

Efficient and scheduled production of pseudopregnant female mice for embryo transfer by estrous cycle synchronization

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Abstract. In embryo transfer experiments in mice, pseudopregnant females as recipients are prepared by sterile mating with vasectomized males. Because only females at the proestrus stage accept males, such females are selected from a stock of animals based on the appearance of their external genital tract. Therefore, the efficiency of preparing pseudopregnant females largely depends on the size of female colonies and the skill of the operators who select females for sterile mating. In this study, we examined whether the efficiency of preparing pseudopregnant females could be improved by applying an estrous cycle synchronization method by progesterone (P4) pretreatment, which significantly enhances the superovulation outcome in mice. We confirmed that after two daily injections of P4 (designated Days 1 and 2) in randomly selected females, the estrous cycles of most females (about 85%) were synchronized at metestrus on Day 3. When P4-treated females were paired with vasectomized males for 4 days (Days 4–8), a vaginal plug was found in 63% (20/32) of the females on Day 7. After the transfer of vitrified-warmed embryos into their oviducts, 52% (73/140) of the embryos successfully developed into offspring, the rate being comparable to that of the conventional embryo transfer procedure. Similarly, 77% (24/31) of females became pregnant by fertile mating with intact males for 3 days, which allowed the scheduled preparation of foster mothers. Thus, our estrous cycle synchronization method may omit the conventional experience-based process of visually observing the vagina to choose females for embryo transfer. Furthermore, it is expected that the size of female stocks for recipients can be reduced to less than 20%, which could be a great advantage for facilities/laboratories undertaking mouse-assisted reproductive technology.

Key words: Embryo transfer, Estrous cycle, Mouse, Pseudopregnant, Recipient

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Assisted reproductive technology (ART) in animals comprises an essential part of the preservation of genetic resources and generation of gene-modified animals. The generation of gene-modified mice, such as transgenic and knockout animals, became successful as early as the 1980s, and the number of gene-modified strains has been exponentially increasing. As a result, the development of time- and cost-effective ART in mice is in high demand [1–3]. Major basic ART in mice includes superovulation, *in vitro* fertilization (IVF), embryo culture, cryopreservation of spermatozoa and embryos, and embryo transfer. The techniques for IVF, embryo culture, and cryopreservation have been extensively improved by the use of chemicals such as methyl- β -cyclodextrin for the capacitation of frozen-thawed spermatozoa [4]. Although the superovulation regimen using equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) has long been used without any

technical improvements, it has been reported recently that the use of anti-inhibin antiserum instead of eCG greatly increases the number of oocytes obtained from superovulated females [5, 6]. Therefore, among the basic ART-related techniques in mice, only the embryo transfer technique has remained unchanged for 40–50 years. The conventional embryo transfer procedures, either oviductal transfer or uterine transfer, are practical enough to obtain mouse pups at high rates. However, the preparation of pseudopregnant females as recipients is an inefficient process, because females at the proestrus stage are selected by visual observation of the vagina before mating with vasectomized males [7]. The precision of this selection depends on the training and experience of the operator. Furthermore, because the length of one estrous cycle in mice is usually 4–5 days, it is necessary to maintain at least 4–5 times the number of females than are actually used for embryo transfer. In addition, when the pups are retrieved by Caesarean section at term, it is also necessary to prepare pregnant females as foster mothers on the same day or a few days before embryo transfer.

For embryo transfer experiments in animals with a complete estrous cycle, recipient females are often prepared by estrous cycle synchronization. In the bovine embryo transfer program, silicon tubes filled with crystalline progesterone (P4) are inserted into the vaginas of recipient cows for approximately 1 week, and then removed to recur their estrous cycle artificially from a luteal phase, followed

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by induction of ovulation by the injection of prostaglandin [8]. Furthermore, in the guinea pig, artificial insemination is conducted following the implantation of a P4 tube for 2 weeks [9]. However, there has been no report of estrous synchronization for embryo transfer in animals with an incomplete estrous cycle, such as mice. Recently, we devised an estrous-synchronization protocol in mice to improve the efficiency of the superovulation regimen [6], and found that most females (93%) at different stages were synchronized to the metestrus stage after two daily injections of P4 [6].

The present study was undertaken to examine whether the same synchronization method could be used to synchronize recipient females for embryo transfer. If this method is applicable, it may reduce the space, effort, and expense necessary for maintaining female stocks and selecting appropriate females for embryo transfer.

Materials and Methods

Animals

C57BL/6J (B6) and ICR mice (CLEA Japan Inc., Tokyo, Japan) were used for the production of 2-cell embryos by IVF and pseudopregnant or pregnant females, respectively. Females were at 10 to 20 weeks of age and males were at 3 to 12 months of age. They were housed with ad libitum food and water under controlled temperature ($24 \pm 1^\circ\text{C}$), humidity ($55 \pm 2\%$), and lighting conditions (daily light period, 0700 to 2100 h). The animal experiments described here were approved by the Animal Experimentation Committee at the RIKEN Tsukuba Institute, and were performed in accordance with the committee's guiding principles.

IVF

Superovulation was performed as previously described [6]. In brief, the mice were injected subcutaneously with 2 mg (0.08 ml per female) of P4 (Progehormon; Mochida Pharmaceutical, Tokyo, Japan) in the evening (1800–2000 h) once a day for 2 days for the synchronization of estrous cycles. The mice were then injected intraperitoneally with anti-inhibin serum (100 μl) (AIS; Central Research, Tokyo, Japan), followed by injection with hCG (5 IU) (Gonotropin; ASKA Pharmaceutical, Tokyo, Japan) 48 h later. IVF was performed with epididymal spermatozoa using a previously described method, with slight modifications [6]. In brief, cumulus-enclosed oocytes were collected and preincubated for 1–1.5 h in 80 μl droplets of human tubal fluid medium (HTF) [10] supplemented with 1.25 mM reduced glutathione [11–14]. Each droplet contained oocytes collected from one female or from one oviduct. Sperm suspensions from the epididymal caudae of male mice (3–12 months old) were suspended in 400 μl of sperm preincubation medium (HTF containing 0.4 mM methyl- β -cyclodextrin [4,15] and 0.1 mg/ml polyvinyl alcohol, but without bovine serum albumin) and incubated at 37°C under 5% CO_2 in humidified air for 45–60 min. At the time of insemination, the preincubated spermatozoa were transferred into the droplets containing oocytes at concentrations of 200–400 spermatozoa/ μl . After 3–4 h, oocytes were separated from spermatozoa and cumulus cells using a fine glass pipette, and transferred into 10 μl droplets of CZB medium [16] containing 5.6 mM glucose, 0.1 mg/ml polyvinyl alcohol, and 3.0 mg/ml bovine serum albumin. They were cultured at 37°C under 5% CO_2 in humidified air for approximately 24 h.

Oocytes that developed into 2-cell embryos with a normal appearance and distinct polar body were considered fertilized.

Embryo cryopreservation by vitrification

For cryopreserving IVF-derived 2-cell embryos, we used an equilibrium vitrification method as reported previously [17], with slight modifications. Briefly, after the embryos were equilibrated in a medium consisting of 5% dimethyl sulfoxide and 5% ethylene glycol in phosphate-buffered medium (PB1) [18] for 3 min, they were transferred with a minimal volume of medium by glass pipette into a cryotube (1.2 ml, SUMILON MS-4501, Sumitomo Bakelite, Tokyo, Japan) containing 50 μl of vitrification medium: 42.5% (v/v) ethylene glycol, 17.3% (w/v) Ficoll, and 1.0 M sucrose in PB1. The cryotube was plunged directly into liquid nitrogen. On the day of embryo transfer, the cryotube was retrieved from the liquid nitrogen and the lid removed. The embryos were warmed to room temperature for 2 min on the bench, and then 850 μl of 0.75 M sucrose/PB1 solution was added slowly at room temperature. Four minutes later, the embryos were recovered on a plastic dish and exposed to 0.25 M sucrose/PB1 solution for 1–2 min. The surviving embryos were transferred to a drop of CZB medium and cultured as described above until embryo transfer.

Embryo transfer

The vitrified-warmed 2-cell embryos produced by IVF were transferred into the oviducts of Day 1 pseudopregnant females of the ICR strain. For anesthesia, 2.5% tribromoethanol (0.014 ml per gram of body weight) was administered by intraperitoneal injection. In the evening on Days 18 and 19, each female was subcutaneously injected with 2 mg of P4 to avoid spontaneous delivery. On the morning of Day 20, the recipient female mice were examined for the number of implantation sites, and live offspring were retrieved by Caesarean section.

Synchronization of the estrous cycle

ICR females used for synchronization of the estrous cycle were caged for at least 1 week as an adaptation period without the addition of other females. We classified their estrous cycle stages based on the morphology and quantity of cells in the vaginal smears at 0900–1000 h as follows [6]: proestrus stage (P), nucleated and few cornified epithelial cells; estrus stage (E), many cornified epithelial cells; metestrus stage (M), many leukocytes and few nucleated epithelial cells; and diestrus stage (D), decreased cell numbers and few leukocytes [7, 19]. For synchronization of the estrous cycle, females were subcutaneously injected with 2 mg (0.08 ml per female) of P4 in the evening (1800–2000 h) once a day for 2 days (designated Days 1 and 2 in this experiment) [6].

Preparation of pseudopregnant and pregnant females by the conventional method

For preparing pseudopregnant or pregnant recipients by the conventional method, the external appearance of the vagina of ICR females was examined. Females showing a gaping vagina and swollen, reddish-pink tissues around the vagina were considered to be at the proestrus stage. They were paired with sexually experienced vasectomized or intact males overnight (from 1400–1700 h). Females

that had a vaginal plug on the following day were used for embryo transfer as recipients.

Additional endocrinological treatments for females

After injections of P4 on Days 1 and 2, some females were treated with an intraperitoneal injection of hCG (5 IU) or gonadotropin-releasing hormone (GnRH) agonist (0.6 mg/0.1 ml/female) (buserelin acetate; Wako Pure Chemical Industries, Osaka, Japan) to induce their exogenous or endogenous luteinizing hormone (LH) surges, respectively, on Day 6 (1800–2000 h). They were immediately paired with sexually experienced vasectomized males. In another group of females, eCG (5 IU) (Peamex; Nippon Zenyaku Kogyo, Fukushima, Japan) or AIS (0.1 ml per female) [5, 6] were intraperitoneally injected on Day 4 with and without injections of P4 to induce follicular development. These females were paired with sexually experienced vasectomized males on the evening of Day 6.

Discontinuous and continuous pairing with and without P4 treatment

To understand the timing of mating with males, females with or without injections of P4 were paired with males for 4 nights, from Day 1 to 5 or Day 4 to 8, respectively, until a vaginal plug was found. They were paired with sexually experienced vasectomized males only during the night (the discontinuous pairing group paired at 1700–1900 h to 0900–1000 h) or during whole days (the continuous pairing group). Some females with and without P4 treatment were continuously paired with sexually experienced intact males for 4 nights in the same way as above, in order to observe the timing of fertile mating that led to pregnancy.

Statistical analyses

We performed statistical analyses to compare the standard method with the other procedures. The mating, pregnancy, implantation, and birth rates were analyzed using Fisher's exact test. Offspring body weights were analyzed using the Mann-Whitney U test. A probability of $P < 0.01$ was considered statistically significant.

Results

Synchronization of the estrous cycle

First, we investigated the estrous cycle stages by the cytology of vaginal smears using 27 ICR females 4 days before the first P4 injection (until Day 0). Of the 108 observations (27 animals \times 4 days), only 14% (15/108) of the mice were cytologically classified as in the proestrus stage. The rates of other stages (estrus, metestrus, and diestrus) were 42, 24, and 20%, respectively. We then examined the distribution of estrous stages after sequential injections of P4 on Days 1 and 2. Most of the animals were at metestrus on Day 3 (85%, 23/27), at metestrus on Day 4 (74%, 20/28), and at diestrus on Day 5 (81%, 22/27) (Table 1), indicating that the estrous cycle in ICR mice could be synchronized simply by injecting P4 twice, as we have reported for B6 females [6]. However, on Day 6, the mice were divided into diestrus (52%, 14/27) and proestrus (30%, 8/27), the stage suitable for mating (Table 1). Therefore, although the rate of proestrus on Day 6 was higher than that of the untreated females described above (14%), the P4 injections alone were not suitable

Table 1. Estrous stages 4 days after the injection of progesterone (P4) on Days 1 and 2 in the ICR strain

Estrous stage	No. (%) of females			
	Day 3	Day 4	Day 5	Day 6
Proestrus	0 (0)	0 (0)	1 (4)	8 (30)
Estrus	3 (11)	0 (0)	0 (0)	3 (11)
Metestrus	23 (85)	20 (74)	4 (15)	2 (7)
Diestrus	1 (4)	7 (26)	22 (81)	14 (52)

for determining the exact timing for mating.

Efficiency of preparing pseudopregnant females and producing offspring following the conventional method

Before devising new protocols for the production of pseudopregnant females in this study, we collected standard data, i.e., the efficiency of preparing pseudopregnant females and the production of offspring by the conventional method. We determined that 2,925 of 15,132 females were in proestrus by the appearance of the vaginal opening during 16 months of routine observations. After mating with vasectomized males, vaginal plugs were found in 62.5% (1,829) of the females on the following day (Tables 2 and 3). When 79 vitrified-warmed B6 embryos were transferred into the oviducts of six pseudopregnant females (13–14 embryos per female), all of the recipient females became pregnant. Of the embryos transferred, 90% (71/79) were implanted and 61% (48/79) developed into term offspring (Tables 2 and 3).

Effects of the endocrinological treatment on the production of pseudopregnant females

We then investigated the mating rates of females after estrous synchronization by P4 injections. After pairing with vasectomized males on Day 6, 42% (10/24) of females had a vaginal plug on the next morning (Day 7). Although not statistically significant, this was lower than the successful mating rate of the standard method (63%) (Table 2). Next, we examined the effects of reagents that could regulate the endocrinological condition of females (Table 2). In the groups treated with hCG or a GnRH agonist on Day 6, which were expected to induce ovulation by an LH surge, the rates of successful mating had not improved (45% and 35%, respectively) (Table 2). However, in females treated with eCG or AIS on Day 4, which were expected to stimulate follicular development, the rates of successful mating increased to 83% (10/12) and 60% (12/20), respectively; this was higher than, or comparable to, that of the standard method (63%) (Table 2). However, when the estrous cycle was not synchronized with P4 injections the mating rates were not improved, even with eCG or AIS treatment (Table 2).

Efficiency of producing offspring after embryo transfer

We also examined birth rates by embryo transfer using recipient females prepared by different protocols. After P4 injections followed by overnight pairing on Day 6, the pregnancy rate (80%, 8/10) and the birth rate (40%, 44/110) were lower than those of the standard method (Table 2). The mating rates were not improved by treatment with hCG or the GnRH agonist. It was noted that

Table 2. Effects of endocrinological treatment on the production of pseudopregnant females and embryonic development after embryo transfer

Injection schedule (Day)						No. (%) of females		No. of embryos transferred	No. (%) of embryos		Body weight of offspring (g)	
1	2	3	4	5	6	With plug	Pregnant		Implanted	Developed to offspring		
Standard (control)						1829/2925 (63)	6/6 (100)	79	71 (90)	48 (61)	1.45 ± 0.02	
P4	P4					10/24 (42)	8/10 (80)	110	88 (80)	44 (40) *	1.42 ± 0.02	
P4	P4					hCG	5/11 (45)	2/5 (40)	28	12 (43) *	5 (18) *	0.99 ± 0.11 *
P4	P4					GnRH agonist	8/23 (35) *	7/8 (88)	92	76 (83)	52 (57)	1.31 ± 0.02 *
						eCG	7/15 (47)	5/7 (71)	70	49 (70) *	17 (24) *	1.25 ± 0.04 *
P4	P4					eCG	10/12 (83)	7/7 (100)	98	67 (68) *	35 (36) *	1.33 ± 0.03 *
						AIS	5/12 (42)	5/5 (100)	70	57 (81)	40 (57)	1.19 ± 0.02 *
P4	P4					AIS	12/20 (60)	12/12 (100)	163	101 (62) *	66 (40) *	1.28 ± 0.02 *

Data for the experimental groups, except body weight, were compared with those for the standard (control) method using Fisher's exact probability test. Body weight is presented as mean ± SEM, and was analyzed using the Mann-Whitney U test. * $P < 0.01$ vs. the corresponding value of the control. P4, progesterone; hCG, human chorionic gonadotropin; eCG, equine chorionic gonadotropin; AIS, anti-inhibin serum; GnRH, gonadotropin-releasing hormone.

hCG significantly impeded conception by significantly decreasing pregnancy, implantation, and birth rates (Table 2). It also resulted in decreased mean body weights of offspring at term (Table 2). Taken together, treatment with hCG or GnRH had no beneficial effects on embryo transfer experiments, at least in combination with estrous synchronization by P4. Treatment of synchronized females with eCG or AIS on Day 4 resulted in a 100% pregnancy rate using the standard method. However, the rates of implantation and offspring birth were significantly decreased by unknown factors (Table 2). This was also the case for nonsynchronized females treated with eCG, while those treated with AIS exhibited rates of implantation and offspring birth that were comparable with those of the standard method (Table 2).

Effects of discontinuous and continuous pairing on the efficiency of producing pseudopregnant females and birth rates after embryo transfer

To investigate the effect of the presence of males and determine the timing of mating, females with or without P4 injections were paired with vasectomized males during an overnight period (discontinuous pairing) or the whole day (continuous pairing) for up to 4 days. In the discontinuous pairing group, the frequency of mating ranged from 17 to 33% for 4 days in females without P4 injections (Fig. 1A, Table 3). With P4 injections, a higher incidence of 48% was found on Day 7 (pairing on Day 6) (Fig. 1B, Table 3), although 39% did not mate with males during the 4-day observation period (Fig. 1B). In the continuous pairing group and in the discontinuous group, there was no mating peak higher than 40% without P4 injections (Fig. 1C, Table 3). The highest mating peak (63%) among all of the experimental groups was observed on Day 7 (pairing on Day 4) in the continuous group with P4 injections (Fig. 1D, Table 3). This rate was comparable with that of the standard method (63%). Moreover, the rates of no mating decreased to 13% (Fig. 1D). In all of the groups examined, the rates of implantation and offspring birth after embryo transfer were comparable with those of the standard method (Table 3). We found that P4-treated females that mated with males on unexpected days (Day 6 or 8) restored the estrous cycle on

the 11th day after mating, indicating that they could be used again from this day on.

Production of pregnant mice using synchronized females

To assess the effectiveness of the same estrous-synchronization method for the production of pregnant females, females with or without P4 injections were continuously paired with intact males for 4 days. The rates of mating were relatively high on Days 2 (31%) and 4 (44%) in females without P4 injections (Fig. 2A). In females injected with P4, mating was confirmed only on Day 6 (19%) and Day 7 (58%) (Fig. 2B). There were no differences between these two groups in the pregnancy rate (P4 (-) 100% vs. P4 (+) 100%) or the mean litter size (P4 (-) 14 ± 1 vs. P4 (+) 15 ± 1) (Table 4). The pregnant periods were also the same (19 days), and there was no difference in the mean body weights of pups between the conventional and present methods (1.72 ± 0.02 g vs. 1.68 ± 0.03 g).

Discussion

The present study was undertaken to ascertain whether pseudo-pregnant or pregnant females could be efficiently produced by the synchronization of the estrous cycle. For this purpose, we attempted to synchronize the estrous cycle of outbred ICR females because they are most frequently used as recipients for embryo transfer and as foster mothers after Caesarean section. Before starting this study, we found that 93% of B6 females were synchronized at the metestrus stage on Day 4 after sequential P4 injections on Days 1 and 2. This treatment, in combination with AIS injections on Days 3 and 4 and an hCG injection on Day 6, resulted in highly efficient superovulation [6]. Because the main purpose of this study was the production of pseudopregnant/pregnant females, it was important to determine the timing of mating with males after estrous synchronization. Therefore, we examined the effects of endocrinological treatments and pairing (caging) with males on the timing of when the females accepted the males. Furthermore, the conventional eCG-hCG regimen for superovulation in females is known to impair subsequent embryonic development [20–22], so we also tested the

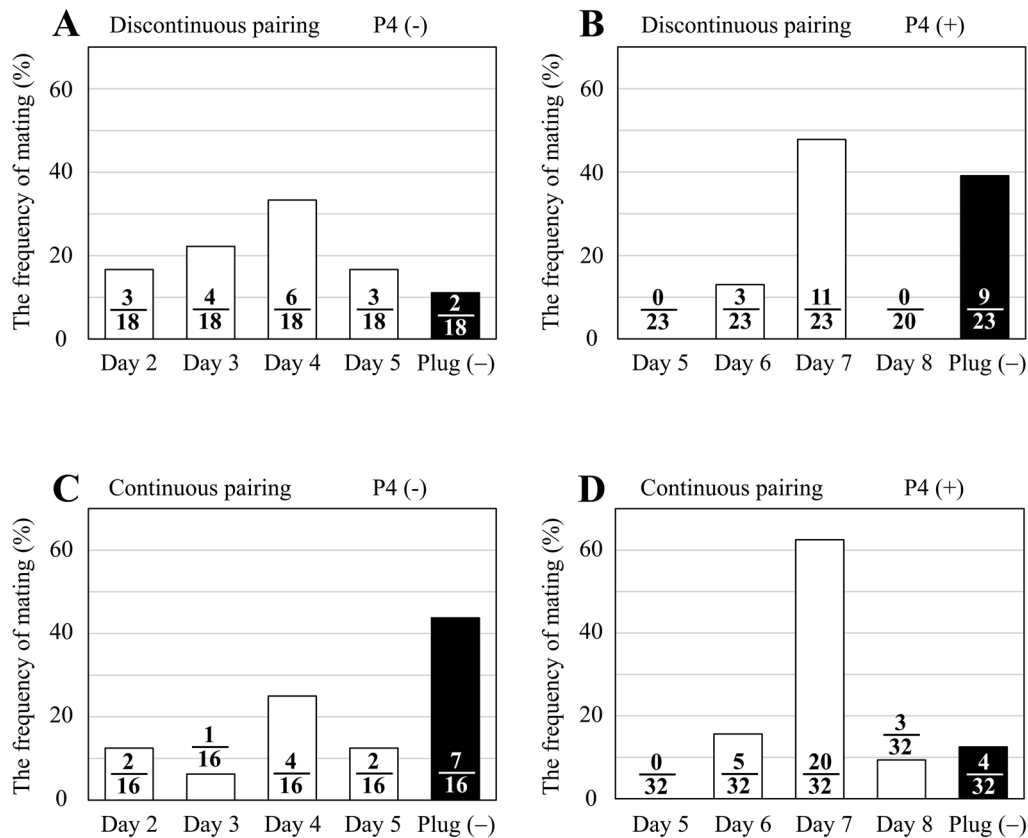


Fig. 1. Distribution of mating rates by discontinuous (A and B) and continuous (C and D) pairing with vasectomized males. Females were treated without (A and C) or with (B and D) progesterone (P4) injections. Values within the bars are the numbers of females that had a vaginal plug out of the total number of paired females.

Table 3. Effects of pairing method and progesterone (P4) on the production of pseudopregnant females and embryonic development after embryo transfer

Pairing method	P4 injections	No. (%) of females		No. of embryos transferred	No. (%) of embryos		Body weight of offspring (g)
		With plug	Pregnant		Implanted	Developed to offspring	
Standard (control)	-	1829/2925 (63)	6/6 (100)	79	71 (90)	48 (61)	1.45 ± 0.02
Discontinuous	-	6/18 (33) *	3/3 (100)	41	38 (93)	26 (63)	1.36 ± 0.03 *
	+	11/23 (48)	10/10 (100)	138	120 (87)	82 (59)	1.37 ± 0.02 *
Continuous	-	4/16 (25) *	n.t.	n.t.	n.t.	n.t.	n.t.
	+	20/32 (63)	10/11 (91)	140	112 (80)	73 (52)	1.40 ± 0.02

Data for the standard method (control) group were the same as those in Table 2. Data for the experimental groups, except body weight, were compared with those for the standard method (control) using Fisher's exact probability test. Body weight is presented as mean ± SEM, and was analyzed using the Mann-Whitney U test. * $P < 0.01$ vs. the corresponding value of the control. n.t., not tested.

efficiency of offspring production after embryo transfer or fertile mating using these estrous-synchronized females. In our assessment, none of the endocrinological treatments, including hCG, GnRH, eCG, or AIS, had any beneficial effects on the efficiency of mating or embryonic development. The hCG treatment, in particular, caused a significant decrease in implantation and birth rates after embryo transfer (Table 2). This result was consistent with those obtained by a

previous study, which reported that hCG injection alone might cause poor embryonic development and low birth weights because of an imbalanced endocrinological environment, particularly in the uterine epithelium [23]. As AIS treatment is known to enhance endogenous follicle-stimulating hormone (FSH) secretion by neutralizing inhibin activity, it induces folliculogenesis more naturally than eCG treatment. Indeed, we could obtain a larger number of high-quality oocytes

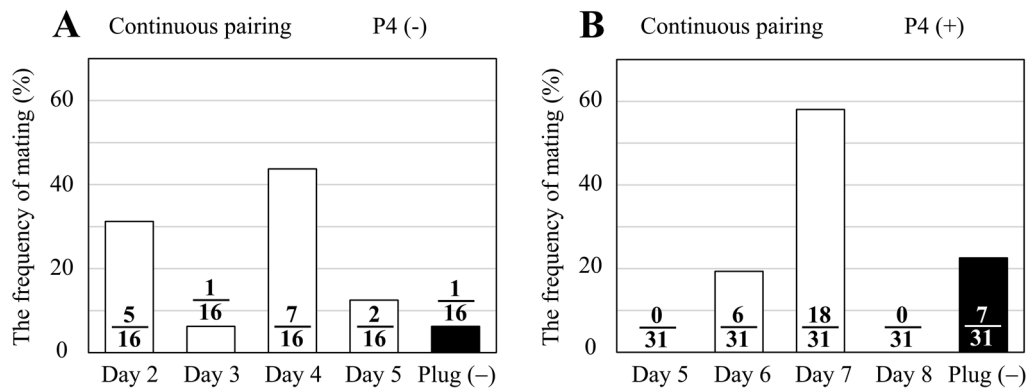


Fig. 2. Distribution of mating rates by continuous pairing with intact males. Females were treated without (A) or with (B) progesterone (P4) injections. Values within the bars are the numbers of females that had a vaginal plug out of the total number of paired females.

Table 4. Results of mating test and litter size by continuous pairing with or without progesterone (P4) injections

P4 injections	No. of females		Litter size
	Plug (%)	Pregnancy (%)	
-	7/16 (44)	7/7 (100)	14 ± 1
+	18/31 (58)	18/18 (100)	15 ± 1

Litter size is presented as mean ± SEM.

by AIS-hCG than by eCG-hCG. Therefore, we expected that AIS treatment following estrous cycle synchronization might result in an efficient production of pseudopregnant females and term offspring after embryo transfer. Unexpectedly, however, AIS treatment did not provide a good protocol in terms of the production of recipient females and offspring birth. Excessive FSH secretion might have systemically compromised the endocrinological condition of the females.

In the last series of experiments, we examined the effect of the males' presence on the receptivity of females, the so-called "Whitten effect". Whitten demonstrated that a mature male introduced into a group of females induces the appearance of a synchronous wave of estrus 3 days later [24]. This phenomenon may be explained by the effects of exposure to male urine that contains androgen-dependent pheromones [25, 26]. Consistent with this, it has been reported that the percentage of estrus on Days 3 and 4 decreases from 79% to 43% by changing from intact to castrated males [27, 28]. Under our experimental conditions, the highest mating rates (63% and 58%) were observed on the 3rd day after pairing with vasectomized and intact males, respectively, supporting the notion of the Whitten effect (Fig. 1D and 2B). Because the vasectomized males were not castrated, they might have influenced the females' estrus cycles as intact males. We found that estrous cycle synchronization by P4 injections increased the rate of females accepting males on Day 7. This was more evident with continuous pairing than discontinuous pairing (63% vs. 48%, respectively). Therefore, for the production of pseudopregnant females, the best result was obtained when P4-injected females were continuously paired with vasectomized males. In the

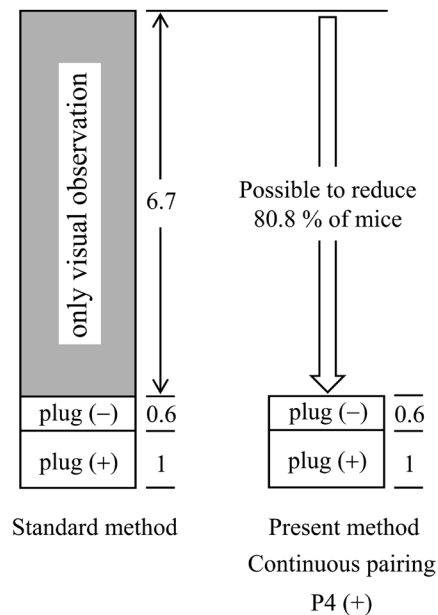


Fig. 3. Schematic representation of the size of a female colony required for the production of pseudopregnant females. Values on the right side of the columns indicate the relative number of females with a vaginal plug (= 1).

discontinuous pairing group, 39% of the females had no vaginal plug during the time of observation. This may be explained by the effect of females, not males, in the same cage, the so-called "Lee-Boot effect". This effect involves longer or more irregular estrous cycles that are caused by pheromonal signals from females in the same cage [29]. The Lee-Boot effect may have been overcome by continuous pairing with males, because the percentage of females with no vaginal plug decreased from 39% to 13%.

The preparation of pregnant females is also important for the successful nursing of newborns after Caesarean section, and is routinely conducted in most facilities that maintain mouse strains.

We found that 77% (24/31) of females successfully mated on Days 6 and 7 in the continuous pairing group after P4 injections on Days 1 and 2 (Fig. 2B). With this high efficiency, our protocol for the scheduled production of pregnant females could be practically introduced to mouse facilities. This method may also be effective for the production of a large cohort of mice of the same age, and to assist reproduction in females with a poor reproductive performance, or aged or prepubertal females. We previously found that P4 followed by AIS-hCG treatment successfully induced ovulation in a 1-year-old mouse [6]; P4 injections may also bring about regular estrous cycles in female mice of a broad range of ages.

The advantages of our scheduled production of pseudopregnant and pregnant females are as follows: firstly, experiments that involve embryo transfer can be securely planned, because the number of females that become pseudopregnant or pregnant can be estimated; secondly, the number of females maintained for embryo transfer can be reduced; and thirdly, the effort of selecting females at the proestrus stage for mating can be omitted. These advantages would collectively save space and the cost of maintaining mouse facilities. Figure 3 schematically shows the size of a female colony necessary for obtaining the required number of recipients. Our newly optimized production method could reduce the size of the female stock by 80.8%, which is in the spirit of the principle of the 3Rs- reduction, refinement, and replacement. Furthermore, researchers should have the same efficiency in preparing recipients, irrespective of their experience.

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