Ultrasound-guided non-surgical embryo collection in the common marmoset

Hidetoshi Ishibashi a,*, Hideyuki H. Motohashi a, Mami Kumon b, Kazuhiro Yamamoto c, Hironori Okada d, Takashi Okada d, Kazuhiko Seki a

a Department of Neurophysiology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan
b Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan
c Division of Laboratory Animals Resources, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan
d Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan

1. Introduction

Experimental primate embryology has been hampered by limited access to embryos. Among primate species, common marmosets have characteristics that make them especially suitable for primate embryology research, e.g., the ovulation of two or more oocytes per ovarian cycle, an early sexual maturity age (approximately 18 months), and a lack of seasonal infertility. An efficient surgical technique for the recovery of uterine stage marmoset embryos has been previously reported [1]. However, the animals require a rest period of two or three ovarian cycles between surgeries, thus limiting the production of embryos by individual animals. In

* Corresponding author at: 4-1-1 Ogawa-Higashi Kodaira, Tokyo 187-8502, Japan. Tel.: +81 42 341 2711; fax: +81 42 346 1754.
E-mail address: ishiba@ncnp.go.jp (H. Ishibashi).

1642-431X/$ – see front matter © 2013 Society for Biology of Reproduction & the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences in Olsztyn. Published by Elsevier Urban & Partner Sp. z o.o. All rights reserved.

http://dx.doi.org/10.1016/j.repbio.2013.02.002
addition, the surgery may induce postsurgical reproductive tract adhesion. A non-surgical technique using uterine flushing that is less stressful to the subject and allows an embryo flush attempt every ovarian cycle has been used successfully to recover primate pre-implantation embryos from humans [2], squirrel monkeys [3], baboons [4,5], and rhesus monkeys [6,7]. However, the small body size of common marmosets (300- to 500-g adult body weight) has delayed the development and use of the non-surgical technique. Thomson et al. [8] were the first to report on non-surgical transcervical cannulation into the marmoset uterus with an otoscope and the collection of preimplantation embryos. However, some concerns remain. First, the technique is difficult to reproduce. Hanazawa et al. [9] had difficulty in reproducing the original technique and instead developed a method based on transabdominal puncture. Second, abdominal palpation alone after the cannulation cannot provide an accurate estimation of the cannula tip location, raising the possibility of damaging the endometrium. Third, the uterus may have small folds or crevices that are incompletely flushed, as the authors themselves noted. Herein, we introduce the use of ultrasonography in marmoset non-surgical embryo flushing to resolve the aforementioned concerns. The first aim of this study was to see whether ultrasonography can make the transcervical cannulation procedure easier. The second aim of this study was to evaluate the repeatability of the flushing experiments. The third aim of this study was to thoroughly flush the uterus by visualizing the degree of the uterine distention.

2. Materials and methods

2.1. Animals

We used common marmoset monkeys (Callithrix jacchus) as subjects. Animal experiments were approved by the ethics committee for primate research of the National Center of Neurology and Psychiatry, Japan. All experiments were conducted in accordance with the institutional guidelines. The marmosets were caged indoors, with the room lights on from 07:00 to 19:00 h, the temperature at 26–28 °C and the humidity at 40–60%. The marmosets were fed 50 g a day of monkey chow (CMS-1M, Clea Japan Inc., Tokyo, Japan) with vitamin supplementation and fruit. Water was provided ad libitum. Paired marmosets were unpaired when they were having behavioral interaction issues or hospitalized when their physical condition was judged to be of concern from a veterinary point of view, and thus, the experiment was not necessarily conducted every ovarian cycle.

Because no pairs had experienced natural delivery before the beginning of this study, we tested new pairings in 2009 and started examining the experimental conditions. We added new pairings in the spring of 2011. The female marmoset age range was 25–86 months, and their body weight range was 300–540 g during the data collection period, i.e., from October 2010 to March 2012.

Blood samples (0.1 mL) were taken from the femoral vein 9, 11, and 13 days after the injection of the prostaglandin F2 alpha analog cloprostenol (Cloprostenol C, Fujita Pharmaceuticals Inc., Tokyo, Japan), and ovulation days were determined by measuring the plasma progesterone concentrations using an enzyme immunoassay (AIA-360, Tosoh Corp., Tokyo, Japan). The day of ovulation (day 0) was designated as the day preceding the day in which the progesterone concentration had risen from basal levels to higher than 10 ng/mL [10]. The animals were subjected to embryo collection on post-ovulation days 4–9. During the middle to late luteal phase, 0.75 μg of cloprostenol were intramuscularly administered to control the ovarian cycle. Blood sampling was conducted only when the female was under pairing.

2.2. Anesthesia

Anesthesia was preceded by food and water deprivation. For the artificial insemination, the female was sedated with an intramuscular administration of a mixture of 0.070 mg of midazolam and 0.014 mg of butorphanol tartrate per kilogram of body weight, and anesthesia was maintained via the inhalation of sevoflurane. For the embryo collection, the anesthesia was induced by an intramuscular administration of a mixture of 0.10–0.30 mg of midazolam, 0.0050–0.015 mg of butorphanol tartrate, and 10–30 mg of ketamine hydrochloride per kilogram of body weight. The animal was placed on a heater-equipped treatment apparatus (custom-made by Natsume Seisakusho Co Ltd., Tokyo, Japan) in the supine position, and the forelimbs and hindlimbs were secured to the apparatus with hook-and-loop fasteners (Fig. 1a and c). Forepaw arterial oxygen saturation and pulse rate were monitored using a pulse oximeter (8600FO, Nonin Medicals Inc., Plymouth, USA). The anesthesia was maintained by the inhalation of sevoflurane vaporized in fresh 80–100% oxygen at 500 mL/min (Narcobit K-100RG, Natsume Seisakusho Co Ltd., Tokyo, Japan) through a face mask that was hung on a flexible coil hanger (Flexible coil hanging set C, Acoma Co., Ltd., Tokyo, Japan). The sevoflurane gas concentration was adjusted such that the pulse rate was maintained between 130 and 220 bpm. Rectal temperature was monitored with a thermometer and a rectal temperature probe (BAT-10 and RET-1, Physitemp Instruments, Inc., Clifton, USA). Additional special monitoring of breathing was conducted with the frequent visual inspection of chest and abdominal movement. The heater controller was set such that the temperature at the contact point of the apparatus surface and skin surface was maintained between 39 and 40 °C.

2.3. Artificial insemination

Females that were incompatible with their paired males (No. 1, 16, 17 in Fig. 3) were artificially inseminated on day –1. Semen was collected by vibratory stimulation of the penis as described previously [11], with minor modifications. Briefly, an electric toothbrush with a frequency of 117 Hz (DB-3, Minimum Corp., Tokyo, Japan) or 100 Hz (Clinica, Lion Corporation, Tokyo, Japan) fitted with a 5.5- or 6.5-mm outer diameter (o.d.) silicone tube was used on unsedated males seated on a marmoset treatment apparatus (CL-4532, Clea Japan Inc., Tokyo, Japan). The procedure lasted less than 5 min. Sperm were suspended in 50 μL of test yolk buffer (90128, IS Japan Co., Ltd., Saitama, Japan) and were checked for motility.
(typically 50–80%), and the whole volume (70–90 µL) was then placed into the vagina of an anesthetized female.

2.4. Embryo collection

Flushing instruments included a blunt tapered 28-G (i.d. 0.17 mm, o.d. 0.36 mm), 118-mm long, inner stainless steel cannula (custom made, Ito Corp., Shizuoka, Japan); a blunt tapered 19-G (i.d. 0.82 mm, o.d. 1.08 mm) or 20-G (i.d. 0.68 mm, o.d. 0.90 mm), 100-mm long, outer stainless steel cannula (custom made, Ito Corp., Shizuoka, Japan); and a 7-cm long, 5- or 6-mm outer diameter glass tube. All instruments were sterilized with a hydrogen peroxide gas plasma sterilizer (Sterrad 50, Advanced Sterilization Products, Irvine, USA) before use. The inner cannula was connected to an extension tube. The other side of the extension tube was connected to 1- and 5-mL syringes via a three-way stopcock (Fig. 1b). The inner cannula was inserted into a 19-G outer cannula. The tube, syringes, and
stop-cock were filled with the medium (SynVitro Flush, Origio a/s, Malov, Denmark). The apparatus described above was kept warm in an incubator until use. The glass tube was inserted into the vagina to distend the internal wall of the vagina and maintain the sterility of the cannulae tips.

The second operator placed a linear ultrasound probe (PLT-1204BT with Xario, Toshiba Medical Systems Corp., Tochigi, Japan) transversally to the uterus to locate the cervix. The first operator aligned the tips of the cannulae, inserted them into the glass tube, and explored the cervix at the center of the circular echogenic signal. Once the inner cannula entered the cervix, the second operator placed the probe longitudinally. The first operator, with the aid of ultrasonography, placed the outer cannula forward to the point just ahead of the internal uterine orifice. When the 19-G outer cannula was too tight to proceed into the cervix, it was replaced by a 20-G outer cannula. We placed the tip of the inner cannula 2–3 mm ahead of the outer cannula (Fig. 1d). While the first operator was holding the cannulae, the second operator inserted a medium collection set. The collection set consisted of a jack and a 50-mm Petri dish or a 75-mm transparent evaporation dish and a doughnut-shaped ring. To avoid pulling out the inner cannula, we tied its base with a thread (Trinitytrachtubeties, Trinitytrachtubeties, USA) to the treatment apparatus. We then checked the cannulae tip locations with ultrasonography and adjusted the height of the jack such that the cannulae and the endometrium were in line. The second operator, with the thumb and index finger, compressed the oviducts against the visceral organs by digital palpation through the abdominal wall. The first operator flushed the uterus slowly over a 1-min period with 1 mL of medium using a 1-mL syringe. When all conditions were appropriate, the medium started dripping from the outer cannula when approximately 0.2 mL of medium was injected. The dripped medium was collected into a dish (Fig. 1c). Then, another 1 mL of medium was loaded from a 5-mL syringe to the 1-mL syringe through the stopcock and used to flush the uterus from the 1-mL syringe. We repeated this process twice more and obtained a total medium flush of 4 mL.

During the initial condition evaluation and set-up period, we found that compressing the oviducts is no longer necessary after 4 mL of flushing, i.e., the medium flows through the outer cannula even with increasing pressure without oviduct compression. Hence, the second operator stopped compressing the oviducts and placed the ultrasound probe longitudinally. The first operator flushed an additional 1 mL of medium from the 5-mL syringe with increasing speed while observing the distention of the uterus using ultrasonography (Fig. 1e). The glass tube and cannulae were removed from the vagina and were washed with additional medium. Some of the collected embryos were incubated in an incubator (BNP-110, Espec Corp., Osaka, Japan) maintained at 38 °C under low-oxygen conditions (90% N₂ + 5% CO₂ + 5% O₂).

### 3. Results

During an 18-month data collection period, we were successful in cannulating the cervix in 167 cases but were not successful for one animal twice in succession. The success rate (167/169) was higher than that (49/54) of otoscope-guided cannulation in a previous report (Fisher’s exact test, two-tailed, p = 0.00999). The minimum and average inter-flush intervals were 21 and 41 days, respectively. In most flushing attempts, the time from the anesthesia induction to the end of the experiment was 25–40 min. During the procedure, the typical pulse rate was 150–200 bpm, oxygen saturation was 95–100%, and rectal body temperature was 37.0–38.5 °C. Table 1 lists the recovery data for ovulation products over 167 flushings. Of the 167 flushings, we recovered at least one ovulation product in 112 flushes. Of the 200 products, 179 were morphologically normal embryos, eight were unfertilized oocytes, one was an empty zona, and 12 were degenerate embryos.

Most of the recovered embryos were 8-cell stage (Fig. 2b) or later, but two were 4-cell stage embryos (Fig. 2a), and one was a 6-cell stage embryo. Although we used most recovered embryos for another study, we examined the viability of three morulae recovered on day 6 flushings (Fig. 2c). These embryos developed into one expanded blastocyst (Fig. 2d) and two hatching blastocysts three days later, indicating that the collected embryos had normal developmental capacity.

The number of flush attempts per animal ranged between one and 17. In the case of Pair 7, we collected embryos in 13

<table>
<thead>
<tr>
<th>Post ovulation day</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8–9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total trials</td>
<td>14</td>
<td>81</td>
<td>50</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>% of successful recovery</td>
<td>50%</td>
<td>65%</td>
<td>74%</td>
<td>79%</td>
<td>50%</td>
</tr>
<tr>
<td>% of successful recovery</td>
<td>10</td>
<td>96</td>
<td>73</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>Ovulation products/successful recovery</td>
<td>1.43</td>
<td>1.81</td>
<td>1.97</td>
<td>1.55</td>
<td>1.00</td>
</tr>
<tr>
<td>Unfertilized oocytes</td>
<td>0</td>
<td>4 (4%)</td>
<td>1 (1%)</td>
<td>3 (18%)</td>
<td>0</td>
</tr>
<tr>
<td>Zona pellucida only</td>
<td>0</td>
<td>1 (1%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Degenerate embryos</td>
<td>0</td>
<td>5 (5%)</td>
<td>7 (10%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4–8 cell stage embryos</td>
<td>7 (70%)</td>
<td>26 (27%)</td>
<td>2 (3%)</td>
<td>1 (6%)</td>
<td>0</td>
</tr>
<tr>
<td>9–16 cell stage embryos</td>
<td>3 (30%)</td>
<td>54 (56%)</td>
<td>33 (45%)</td>
<td>4 (24%)</td>
<td>0</td>
</tr>
<tr>
<td>Morula stage embryos</td>
<td>0</td>
<td>5 (5%)</td>
<td>23 (32%)</td>
<td>6 (35%)</td>
<td>0</td>
</tr>
<tr>
<td>Blastocyst stage embryos</td>
<td>0</td>
<td>1 (1%)</td>
<td>7 (10%)</td>
<td>3 (18%)</td>
<td>4 (100%)</td>
</tr>
</tbody>
</table>

Ovulation products: any products resulted from ovulation, including unfertilized oocytes, broken zona pellucida, and embryos. Successful recovery: trials in which any ovulation products were collected.
attempts, including the last four trials in succession (Fig. 3). Similarly, successful recovery was obtained after more than eight repeated flushing attempts in Pairs 3, 5, 8, 10, 13, 14, and 15, indicating that the experimental load was tolerable and that repeated embryo collection using the current method is reasonably possible for marmosets.

4. Discussion

The higher success rate of cannulation in this report compared with that observed in the previous report [8] suggests that cannulation is easier with ultrasonography than with otoscopy and that the ultrasound-guided technique will thus enable more groups to participate in primate embryology research.

Our non-surgical procedure did not produce any residual damage to the animal’s reproductive health. Repeated uterine flushing did not appear to affect the cycle length, hormone parameters, ovulation, integrity of the reproductive tract, or capacity to conceive (data not shown). Furthermore, repeated collections did not appear to affect either the quality or quantity of the embryos produced. We have not yet found a practical limit on the frequency of use for donor females. An additional strength of using ultrasonography is that it produces reduced structural or physiological disruption of the uterine endometrium, which can otherwise possibly reduce the pregnancy rate [12].

The number of ovulation products per successful recovery reached 1.97 by day 6 of flushing. The average number of corpora lutea previously reported is 2.3 [1] or 2.1 [8]. Using 2.2 corpora lutea per animal as an estimate, the recovery of 73 ovulation products in 37 flushing corresponds to a recovery rate of 90%, suggesting that thorough flushing was accomplished using our technique, which was likely due to the distention of the uterus during the flushing. The rates of

Fig. 2 – Images of embryos recovered from the common marmoset. (a) A 4-cell embryo recovered on day 5 flushing. (b) An 8-cell embryo recovered on day 5 flushing. (c) Morula-stage embryos recovered on day 6 flushing. (d) The blastocyst-stage embryo after a three-day culture of the embryo recovered on day 6 flushing. Scale bar: 200 μm, applies to all panels.

Fig. 3 – Individual embryo collection throughout the data collection period. The open circles represent successful recoveries, and solid circles represent unsuccessful recoveries. The numbers to the right denotes the female number.
successful recovery on days 4 and 5 were lower than that on day 6, suggesting that the embryos were passing into the uterus from the oviducts around days 4 and 5. The numbers of ovulation products per successful recovery on days 4 and 5 were also lower than on day 6. It is likely that the timing of passing into the uterus is not necessarily synchronous among two or more embryos, e.g., one embryo is present in the uterus while another remains in the oviduct. The number of ovulation products per successful recovery was also low on day 7 and later. The size of the embryo increases as it develops. The typical diameter of the morula on day 6 was 160 μm (Fig. 2c), and after a three-day culture, it was 200 μm (Fig. 2d). Because the average clearance between two cannulae is 160 or 230 μm depending on the size of the outer cannula, the embryos on day 7 or later are more likely to stick at the tip of the outer cannula than are the embryos on day 6. If this is the case, it is better to introduce a thinner outer cannula to collect blastocyst stage embryos.

One finding we had not expected before starting the study was the recovery of the early embryos. According to Summers et al. [1], 10-cell stage or earlier stage embryos can only be obtained from the oviducts on day 3 (day 4 in their terminology) or earlier, and 10- to 20-cell stage embryos can only be obtained from the uterus on days 4 and 5. Our data indicate that 8-cell stage or earlier stage embryos can be obtained by non-surgical uterine flushing. Our oviduct compression may have squeezed the embryo out to the uterus, combined with the possible difference in the estimation of ovulation day. The latter is possible because the measurement systems are based on enzyme immunoassays using antibodies raised against human antigens, and thus, the affinity may differ for marmoset antigens. Furthermore, 83% (80/96) of the embryos were 16-cell stage or earlier when collected on day 5 in our study. Because Thomson et al. [8] performed embryo collection on day 8 and Hanazawa et al. [9] did not describe data on a daily basis, our study is the first to demonstrate that early stage embryos can be obtained stably and frequently by day 4 or 5 using non-surgical uterine flushing. In conclusion, the ease and effectiveness of this novel ultrasound-guided technique will enable more groups to participate in marmoset embryology research and facilitate progress in this field.

Acknowledgements

This work was supported by The Research Grant (20-10) for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare, and an Intramural Research Grant (23-9) for Neurological and Psychiatric Disorders from the NCNP. We are also thankful for the generous support and encouragement from Drs. Katsuki Nakamura, Keiji Wada, Shin’ichi Takeda, and Shinichi Kohsaka.

References


