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—Original Article—

Improvement of a twice collection method of mouse oocytes by surgical operation

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Abstract. Mouse oocytes are generally collected after euthanasia. However, if oocytes were collected without euthanasia, then mice could be used to collect oocytes again after recovery. This condition is especially useful for mice that are genotypically rare. In this study, we examined the reusability of mice after collecting oocytes via a surgical operation. When oocytes were collected using medetomidine/midazolam/butorphanol combination anesthesia and examined for the quality of oocytes after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), they could develop to full term at the same rate as controls. When oocytes were collected from those mice a second time, the average number of oocytes was reduced by nearly 1/3. However, the blastocyst and offspring rates of those oocytes after IVF or ICSI were the same as those of the control regardless of the recovery day period. Although germinal vesicle (GV) oocytes can be collected from all reused mice, the final number of offspring did not increase. Interestingly, when oocytes were collected from the front position of the ampulla, 76% of the oviducts possessed oocytes after reuse, and the average number of oocytes significantly increased to a level comparable to that of the control. Finally, we examined whether reused mice can be used as recipient females, and then healthy offspring were obtained similarly as the control recipients. In conclusion, we provide a new method to collect a sufficient number of oocytes from reused mice without concern.

Key words: Intracytoplasmic sperm injection (ICSI), *In vitro* fertilization (IVF), Oocyte, Ovulation, Reuse

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In mice, oocytes are usually collected after euthanasia. If the mouse is unique and irreplaceable but shows an infertile phenotype, such as naturally mutated mice, assisted reproductive technology is required to produce the next generation [1, 2]. However, due to several reasons, sometimes, oocytes unexpectedly fail to fertilize *in vitro* with spermatozoa. If oocytes could be collected from a particular mouse without euthanasia, then that mouse could be used again to collect oocytes after it recovers from the damage of the surgical operation. In addition, from the viewpoint of animal welfare, it is required to reduce the number of used mice. For these reasons, oocyte collection by surgical operation is beneficial.

Recently, Byers *et al.* reported that when oocytes were collected using the surgical oocytes retrieval (SOR) method, second ovulated oocytes (Second-oocyte) can be collected from previously used mice (reuse mouse), but the number was decreased to about 30% compared with the number of first ovulated oocytes (First-oocyte) [3]. Importantly, the Second-oocytes collected from reused mice showed a normal developmental potential after *in vitro* fertilization (IVF). Because IVF sometimes fails, the most reliable method to generate offspring from oocytes *in vitro* is intracytoplasmic sperm injection (ICSI) rather than IVF [4]. However, to perform ICSI, sperms are injected into oocytes by drilling a hole in the oocyte membrane, which causes more damage to oocytes than IVF, and some oocytes are died after ICSI by this damage [5]. Generally, the tolerance of oocytes to ICSI varies and depends on the oocyte quality or mouse strain [6, 7]. Therefore, it is important to know whether Second-oocytes derived from reused mice are of sufficient quality and if they can be used for ICSI. In addition, the SOR method requires that the incision in the ampulla region is closed by tissue adhesives [3], which increases the total surgical operation time and delays the time before IVF or ICSI can be performed after oocyte collection. For the practical use of the surgical collection of oocytes, we have to develop a simpler method that yields an increased number of Second-oocytes from reused mice.

Anesthesia is also an important factor for oocyte collection because it affects the quality of oocytes [3]. Until recently, ketamine, pentobarbital sodium (Nembutal), or tribromoethanol (Avertin) were used for murine anesthesia in experiments. However, some of these agents require a narcotic handling license for their use and possession[6]. Moreover, due to animal welfare, it was recommended to use a combination of medetomidine, midazolam, and butorphanol (MMB) anesthetic agents worldwide to reduce damage to the body of animals, but it is not yet known whether MMB causes any damage to the oocytes *in vivo* [6, 8].

Therefore, in this study, we examined the normality of the First-oocytes obtained by surgical collection with MMB anesthesia, the

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developmental potential of Second-oocytes after IVF or ICSI, and the recovery period of mice after the surgical operation. To increase the number of Second-oocytes, we aimed to improve the surgical method at the time of the first collection or collect immature oocytes from the ovaries of reused mice. Finally, we also examined whether the reused female mice can be used as recipients for embryo transfer.

Methods

Animal

ICR female mice and ICR male mice were obtained from SLC Inc (Hamamatsu, Japan) and bred in our mouse facility. The mice selected were collected at 8 to 12 weeks of age for experimental control purposes or for surgical oocyte collection. For the second oocyte collection or for reusability purposes as a recipient female, the selected mice first operated on were used at 10 to 30 days or 1 to 2 months after first oocyte collection, respectively. The surrogate pseudopregnant ICR females, which were used as recipients of the embryos, were mated with vasectomized ICR males, whose sterility had been previously demonstrated. On the day of the experiment or after all experiments were completed, the mice were euthanized by cervical dislocation and were used in the experiments described below. All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation of the University of Yamanashi.

Oocyte collection after euthanasia (control and Second-oocytes)

Female mice were superovulated by the injection of 7.5 IU of equine chorionic gonadotropin (eCG), followed by 7.5 IU of human chorionic gonadotropin after 46–48 h. Cumulus-oocyte complexes (COCs) were collected from the oviducts of females 14–16 h later and placed in a Falcon dish containing HEPES-CZB media [5]. To disperse the cumulus, COCs were transferred into a 50 µl droplet of a HEPES-CZB medium containing 0.1% bovine testicular hyaluronidase for 3 min. Cumulus-free oocytes were washed twice and moved to a 20 µl droplet of CZB medium [9] for culture.

Oocyte collection by surgical operation (First-oocytes)

Female mice were superovulated similarly as controls. To collect oocytes, superovulated female mice were anesthetized (body weight (g) × 0.01 ml MMB) [10], and the ovary/oviduct was pull out from body without killing the mice (Fig. 1). Using stereomicroscopy, a part of the ampulla of the removed oviducts was cut approximately 0.1–0.2 mm depth using micro scissors. The COCs were immediately popped out from the slit spontaneously, and then were collected using fine tweezers. In Exp. 2 and Exp. 3 (Fig. 1), the cutting position of ampulla was not decided, and around the middle of the ampulla was the typical location chosen to cut. After COCs collection, the ovary/oviduct was resituated in the body, the muscle and skin were sutured, and an equal amount of atipamezole was injected. Until awakening (usually after 10–30 min), mice were kept warm at 38°C using a heater.

Preparation of spermatozoa

Caudae epididymides were collected from ICR male mice, and the ducts were severed with sharp scissors. A few drops of the dense spermatozoa mass were then placed into a dish containing 200 µl HTF medium [11], which was then incubated at 37°C in 5% CO_2 for 1 h.

IVF

After the sperm collection, sperm was incubated for 1 h at 37°C in 5% CO_2, and ~5–10 µl of the sperm culture solution were added with a micropipette into the 200 µl HTF drop containing the previously collected COCs. After insemination for 5–6 h, pronucleus formation was verified, and zygotes were washed several times with a 20 µl CZB drop and cultured in CZB medium at 37°C and 5% CO_2.

ICSI

ICSI was performed as previously described [12, 13]. Briefly, for the microinjection of spermatozoa, 1–2 µl of the sperm culture solution were directly moved to the injection chamber. The application of several piezo pulses was used to separate the spermatozoa head from the tail, and the head was then injected into the oocyte. The oocytes that survived ICSI were incubated in the CZB medium at 37°C with 5% CO_2. Pronucleus formation was verified 6 h after ICSI.

Embryo transfer

Embryo transfer to oviducts: Embryos at the two-cell stage were transferred to the oviducts of a day 0.5 pseudopregnant mouse that had been mated with a vasectomized male the night before transfer. On day 18.5 of gestation, offspring were delivered via cesarean section.

Embryo transfer to the uteri of reused mice: four- or eight-cell stage embryos were flashed from the oviducts/uteri of normal superovulated female mice 2 days after being mated with males and used the next day. The embryos that developed to morulae or blastocysts were transferred to the uteri of a day 2.5 pseudopregnant reused mouse, which were also used at 10 to 30 days after the surgical operation. On day 18.5 of gestation, offspring were delivered via cesarean section.

Germinal vesicle (GV)-stage oocyte collection and in vitro maturation

The collection of GV-stage oocytes and oocyte maturation were performed as previously described [14, 15]. Briefly, GV-stage oocytes were collected from the ovaries of ICR female mice at 46–48 h after a 7.5 IU intraperitoneal injection of eCG. Blood and fat on the collected ovaries were removed. The ovary was dissected using a 26-gauge needle, and the GV oocytes with cumulus cells were collected in HEPES-CZB. The cumulus cells were denuded by pipetting. Abnormal (small, dark, and distorted) immature oocytes were removed. Denuded GV-stage oocytes were cultured for 16 h in 50 µl of IVM medium (αMEM supplemented with 5% fetal bovine serum). Only oocytes that developed into metaphase II (MII) oocytes were collected and subjected to IVF.

Examination of the appropriate cut position of the ampulla at the time of First-oocyte collection

At the time of First-oocyte collection under anesthesia, we defined the ovary side of the ampulla of oviducts as “front”, the uterus side as “rear”, and the middle of them as “middle” (Fig. 1). Once the mice recovered, we tried to collect oocytes again after euthanizing them.

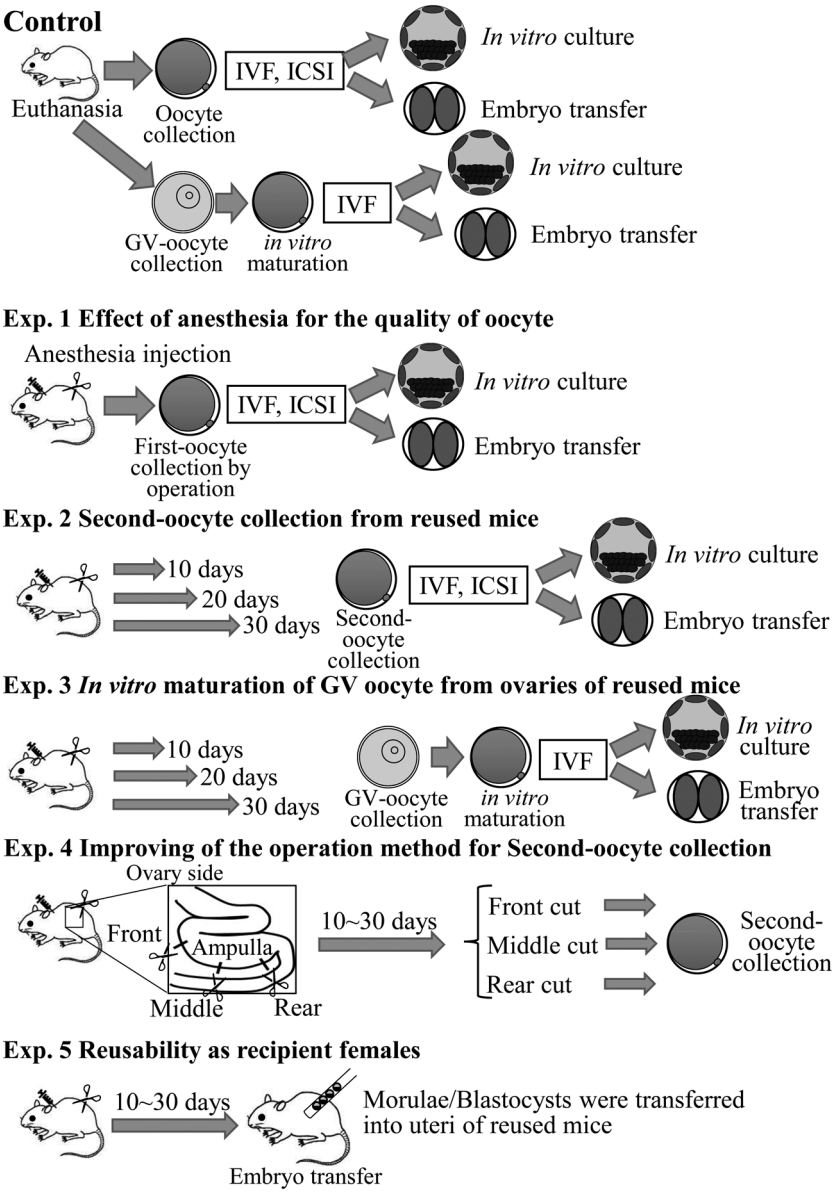

Fig. 1. Schematic diagram of the surgical operation and examination of the quality of First- and Second-oocytes. As a control, oocytes were collected after euthanasia as usual. Exp. 1: First-oocytes were collected via surgery under anesthesia, and their quality was examined by *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Then, fertilized embryos were cultured up to 4 days, or on the next day, two-cell stage embryos were transferred into recipient females. Exp. 2: 10–30 days after surgical operation, Second-oocytes were collected by euthanasia, and their quality was examined as in Exp. 1. Exp. 3: 10–30 days after surgical operation, germinal vesicle (GV)-stage oocytes were collected from the ovaries and matured *in vitro*. Once these oocytes matured to the MII stage, they were used as in Exp. 1. Exp. 4: To prevent the adhesion of oviducts, the cutting position of the ampulla was changed, and the number of Second-oocytes was examined. Exp. 5: Reused mice were used as recipient mice at 10–30 days after surgical operation. Morulae/blastocysts were transferred into the uteri of pseudopregnant reused mice.

Statistical analysis

The number of oocytes was evaluated using Student's *t*-test. Blastocyst formation, birth rates and the success rates of the oocytes collection were evaluated using Pearson's chi-squared test. Statistically significant differences between variables were determined at $P < 0.01$.

Results

Quality of First-oocytes collected via an operation

When IVF was performed and the developmental rate to the blastocyst stage or full term was evaluated, there was no significant difference between the control oocytes collected without operation (blastocyst: 90%, full term: 73%) and First-oocytes collected via a

surgical operation (blastocyst: 89%, full term: 60%) (Tables 1 and 2). Similar to IVF, when ICSI was performed, there was no significant difference between the control oocytes (blastocyst: 66%, full term: 59%) and First-oocytes (blastocyst: 73%, full term: 55%). These results clearly showed that there was no effect of MMB anesthesia treatment on the quality of oocytes after collection. In these experiments, 59 mice were used to collect First-oocytes via a surgical operation, but in most cases, those oocytes were subjected to IVF or ICSI and *in vitro* culture to the blastocyst stage or embryo transfer. Therefore, we could not show the number of used mice in these tables.

Number and quality of Second-oocytes collected from reused mice

As shown in Table 3, 47–52% of the reused mice had no COCs in either oviducts, 35–45% of the reused mice had COCs in only the oviduct on one side, and 9–12% of the reused mice had COCs in oviducts on both sides. Therefore, the total number of oocytes from the reused mice was significantly reduced by nearly 1/3 irrespective of the recovery period. However, compared with that of the ovulated oocytes, the average number of oocytes was comparable between the control and reused mice (control mouse: 15.7 oocytes *vs*. reuse mouse: 16.1–17.6 oocytes) (Table 3).

When IVF was performed, and the developmental rate up to the blastocyst stage or full term of the subjects was evaluated, although some mice demonstrated a significant difference between the second oocytes and control oocytes, the difference was not notable except for 10 days of the recovery period for full term mice (Tables 4 and 5). On the other hand, when ICSI was performed and the developmental rate to the blastocyst stage or full term was evaluated, there was no significant difference between the second oocytes and control oocytes regardless of the recovery period duration (Tables 4 and 5). This result shows that although the number of Second-oocytes from the reused mice was reduced, the quality of those oocytes was not inferior to the control oocytes, and the recovery period was not affected.

Number and quality of GV-stage oocytes from the reused mice

We attempted to collect GV-stage oocytes from ovaries because those immature oocytes can be collected without being affected by

Table 1. *In vitro* developmental potential of oocytes collected via surgical operation with anesthesia after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI)

Exp.	Collection method	No. of used mice *	No. of used oocytes	No. of oocytes surviving after ICSI	No. (%) ** of oocytes with 2PN formed	No. of embryos developed to			
						2 cell	4–8 cell	Morula	Blastocyst (%) **
IVF	Control	17	376	-	353 (93.9) [a]	348	335	325	319 (90.4) [a]
	Operation	42	1022	-	963 (94.2) [a]	939	894	861	852 (88.5) [a]
ICSI	Control	20	286	214	198 (92.5) [b]	192	165	144	130 (65.7) [b]
	Operation	17	294	218	204 (93.6) [b]	195	175	160	148 (72.5) [b]

Some experimental groups were not performed with control at the same time. PN: Pronuclei. * The data of oocytes collected from these mice were used not only in this table but also in Table 2. ** Identical letters indicate no significant differences by Pearson's chi-squared test ($P > 0.01$).

Table 2. Effect of anesthesia on the full-term development of First-oocytes after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI)

Exp.	Collection method	No. of used oocytes	No. of oocytes surviving after ICSI	No. (%) * of oocytes with 2PN formed	No. of embryos developed to 2 cell	No. of transferred embryos (no. of recipients)	No. (%) * [min–max] of offspring
IVF	Control	141	-	139 (98.6) [a]	139	89 (8)	65 (73.0) [a] [33.3–100]
	Operation	159	-	148 (93.1) [a]	147	72 (6)	43 (59.7) [a] [40.0–81.8]
ICSI	Control	141	82	82 (100) [b]	80	70 (7)	41 (58.6) [b] [28.6–70.0]
	Operation	141	114	111 (97.4) [b]	111	99 (7)	54 (54.5) [b] [30.0–88.9]

PN: Pronuclei. * Identical letters indicate no significant differences by Pearson's Chi-square test ($P > 0.01$).

Table 3. Number and normal rate of Second-oocytes collected from the reused mice

Recovery period after surgery	No. of used mice (oviducts)	No. (%) of mice with COCs contained in oviducts			No. of oocytes		Average no. of normal oocytes	
		Without COCs	Only 1 oviduct	Both oviducts	Total	Normal oocytes (%) [average] *	Per mouse with COCs	Per oviduct contained COCs
Control	29 (58)	0	0	29 (100.0)	1150	910 (79.1) [31.4] [a]	31.4	15.7
10 days	46 (92)	23 (50.0)	19 (41.3)	4 (8.7)	550	476 (86.5) [10.3] [b]	20.7	17.6
20 days	53 (106)	25 (47.2)	24 (45.3)	5 (9.4)	614	567 (92.3) [10.7] [b]	19.6	16.7
30 days	65 (130)	34 (52.3)	23 (35.4)	8 (12.3)	681	581 (85.3) [8.9] [b]	18.7	16.1

COCs: Cumulus oocytes complexes. * Different letters indicate significant differences by *t*-test ($P < 0.01$).

Table 4. *In vitro* development of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) embryos derived from Second-oocytes

Exp.	Recovery period after surgery	No. of used oocytes	No. of oocytes surviving after ICSI	No. (%) * of oocytes with 2PN formed	No. of embryos developed to			
					2 cell	4–8 cell	Morula	Blastocyst (%) *
IVF	Control	376	–	353 (93.9) [ab]	348	335	325	319 (90.4) [ab]
	10 days	222	–	214 (96.4) [a]	213	213	206	202 (94.4) [a]
	20 days	194	–	182 (93.8) [ab]	180	176	168	154 (84.6) [bc]
	30 days	189	–	169 (89.4) [b]	161	158	146	131 (77.5) [c]
ICSI	Control	286	214	198 (92.5) [c]	192	165	144	130 (65.7) [d]
	10 days	70	64	59 (92.2) [c]	57	53	43	36 (61.0) [d]
	20 days	107	81	78 (96.3) [c]	76	69	61	59 (75.6) [d]
	30 days	97	81	79 (97.5) [c]	78	73	64	57 (72.2) [d]

Some experimental groups were not performed with control at the same time. PN: Pronuclei. * Different letters indicate significant differences by Pearson's Chi-square test ($P < 0.01$).

Table 5. Full-term development of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) embryos derived from Second-oocytes

Exp.	Recovery period after surgery	No. of used oocytes	No. of oocytes surviving after ICSI	No. (%) * of oocytes with 2PN formed	No. of embryos developed to two cells	No. of transferred embryos (no. of recipients)	No. (%) * [min–max] of offspring
IVF	Control	141	-	139 (98.6) [a]	139	89 (8)	65 (73.0) [a] [33.3–100]
	10 days	71	-	71 (100) [a]	71	51 (6)	24 (47.1) [b] [20.0–63.6]
	20 days	30	-	25 (83.3) [b]	24	24 (3)	22 (91.7) [a] [87.5–100]
	30 days	29	-	27 (93.1) [ab]	27	27 (3)	22 (81.5) [a] [62.5–90.0]
ICSI	Control	141	82	82 (100) [c]	80	70 (7)	41 (58.6) [b] [28.6–70.0]
	10 days	41	24	22 (91.7) [c]	20	17 (2)	11 (64.7) [b] [54.5–83.3]
	20 days	83	46	44 (95.7) [c]	36	32 (4)	22 (68.8) [b] [40.0–75.0]
	30 days	43	23	22 (95.7) [c]	22	21 (3)	12 (57.1) [b] [20.0–100]

Some experimental groups were not performed with control at the same time. PN: Pronuclei. * Different letters indicate significant differences by Pearson's Chi-square test ($P < 0.01$).

oviduct adhesions. As a result, GV-stage oocytes were collected from all reused mice, and the number of oocytes per mouse was similar to the control irrespective of the recovery periods (27–30 GV-stage oocytes from reused mice *vs.* 31 GV-stage oocytes from the control) (Table 6). The *in vitro* maturation rates of these GV-stage oocytes to MII stage oocytes, fertilization rate *in vitro*, and developmental rate to the blastocyst or full term were not significantly different compared to the control mice, regardless of the recovery period of the reused mice. A significant difference was observed only in the rate of PN formation between 20 days and 30 days of the recovery period.

Prevent the adhesion of oviducts by improving the cutting position of the ampulla

As shown in Table 3, when the oviducts contained COCs, the number of oocytes was similar to the control (16–18 *vs*. 16), suggesting that the lower number of oocytes from reused mice is due to the adhesion of the oviduct rather than the reduced number of oocyte ovulation from the ovaries. Therefore, we tried to reduce the number of adhesions in the oviduct. When the tool used to cut the ampulla was changed from scissors to needles or the cutting depth was modified, there was no improvement (data not shown). Next, we attempted to determine the best position of the ampulla for cutting. When the middle or rear position of the ampulla was cut, COCs were collected, and the mice recovered from the operation, Second-oocytes were collected from 25–31% of the oviducts derived from the reused mice. However, when COCs were collected after cutting the front position of the ampulla, Second-oocytes were obtained from 76% of the oviducts derived from the reused mice, which was significantly higher than that of the other positions (Table 7). These collected Second-oocytes were used for the above experiments (Tables 4 and 5), which demonstrated that the quality of oocytes collected from the front position of the ampulla was normal.

Embryo transfer into the reused mice as recipient females

Finally, we examined whether reused mice can be used as recipient females. In this study, morulae/blastocysts were transferred into the uterus because the transfer of the embryo to the oviduct was difficult due to the adhesion of the oviduct as described above. As a result, there was no adverse effect on the birth rate of offspring in reused recipients compared with usual recipients (49 *vs*. 42%, respectively) (Table 8). These results suggest that the reused mice can be used as usual recipients.

Discussion

In this study, we demonstrated that the Second-oocytes from the

Table 6. Blastocyst and full-term development of *in vitro* fertilization (IVF) embryos derived from germinal vesicle (GV)-stage oocytes of reused mice

Recovery period after surgery	No. of used mice	No. (average) * of GV oocytes	No. (average) * [%] of MII oocytes after IVM	No. of used oocytes	No. (%) ** of oocytes with 2PN formed	No. of embryos developed to				No. of transferred embryos (no. of recipients)	No. (%) ** [min–max] of offspring
						2 cell	4–8 cell	Morula	Blastocyst (%) **		
Control	16	493 (30.8) [a]	381 (23.8) [a] [77.3]	264	156 (59.1)	143	111	87	69 (44.2) [a]	–	–
				66	48 (72.7)	44	–	–	–	44 (4)	13 (29.5) [a] [22.2–33.3]
10 days	12	327 (27.3) [a]	203 (16.9) [a] [62.1]	155	95 (61.3)	89	64	43	40 (42.1) [a]	–	–
				47	31 (66.0)	30	–	–	–	30 (2)	13 (43.3) [a] [31.3–57.1]
20 days	14	425 (30.4) [a]	302 (21.6) [a] [71.1]	281	167 (59.4) [a]	155	116	94	78 (46.7) [a]	–	–
				19	16 (84.2) [b]	16	-	–	–	16 (1)	5 (31.3) [a] [31.3]
30 days	27	733 (27.1) [a]	552 (20.4) [a] [75.3]	426	267 (62.7)	240	170	115	97 (36.3) [a]	–	–
				88	66 (75.0) [b]	63	–	–	–	63 (4)	18 (28.6) [a] [15.0–63.6]

Some experimental groups were not performed with control at the same time. PN: Pronuclei. * Identical letters indicate no significant differences by *t*-test (P > 0.01). ** Identical letters indicate no significant differences by Pearson's Chi-square test (P > 0.01).

Table 7. Success rates of Second-oocyte collection by cutting the position at the First-oocyte collection

Cutting position	No. of oviducts	No. (%) * of oviducts contained COCs	No. (average) ** of normal oocytes collected ***
Control	12	12 (100) [a]	199 (16.6) [a]
Front	17	13 (76.5) [a]	230 (13.5) [a]
Middle	13	4 (30.8) [b]	66 (5.1) [b]
Rear	40	10 (25.0) [b]	190 (4.8) [b]

COCs: Cumulus oocytes complexes. Total 41 mice were used in this experiment. For some mice, the ampulla was cut at different positions in the same individual.* Different letters indicate significant differences by Pearson's Chi-square test (P < 0.01). ** Different letters indicate significant differences by *t*-test (P < 0.01). *** Number of oocytes collected per an oviduct.

Table 8. Embryo transfer into the reused mice as recipients

Exp.	No. of transferred embryos (no. of recipients)	No. (%) * of offspring	Min–Max
Control	73 (7)	31 (42.5) [a]	16.7–72.7
Reuse **	106 (7)	52 (49.1) [a]	22.2–83.3

* Identical letters indicate no significant differences by Pearson's Chi-square test (P > 0.01). ** Reuse indicates the mice recovered for 10–30 days from First-oocytes collection were used as recipients without distinction.

reused mice have enough potential to be used for other experiments without considering the effect of anesthesia on the First-oocytes. In addition, we successfully increased the number of Second-oocytes from the reused mice by improving the surgical operation method.

First, the effects of anesthesia on the First-oocytes were examined because MMB was recently recommended for anesthesia in mice [10]. This mixed anesthesia is better according to the animal welfare guidelines, but its effect on oocytes is not yet clear. In this study, we clearly demonstrated that MMB did not detrimentally affect the quality or developmental potential of oocytes after IVF or ICSI, suggesting that MMB can be used for oocyte collection without any concern.

Importantly, the quality of these Second-oocytes was similar to that of the First-oocytes and controls. In general, ICSI causes damage to the oocyte membrane, and a relatively strong tolerance of the oocytes is required compared with IVF [5]. However, when the Second-oocytes were used for not only IVF but also ICSI, they fertilized and developed to full term with a high success rate that was the same as the controls. Thus, we conclude that the quality of Second-oocytes is comparable to that of the control and that these oocytes can be used for other experiments without any concern.

Similarly, when we collected GV-stage oocytes from the reused mice and matured them *in vitro*, the developmental potential after IVF was not different between secondary collected GV-stage oocytes and control GV-stage oocytes. However, although we could collect GV-stage oocytes from all reused mice, this method required IVM, and in general, the maturation rate of GV-stage oocytes to the MII stage was low [14], and the quality of those *in vitro* matured oocytes was lower than that of naturally matured and ovulated MII oocytes [16]. These results suggest that although GV-stage oocytes can be collected from all reused mice, the final number of blastocysts or full-term oocytes was not increased compared with mature oocytes collected from reused mice due to the difficulty of IVM and lower quality of matured oocytes.

In addition, when the reused mice were used 10 days after surgical operation, the number of Second-oocytes was not different compared with mice that recovered for 20 or 30 days after surgical operation. This finding suggests that even though the effects of the hormone may remain in the body of the reused mice, it did not affect the ability of reused mice to ovulate the Second-oocytes. Thus, there is no need to maintain reused mice for a longer period until use, which reduces the cost of maintenance fees.

However, the most significant limitation of this method is the lower number of second-oocytes collected from the reused mice. When the reused mice were superovulated, approximately 50% of them ovulated with relatively the same number of oocytes irrespective of the recovery period after the operation (Table 3), which suggests that the capacity of the mice ovary for superovulation was not lose. The remaining ampulla did not contain any oocytes, most likely due to the adhesions of the oviducts. Byers *et al.* reported that the closure of the ampulla incision is a critical step in the SOR method to ensure

that oocytes produced in subsequent ovulations will be contained within the oviduct [3]. In the absence of a tissue adhesive, oocytes were not present in the oviduct. They conclude that a tissue adhesive method must be used to close the ampulla incision to ensure that future oocytes will be contained in the oviduct. However, in our study, when the surgical operation was performed to collect First-oocytes, we did not close the ampulla incision after the operation, which suggests that a tissue adhesive method is not essential (Table 3). When we collected COCs via a surgical operation, the ampulla was cut as minimally as possible, which may reduce damage and allow for spontaneous healing.

This result suggests to us that if we can improve the collection method of COCs from the ampulla by reducing damages, then the number of ampulla containing COCs would increase. Before starting this experiment, we thought that the rear position (close to the uteri) of the ampulla is better to cut for COCs collection than the front (close to the ovary) because even if adhesion occurs in the ampulla, COCs can still be inside the oviduct. However, in contrast to our expectation, when COCs were collected from the front of the ampulla, nearly 80% of the oviducts possessed COCs, and the number of the Second-oocytes from the reused mice significantly increased to a number that was similar to the control (Table 7). The reason is unclear, but it is known that the mammalian oviduct, especially in the close to the uteri position, acts as the site of sperm storage by adhesion with spermatozoa [17], which may suggest that adhesion of oviduct is varies by site. More examination is required to understand this phenomenon.

Finally, we also examined whether reused mice can be used as recipients. When morulae/blastocysts were transferred to the uterus, healthy offspring were obtained with a success rate similar to that of the control, which suggests that reused mice can be used not only as oocyte donors but also as recipient females.

In conclusion, although this method raises an ethical problem because it increases the burden to a single mouse, the number of mice used in the experiment can be reduced by nearly half (59%). This method requires a surgical operation before the start of the experiment, but it takes less than 30 min. The recovery period of operated mice is only 10 days, and then mice can be reused any number of days later. Furthermore, the number of Second-oocytes is increased by up to 81% compared with usual mice. Thus, this method has several advantages, including reducing the number of mice and cost. In addition, if the mouse is genetically rare and irreplaceable, then the benefits of the surgical collection of oocytes are greater, even when considering the burden of surgery.

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