in vitro* culture (Fig. 2E–F) and those were also found to be vitrified. The remaining embryos stopped developing at the 6-cell-stage.

The sum results of ICSI and *in vitro* culture of embryos in Experiments 1 and 2 are shown in Table 2.

Embryo transfer

The vitrified blastocysts were thawed and transferred to three recipient monkeys. One recipient monkey underwent a sham operation because the embryo obtained from Experiment 1 was lost during the thawing procedure. The remaining two embryos were technically transferred to the other two recipients, but no pregnancies resulted.

Discussion

Induction of follicular development in immature female squirrel monkeys and sperm collection

The sexual maturity of squirrel monkeys is 2.5 to 3.5 years old; however, squirrel monkeys breed seasonally, so there is a lot of individual variation [21]. Although Experiment 1 was not carried out during the breeding season and an immature female was used, many COCs were obtained, possibly due to hormone administration.

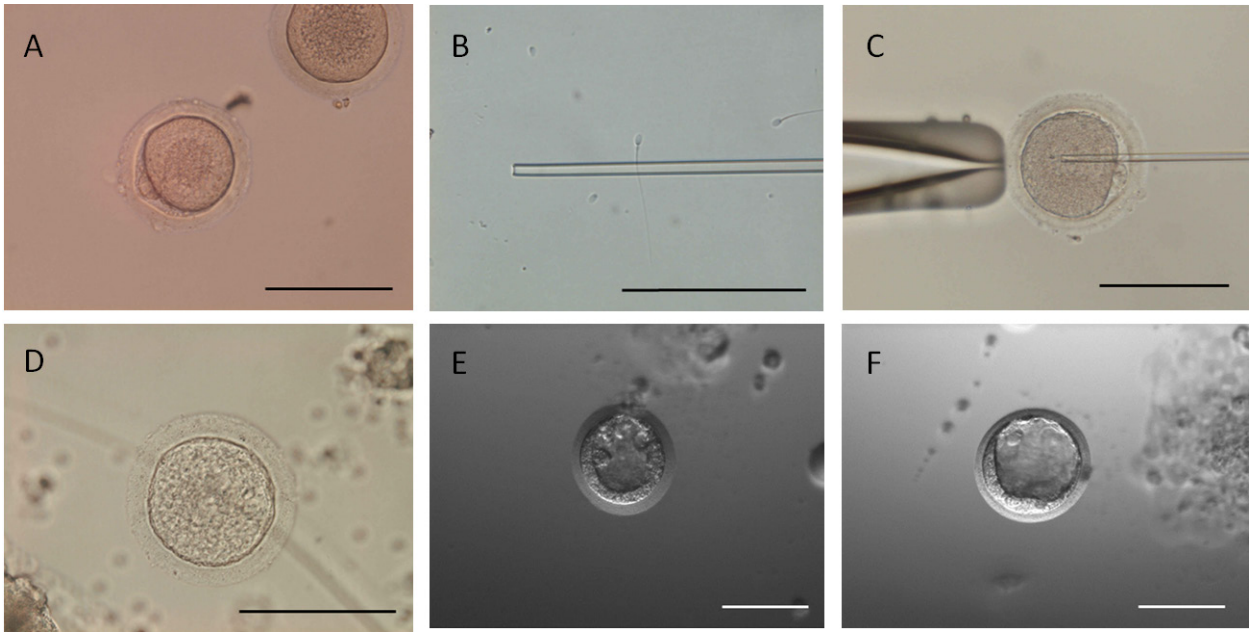

Fig. 2. A–F) The process of intracytoplasmic sperm injection (ICSI) in the squirrel monkey oocyte (A–C) and embryo development to the blastocyst-stage (D–F). Scale bar: 100 µm. A) The squirrel monkey oocyte released the first polar body and matured *in vitro* within a short time in Experiment 2. B) Squirrel monkey spermatozoa in Experiment 2. C) The precise moment of ICSI into the squirrel monkey oocyte in Experiment 2. D) A fertilized squirrel monkey oocyte showing two pronuclei in Experiment 2 (at 19 h after ICSI). E) The squirrel monkey blastocyst developed upon *in vitro* culture for 173 h after ICSI in Experiment 2. F) The other squirrel monkey blastocyst developed upon *in vitro* culture for 189 h after ICSI in Experiment 2.

Table 2. Results of *in vitro* culture of oocytes fertilized successfully using intracytoplasmic sperm injection (ICSI) in Experiments 1 and 2

Experiment No.	No. of oocytes		Results of embryo culture (culture hours after ICSI)
	Performed using ICSI	Fertilized using ICSI	
1	2	1	1: Blastocyst (140 h)
2	12	4	1: Blastocyst (173 h) 1: Blastocyst (189 h) 1: Stopped at 6 cells 1: Not cleaved

ICSI: intracytoplasmic sperm injection.

In Experiment 2, modified administration of a combination of eCG and inhibin antiserum (in accordance with the method of Takeo *et al*. [20]) and the originally modified method for administration of hCG to immature female squirrel monkeys led to successful induction of follicular development, resulting in a total of 32 COCs being obtained from each unilateral ovary. During IVM of follicular oocytes over a short period of time, 12 oocytes reached maturity with the second meiotic division. These results demonstrated that the administration of a combination of eCG and inhibin antiserum resulted in superior follicle development. In our previous reports [6–8] related to owl monkeys and squirrel monkeys, ovaries and testes were removed to obtain gametes during the dissection of monkeys used for other experiments; however, dissected individuals could not be obtained when the present study was conducted, due to time constraints. Although in the Common Marmoset, which is as small as the squirrel monkey, Takahashi *et al*. [17] showed that oocytes could be retrieved from the ovaries through laparotomy under anesthesia, following which the birth of an offspring was possible through ICSI, in the present study, we had to consider experimental plans and perform them within a limited time frame at the Amami laboratory, under the constraint of having limited number of the animals that were usable in the experiments. Thus, we planned such that we used immature females without affecting the original experiments planned at the Amami laboratory, while also maintaining reproductive capacity in the future. Therefore, to collect oocytes while maintaining reproductive capacity, we removed the unilateral ovary from a female. Since squirrel monkeys are seasonal breeders, the timing of sexual maturity in females varies among individuals [21]; therefore, we selected immature females that had not yet entered estrus. We attempted a method to obtain more mature oocytes from immature mice by administering hormones [19, 20] to immature female squirrel monkeys; using this method, we succeeded in collecting oocytes from the ovaries.

On the other hand, it has been shown in African green monkeys [22] and baboons [23] that sperm collection can be carried out by means of electrical stimulation. We also tried to use sperm ejaculated by means of electrical stimulation in squirrel monkeys, but the obtained semen soon coagulated and could not be used for experiments. Therefore,

we had to choose to remove a unilateral testis to collect epididymal sperm from the tail epididymis. Nevertheless, if we could study sperm ejaculation by means of electrical stimulation in more detail, it would serve as a less invasive method to study reproduction of squirrel monkeys in the future.

Recovery and IVM of follicular oocytes

Most of the oocytes, which were in the MI-stage when they were collected from follicles, matured to the MII-stage after a short period of IVM for 25 h. The reason many of the collected follicle oocytes were in the MI-stage in the present study was attributed to treatment with hCG. Matsumoto *et al.* observed only a few mature oocytes after IVM in HTF medium for 22–23 h. The oocytes were recovered from follicles in squirrel monkeys without any hormone treatment and exhibited poor maturation rates: 40.0% (4/10) in the 'excellent' grade and 4.5% (1/22) in the 'good' grade, compared to 83.3% (5/6) in the 'excellent' grade and 50.0% (10/20) in the 'good' grade in the owl monkey oocytes [6]. In a previous report by Matsumoto *et al.* [6], mature female squirrel monkeys were used without hormones, while in the present study, we used immature females treated with hormones. Administration of eCG + inhibin antiserum and hCG in the present study resulted in higher rates of oocyte maturation than those observed in the study by Matsumoto *et al.* [6].

Successful IVM of rhesus monkey GV-stage oocytes using CMRL 1066 medium has been reported [24], in addition to successful IVM of rhesus monkey GV-stage oocytes using CMRL 1066 and hamster embryo culture medium version 9 [25]. Yet, our experiment on IVM with CMRL 1066 was not successful. These previous studies were conducted using GV-stage oocytes collected from the ovaries of monkeys without hCG treatment. In contrast, the GV-stage oocytes in the present study did not mature *in vitro*. The oocytes in the present study were collected as GV-stage oocytes, despite being stimulated by hCG after superovulation. Therefore, it may be presumed that the oocytes had no ability to mature during IVM. Furthermore, it was considered that assessing morphologic development based on attached layers of cumulus cells after administration of hCG is useful for judging maturity.

Fertilization using ICSI

In the present study, the piezo method was selected for ICSI, similar to a previous study on owl monkeys [8]. It has been reported that the piezo method has a lower degeneration rate than ICSI using pipettes with spikes, which is common in human ICSI [26–28]. In mouse oocytes, which are small and frangible, spiked ICSI degenerates most of the oocytes, but the piezo method is effective [29] and has been successfully performed in mouse cloning by means of somatic cell nuclear transfer [30].

These data suggest that the piezo method is adequate and causes less damage to oocytes than the spike method. The 80.7 µm median size of squirrel monkey oocytes without the zona pellucida is smaller than the 98.2 µm median size of owl monkey oocytes (112.0 µm *vs.* 145.7 µm with the zona pellucida) [6], and squirrel monkey sperm are larger than owl monkey sperm (76.8 µm *vs.* 55.1 µm) [7]. Because we had to insert larger sperm into smaller oocytes during squirrel monkey ICSI compared to owl monkey ICSI, we performed piezo-ICSI on squirrel monkey oocytes and obtained four fertilized oocytes and eight degenerated oocytes. This high rate of degeneration may have been due to the fragility of the oolemma that we noted when performing ICSI.

Time of pronuclei appearance

The success of fertilization of the oocytes was determined by checking for the presence of two pronuclei within the cytoplasm by means of microscopic observation, 19 h after ICSI. As we reported previously in a study involving ICSI of owl monkey embryos, the presence of pronuclei could not be confirmed by observation at 17.5 h after ICSI [8]. In a study involving rhesus monkeys, fertilization was confirmed based on the presence of pronuclei 10 to 12 h after ICSI [31]. In a study of squirrel monkey embryos, Kuehl *et al.* [11] observed the first cleavage 16.2 h after insemination, whereas Yeoman *et al.* [9] reported that while some embryos had two pronuclei at 16–18 h after insemination, some did not. Pronuclear disappearance in squirrel monkey embryos may be slower than that in the embryos of owl monkeys. In our study, pronuclei were identified 19 h after ICSI, and IVF and ICSI may differ in terms of the mechanism of fertilization and timing. In humans, it has been reported that pronuclei appear sooner with ICSI than with IVF [32]. However, the results of the present study are opposite to those of Kuehl *et al.* [11] and Yeoman *et al.* [9]. We speculate that other factors, such as culture medium and culture atmosphere, may be involved in the progression of fertilization.

In vitro culture of embryos after successful ICSI

One of the major factors that determine whether any embryo obtained using ICSI grows to the blastocyst-stage may be the selection of the culture medium. Therefore, the selection of an appropriate culture medium was an early priority in the present study. We succeeded in obtaining blastocysts by culturing oocytes in CMRL 1066 medium supplemented with 10% FBS. Yeoman *et al*. compared culture results obtained by adding bovine serum albumin (BSA), squirrel monkey serum, and human follicular fluid to Ham's F-10 medium, but there was no difference in growth in each group, and no blastocysts were obtained [9]. In our previous study, KSOM with 0.1% BSA was used for the culture of owl monkey embryos, but no blastocysts were obtained [8]. Thus, we concluded that selection of an appropriate culture medium is important for blastocyst development, and replaced the KSOM used in our previous study [8] with CMRL 1066 medium in the present study, which reportedly enables the production of rhesus monkey blastocysts [18]. In addition, embryos were cultured with cumulus cells removed from around the ovum in the present study, and a co-culture method using cumulus cells was adapted for successful blastocyst production. We believe that if we would repeat ICSI in the owl monkey for embryo production, we could succeed in blastocyst production using CMRL 1066 medium and co-culture with cumulus cells.

CMRL-1066 has been used for common marmosets [33], and CMRL-1066 co-cultured with BRL cells has been used in cynomolgus monkeys [34], African green monkeys [22], and baboons [23], all of which were successfully cultured to the blastocyst-stage. In a report on cynomolgus monkeys [34], 22.7% of oocytes developed into blastocysts. Gilchrist *et al.* [33] reported a blastocyst attainment rate of 7% in common marmoset embryos using CMRL-1066, while Takahashi *et al.* [17] reported blastocyst development rates of 35.4% for ICSI embryos and 39.2% for IVF embryos using human culture medium. Therefore, it is possible to further improve the results of the present study by improving the culture medium.

In the present study, the blastocoel was well-formed; however, the number of cells might have been low, although it had not been completely counted. For example, it is possible that the number of cells might be low compared to the photograph of blastocyst development in rhesus monkeys [35].

Blastocyst transfer

When the abdomen of pseudopregnant females was opened for embryo transfer in the present study, the uterus and fallopian tubes were found to be swollen and reddish in appearance, thus making the pseudopregnancy effect due to hormone administration obvious. The fallopian tubes were finely dissected, and blastocysts were transferred using a pipette tip; however, no pregnancies occurred. It is not clear why our procedure was unsuccessful; however, one possible reason is that the artificially induced pseudopregnant state was inadequate. Another possibility is that the transferred blastocysts were arrested *in utero* for unknown reasons. To obtain a successful monkey birth, further improvements in methodology are needed to overcome these issues. For example, Balmaceda *et al.* [36] laparotomized synchronized recipients of cynomolgus monkeys and transferred the early embryos to the fallopian tubes using a plastic tip. Wolf *et al.* [37] performed laparotomy of rhesus monkeys during the spontaneous ovulation cycle and transferred the early embryos to the fallopian tubes, resulting in successful pregnancy and birth. Torii *et al.* [38] obtained a newborn baby of a Japanese monkey by laparoscopically transferring the early embryo to the fallopian tubes *via* the fimbria. In addition, several successful studies have also reported the following: an embryo transfer into the fallopian tube with a micro glass capillary through an open abdomen in a cynomolgus monkey (miscarriage after pregnancy established) [34], laparoscopic embryo transfer and transvaginal intrauterine transfer using a small catheter in the baboons [23], and non-surgical embryo transfer of common marmosets with a transvaginal catheter (outer tube made of Fluon® Ethylene tetrafluoroethylene and inner tube made of polyethylene) under transabdominal ultrasound observation [17]. These embryo transfer methods should also induce success in squirrel monkeys.

In conclusion, the present study is the first to successfully produce ICSI-derived blastocysts from MII oocytes obtained by hormone administration (a combination of eCG+inhibin antiserum and hCG) and IVM in immature squirrel monkeys.

Conflict of interests: The authors declare that there are no conflicts of interest.

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References

1. **Coutinho LN, de Brito MB, Monteiro FO, de Andrade RS, da Conceição ME, Feliciano MR, de Castro PH, Teixeira PP, Vicente WR.** Analysis of follicular events in owl monkeys (*Aotus azarai infulatus*) using B-mode and Doppler ultrasound. *Theriogenology* 2013; **80**: 99–103. [Medline] [CrossRef]
2. **Yeoman RR, Williams LE, Aksel S, Abee CR.** Mating-related estradiol fluctuations during the estrous cycle of the Bolivian squirrel monkey (Saimiri boliviensis boliviensis). *Biol Reprod* 1991; **44**: 640–647. [Medline] [CrossRef]
3. **Tachibana T, Kusakabe KT, Osaki S, Kuraishi T, Hattori S, Yoshizawa M, Kai C, Kiso Y.** Histocytological specificities of adrenal cortex in the New World Monkeys, *Aotus lemurinus* and *Saimiri boliviensis*. *J Vet Med Sci* 2016; **78**: 161–165. [Medline] [CrossRef]
4. **Gysin J, Hommel M, da Silva LP.** Experimental infection of the squirrel monkey (*Saimiri sciureus*) with Plasmodium falciparum. *J Parasitol* 1980; **66**: 1003–1009. [Medline] [CrossRef]
5. **Delpeut S, Sawatsky B, Wong XX, Frenzke M, Cattaneo R, von Messling V.** Nectin-4 interactions govern measles virus virulence in a new model of pathogenesis, the squirrel monkey (*Saimiri sciureus*). *J Virol* 2017; **91**: e02490–e16. [Medline] [CrossRef]
6. **Matsumoto T, Isobe K, Kusakabe KT, Kuraishi T, Hattori S, Nakazato C, Matsumoto H, Fukui E, Kuwahata A, Ochi M, Kiso Y, Kai C, Yoshizawa M.** Morphological characterization and *in vitro* maturation of follicular oocytes from the owl monkey (*Aotus lemurinus*). *J Mamm Ova Res* 2015; **32**: 103–108. [CrossRef]
7. **Nakazato C, Yoshizawa M, Isobe K, Kusakabe KT, Kuraishi T, Hattori S, Matsumoto H, Fukui E, Kuwahata A, Ochi M, Kiso Y, Kai C.** Morphological characterization of spermatozoa of the night monkey. *J Mamm Ova Res* 2015; **32**: 37–40. [CrossRef]
8. **Watanabe H, Matsumoto T, Nishi M, Kusakabe KT, Kuraishi T, Hattori S, Matsumoto H, Fukui E, Kuwahata A, Ochi M, Kiso Y, Kai C, Yoshizawa M.** Intracytoplasmic sperm injection into oocytes matured in vitro and early embryonic development in the owl monkey (*Aotus lemurinus*). *Reprod Med Biol* 2015; **15**: 183–186. [Medline] [CrossRef]
9. **Yeoman RR, Helvacioglu A, Williams LE, Aksel S, Abee CR.** Restoration of oocyte maturational competency during the nonbreeding season with follicle-stimulating hormone stimulation in squirrel monkeys (*Saimiri boliviensis boliviensis*). *Biol Reprod* 1994; **50**: 329–335. [Medline] [CrossRef]
10. **Kuehl TJ, Dukelow WR.** Maturation and in vitro fertilization of follicular oocytes of the squirrel monkey (*Saimiri sciureus*). *Biol Reprod* 1979; **21**: 545–556. [Medline] [CrossRef]
11. **Kuehl TJ, Dukelow WR.** Time relations of squirrel monkey (*Saimiri sciureus*) sperm capacitation and ovum maturation in an in-vitro fertilization system. *J Reprod Fertil* 1982; **64**: 135–137. [Medline] [CrossRef]
12. **Gould KG, Cline EM, Williams WL.** Observations on the induction of ovulation and fertilization in vitro in the squirrel monkey (*Saimiri sciureus*). *Fertil Steril* 1973; **24**: 260–268. [Medline] [CrossRef]
13. **Steptoe PC, Edwards RG.** Birth after the reimplantation of a human embryo. *Lancet* 1978; **2**: 366. [Medline] [CrossRef]
14. **Cohen J, Simons RF, Fehilly CB, Fishel SB, Edwards RG, Hewitt J, Rowlant GF, Steptoe PC, Webster JM.** Birth after replacement of hatching blastocyst cryopreserved at expanded blastocyst stage. *Lancet* 1985; **1**: 647. [Medline] [CrossRef]
15. **Palermo G, Joris H, Devroey P, Van Steirteghem AC.** Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992; **340**: 17–18. [Medline] [CrossRef]
16. **Hosoi Y, Torii R, Fujinami N, Matsumoto K, Saeki K, Iritani A.** Fertilization by intracytoplasmic sperm injection and subsequent embryo development in vitro blastocysts in Japanese monkey (*Macaca fuscata*). *J Mamm Ova Res* 2003; **20**: 34–40. [CrossRef]
17. **Takahashi T, Hanazawa K, Inoue T, Sato K, Sedohara A, Okahara J, Suemizu H, Yagihashi C, Yamamoto M, Eto T, Konno Y, Okano H, Suematsu M, Sasaki E.** Birth of healthy offspring following ICSI in in vitro-matured common marmoset (*Callithrix jacchus*) oocytes. *PLoS One* 2014; **9**: e95560. [Medline] [CrossRef]
18. **Schramm RD, Bavister BD.** Development of in-vitro-fertilized primate embryos into blastocysts in a chemically defined, protein-free culture medium. *Hum Reprod* 1996; **11**: 1690–1697. [Medline] [CrossRef]
19. **Takeo T, Nakagata N.** Superovulation using the combined administration of inhibin antiserum and equine chorionic gonadotropin increases the number of ovulated oocytes in C57BL/6 female mice. *PLoS One* 2015; **10**: e0128330. [Medline] [CrossRef]
20. **Takeo T, Mukunoki A, Nakagata N.** Ovulation of juvenile, mature, and aged female C57BL/6 mice following coadministration of inhibin antiserum and equine chorionic gonadotropin. *Theriogenology* 2019; **135**: 1–6. [Medline] [CrossRef]
21. **Goldschmidt B, Mota-Marinho A, Araújo-Lopes C, Brück-Gonçalves MA, Matos-Fasano D, Ribeiro-Andrade MC, Ferreira-Nascimento LW, Simmer-Bravin J, Monnerat-Nogueira D.** Sexual dimorphism in the squirrel monkey, *Saimiri sciureus* (Linnaeus, 1758) and *Saimiri ustus* (I. Geoffroy, 1844) (Primates, Cebidae). *Braz J Biol* 2009; **69**: 171–174. [Medline] [CrossRef]
22. **Shimozawa N, Nakamura S, Takahashi I, Hatori M, Sankai T.** Characterization of a novel embryonic stem cell line from an ICSI-derived blastocyst in the African green monkey. *Reproduction* 2010; **139**: 565–573. [Medline] [CrossRef]
23. **Simerly CR, Castro CA, Jacoby E, Grund K, Turpin J, McFarland D, Champagne J, Jimenez JB Jr, Frost P, Bauer C, Hewitson L, Schatten G.** Assisted reproductive technologies (ART) with baboons generate live offspring: a nonhuman primate model for ART and reproductive sciences. *Reprod Sci* 2010; **17**: 917–930. [Medline] [CrossRef]
24. **Morgan PM, Warikoo PK, Bavister BD.** In vitro maturation of ovarian oocytes from unstimulated rhesus monkeys: assessment of cytoplasmic maturity by embryonic development after in vitro fertilization. *Biol Reprod* 1991; **45**: 89–93. [Medline] [CrossRef]
25. **Zheng P, Wang H, Bavister BD, Ji W.** Maturation of rhesus monkey oocytes in chemically defined culture media and their functional assessment by IVF and embryo development. *Hum Reprod* 2001; **16**: 300–305. [Medline] [CrossRef]
26. **Huang T, Kimura Y, Yanagimachi R.** The use of piezo micromanipulation for intracytoplasmic sperm injection of human oocytes. *J Assist Reprod Genet* 1996; **13**: 320–328. [Medline] [CrossRef]
27. **Yanagida K, Katayose H, Yazawa H, Kimura Y, Konnai K, Sato A.** The usefulness of a piezo-micromanipulator in intracytoplasmic sperm injection in humans. *Hum Reprod* 1999; **14**: 448–453. [Medline] [CrossRef]
28. **Hiraoka K, Kitamura S.** Clinical efficiency of Piezo-ICSI using micropipettes with a wall thickness of 0.625 µm. *J Assist Reprod Genet* 2015; **32**: 1827–1833. [Medline] [CrossRef]
29. **Kimura Y, Yanagimachi R.** Intracytoplasmic sperm injection in the mouse. *Biol Reprod* 1995; **52**: 709–720. [Medline] [CrossRef]
30. **Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R.** Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 1998;

394: 369–374. [Medline] [CrossRef]

31. **Hewitson L, Takahashi D, Dominko T, Simerly C, Schatten G.** Fertilization and embryo development to blastocysts after intracytoplasmic sperm injection in the rhesus monkey. *Hum Reprod* 1998; **13**: 3449–3455. [Medline] [CrossRef]
32. **Nagy ZP, Janssenswillen C, Janssens R, De Vos A, Staessen C, Van de Velde H, Van Steirteghem AC.** Timing of oocyte activation, pronucleus formation and cleavage in humans after intracytoplasmic sperm injection (ICSI) with testicular spermatozoa and after ICSI or in-vitro fertilization on sibling oocytes with ejaculated spermatozoa. *Hum Reprod* 1998; **13**: 1606–1612. [Medline] [CrossRef]
33. **Gilchrist RB, Nayudu PL, Hodges JK.** Maturation, fertilization, and development of marmoset monkey oocytes in vitro. *Biol Reprod* 1997; **56**: 238–246. [Medline] [CrossRef]
34. **Ogonuki N, Tsuchiya H, Hirose Y, Okada H, Ogura A, Sankai T.** Pregnancy by the tubal transfer of embryos developed after injection of round spermatids into oocyte cytoplasm of the cynomolgus monkey (*Macaca fascicularis*). *Hum Reprod* 2003; **18**: 1273–1280. [Medline] [CrossRef]
35. **Zhou Q, Yang SH, Ding CH, He XC, Xie YH, Hildebrandt TB, Mitalipov SM, Tang XH, Wolf DP, Ji WZ.** A comparative approach to somatic cell nuclear transfer in the rhesus monkey. *Hum Reprod* 2006; **21**: 2564–2571. [Medline] [CrossRef]
36. **Balmaceda JP, Pool TB, Arana JB, Heitman TS, Asch RH.** Successful in vitro fertilization and embryo transfer in cynomolgus monkeys. *Fertil Steril* 1984; **42**: 791–795. [Medline] [CrossRef]
37. **Wolf DP, Vandevoort CA, Meyer-Haas GR, Zelinski-Wooten MB, Hess DL, Baughman WL, Stouffer RL.** In vitro fertilization and embryo transfer in the rhesus monkey. *Biol Reprod* 1989; **41**: 335–346. [Medline] [CrossRef]
38. **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. [Medline] [CrossRef]