High-Yield Superovulation in Adult Mice by Anti-Inhibin Serum Treatment Combined with Estrous Cycle Synchronization 1

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ABSTRACT

Producing many mature oocytes is of great importance for assisted reproductive technologies. In mice, superovulation by consecutive injections of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) has been the gold standard for oocyte collection. However, the yield of mature oocytes by this regimen can fluctuate according to the stage of the estrous cycle, strain, and age. Therefore, our objective was to develop a high-yield superovulation protocol to collect higher numbers of oocytes from adult female mice of different strains and ages. First, we aimed to synchronize the estrous cycle using C57BL/6 (B6) female mice. Most (93%) were synchronized to metestrus after two daily injections of progesterone. Second, we found that with the injection of anti-inhibin serum (AIS) instead of eCG, the mean number of ovulated oocytes almost doubled (21 vs. 41 per mouse). Third, by combining estrous cycle synchronization with two AIS injections, we obtained 62 oocytes per mouse, about three times that with the eCG-hCG protocol. Importantly, this approach increased the proportion of mice that ovulated >25 oocytes from about 40% (eCG-hCG) to 90%. The same protocol was also effective in other inbred (BALB/cA), outbred (ICR), and hybrid (B6D2F1) strains. In addition, B6 female mice aged over 1 yr ovulated 1.8-fold more oocytes by this protocol. Thus, estrous cycle synchronization followed by AIS-hCG yielded a broadly applicable, highly efficient superovulation. This protocol should promote the effective use of invaluable female mouse strains and decrease the numbers of animals euthanized.

estrous cycle, inhibin, in vitro fertilization, mouse, superovulation

INTRODUCTION

Collecting sufficient oocytes from either ovaries or oviducts is a critical step for assisted reproductive technologies (ARTs), including in vitro fertilization (IVF), intracytoplasmic sperm injection, and cloning by somatic cell nuclear transfer. In most small animal species such as rodents and rabbits, mature metaphase II (MII) oocytes can be collected freshly from the oviducts following superovulation treatments. Therefore, many efforts have been made to optimize practical superovulation regimens for these species. Such protocols usually consist of two steps: hyperstimulation of follicular development and the induction of ovulation. In mice, the most prevailing superovulation regimen involves an injection of equine chorionic gonadotropin (eCG) to stimulate follicular development, followed by an injection of human chorionic gonadotropin (hCG) for inducing ovulation. However, the number of oocytes collected by this method varies with the age, the genetic background (mouse strain) [1–3], and the stage of the estrous cycle of the females.

Concerning the genetic background, it is known that the efficiency of superovulation is generally lower in inbred strains than in outbred or hybrid strains [4, 5]. It is probable that exogenous eCG collected from pregnant mares is not always effective for some strains of mice. In theory, this obstacle could be overcome by increasing the endogenous levels of follicle-stimulating hormone (FSH). This can be achieved by reducing the serum level of inhibin, which inhibits the production of FSH by the pituitary gland. Passive immunization using anti-inhibin serum (AIS) has been applied successfully to several mammalian species including golden hamsters [6], cows [7], horses [8], guinea pigs [9], mice [10], rats [11], and goats [12]. We have also reported that AIS treatment was more effective than conventional eCG treatment in 20 of 37 wild-derived mouse strains, especially those belonging to *Mus musculus molossinus*, which had been difficult to superovulate by eCG-hCG [13, 14].

The effect of the stage of the estrous cycle on superovulation efficiency has been studied extensively using rats, which show regular 4-day estrous cycles [11, 15]. In mice, it was reported that the stage of the estrous cycle affected the number and quality of oocytes in the DD and ddY strains [5, 10]. According to Shi et al. [9], guinea pigs could be induced to undergo synchronized ovulation by subcutaneous implantation of a tube filled with crystalline progesterone for 4 wk. This progesterone treatment inhibited ovulation in cycling guinea pigs, and ovulation occurred within 5–6 days after removal of

the progesterone-filled tube [16]. Because the guinea pig is an animal with complete estrous cycles with a regular luteal phase, it is uncertain whether animals with incomplete estrous cycles such as mice can also be synchronized using hormonal treatment. One study was conducted to synchronize female mice with combined administrations of progesterone and prostaglandin [17]. However, because the results were assessed by fertile mating with male mice and the births of offspring, whether the method could be used for oocyte collection following superovulation treatment remains unclear.

In the present study, we sought to develop effective protocols for superovulating adult female mice of different ages and strains. First, we established a simple method for estrous cycle synchronization and then assessed whether the female mice thus synchronized responded well to eCG-hCG or AIS-hCG treatments. Subsequently, to evaluate the functional normality of the oocyte thus prepared, we undertook IVF and embryo transfer experiments. The high-yielding superovulation protocols established in this study could increase the efficacy of reproductive engineering experiments, especially when genetically valuable or low-responder female strains are used. From an animal ethics perspective, a reduction in the numbers of female mice that needed to be euthanized per experiment would also be a benefit of our new superovulation protocol.

MATERIALS AND METHODS

Animals

Most experiments in this study were performed using young animals (10–20 wk of age) at the RIKEN BioResource Center. The mouse strains used were C57BL/6J, BALB/cA, ICR (CLEA Japan Inc., Tokyo, Japan), and B6D2F1 (Japan SLC Inc., Shizuoka, Japan). They were housed under controlled lighting conditions (daily light period, 0700–2100 h). Experiments on aged mice were performed at the Tokyo Metropolitan Institute of Gerontology. C57BL/6N female mice (Japan SLC) were maintained under a 12L:12D cycle (daily light period, 0800–2000 h) until they were used for experiments at 46 and 62–63 wk of age. Experiments were approved by the Animal Experimentation Committees of the RIKEN Tsukuba Institute and the Tokyo Metropolitan Institute of Gerontology, and were performed in accordance with the committees' guiding principles.

Superovulation

The mice were injected intraperitoneally with eCG (5 IU) (Peamex; Nippon Zenyaku Kogyo, Fukushima, Japan) or AIS (100 μl) followed by injection with hCG (5 IU) (Gonatropin; ASKA Pharmaceutical, Tokyo, Japan) 48 h later. Mature MII oocytes were collected from the oviducts 16–17 h after hCG injection. The AIS was obtained from a castrated goat immunized against [Tyr30]-inhibin α(l–30)-NH$_2$ conjugated to rabbit serum albumin as reported previously [18]. The titer of the antiserum was determined in our previous report [19]. The serum had a titer of 1:1 000 000 as defined by final dilution of the antiserum required to bind 50% of ^{125}I-labeled bovine 32-kDa inhibin. AIS is available to researchers from our laboratories upon request. The numbers of ovulated oocytes were counted with classifications based on their morphology; thus, oocytes with a fragmented or small ooplasm ($\leq$60 μm diameter) or expanded zona pellucida were classified as abnormal.

Synchronization of Estrous Cycles

We classified females into each stage of the estrous cycle based on the following criteria for the morphology and quantity of cells found in vaginal smears taken daily at 0900–1100 h [20]. The proestrus stage showed nucleated and few cornified epithelial cells, the estrus stage showed many cornified epithelial cells, the metestrus stage showed many leukocytes and few nucleated epithelial cells, and the diestrus stage showed decreased cell numbers and few leukocytes. Ten female mice at each stage were injected subcutaneously with 2 mg (0.08 ml per female) of progesterone in the evening (1800–2000 h) once a day for 2 days (designated Days 1 and 2 of these experiments; abbreviated to 2P4 treatment) and were examined sequentially for the stage of the estrous cycle on Days 2–4.

Radioimmunoassay for FSH

To measure the circulating FSH levels, C57BL/6J female mice were injected with progesterone on Days 1 and 2, followed by injection with either AIS on Day 4 (2P4-AIS), AIS on Days 3 and 4 (2P4-2AIS), or nonimmune goat serum on Day 4 (control; 2P4-NGS). Blood samples were collected under anesthesia at 1600–1700 h on Days 5 and 6. They were placed into heparinized centrifuge tubes and centrifuged at $1700 \times g$ for 30 min at 4°C. Plasma was separated and stored at −40°C until assayed for FSH. Plasma concentrations of FSH were measured using a National Institute of Diabetes and Digestive and Kidney Diseases heterologous double-antibody radioimmunoassay kit (National Institutes of Health, Bethesda, MD) for rat FSH as described previously [21]. Iodinated preparation was FSH-I-5, and the antiserum used was anti-rat FSH-S-11. Results were expressed in terms of National Institute of Diabetes and Digestive and Kidney Diseases rat FSH-RP-2.

IVF

This was performed with slight modifications using frozen-thawed epididymal spermatozoa as described previously [14, 22]. In brief, cumulus-enclosed oocytes were collected and preincubated for 1–1.5 h in 80 μl droplets of human tubal fluid medium [23] supplemented with 1.25 mM reduced glutathione [24, 25]. Each droplet contained oocytes collected from one female or from one oviduct. Sperm suspensions from the epididymal caudae of male mice (3- to 12-mo-old) were suspended in 400 μl of sperm preincubation medium (human tubal fluid containing 0.4 mM methyl-β-cyclodextrin [26, 27] 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 [28] 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 normal-appearing 2-cell embryos with a distinct polar body were considered to have been fertilized.

Assessment of Zona Pellucida Hardening

To test whether AIS induced zona pellucida hardening that might cause fertilization failure, we performed a chymotrypsin digestion test using oocytes obtained by superovulation with AIS-hCG, eCG-hCG, or hCG alone. Collected oocytes were freed from cumulus cells with PB1 solution [29] containing 0.1% hyaluronidase. Seven cumulus-free oocytes were placed in a 1 μl droplet of 1% α-chymotrypsin in PB1 at room temperature (22°C–25°C) and the time for complete digestion of the zona was recorded [30].

Embryo Transfer

The 2-cell embryos produced by IVF were transferred into the oviducts of Day 1 pseudopregnant ICR strain female mice previously mated with vasectomized male mice. In the evenings of Days 18 and 19 of pregnancy, each mouse was injected subcutaneously with 2 mg of progesterone (in 0.08 ml) to prevent spontaneous delivery. On the morning of Day 20, they were examined for the numbers of implantation sites and live offspring by Caesarian section after euthanasia.

Statistical Analysis

The total numbers of oocytes collected from each mouse, the rates of fertilization, and the zona pellucida dissolution time were evaluated statistically by one- or two-way analysis of variance (ANOVA) using IBM SPSS software (version 21; IBM Corp., Armonk, NY), as appropriate. Associations between the total numbers of oocytes per insemination droplets and fertilization rates were analyzed using the Pearson correlation coefficient. Birth rates were analyzed using chi square tests. Other statistical analyses are described as appropriate in the text or table footnotes. Those parameters calculated as percentages were subjected to arcsine transformation before performing ANOVA, and $P < 0.05$ was considered statistically significant.

TABLE 1. Results of superovulation induced by injecting eCG or anti-inhibin serum (AIS) at each estrous stage in the C57BL/6J strain.[a]

Estrous stage	Superovulation	No. of females ovulated/tested (%)	Mean ± SEM no. of oocytes per female[b]			
			Total	Normal	Abnormal	Normality (%)
Proestrus	eCG-hCG	5/7 (71)	11 ± 4	9 ± 3	2 ± 1	79 ± 7
	AIS-hCG	5/6 (83)	16 ± 4	16 ± 4	0 ± 0	100 ± 0
Estrus	eCG-hCG	5/6 (83)	30 ± 7	26 ± 6	5 ± 1	85 ± 1
	AIS-hCG	7/7 (100)	51 ± 4	50 ± 4	1 ± 0	99 ± 1
Metestrus	eCG-hCG	6/7 (86)	23 ± 5	16 ± 3	7 ± 3	75 ± 7
	AIS-hCG	6/6 (100)	59 ± 7	58 ± 7	2 ± 0	98 ± 1
Diestrus	eCG-hCG	7/7 (100)	23 ± 3	19 ± 3	4 ± 1	81 ± 4
	AIS-hCG	6/6 (100)	38 ± 10	35 ± 11	3 ± 1	81 ± 9

[a] Two-way ANOVA was performed to analyze the total numbers of oocytes and the normality rates. Results of post hoc test: total no. oocytes, eCG < AIS and proestrus < estrus, metestrus, and diestrus; normality, eCG < AIS and diestrus < proestrus and estrus (see also Supplemental Table S1).
[b] There was no significant interaction between estrous stage and superovulation regimen (see also Supplemental Table S1).

RESULTS

Effects of Estrous Cycle Stage and Superovulation Regimen on Superovulation Efficiency

We first examined the effect of the estrous cycle stage and the type of superovulation treatment on the number of ovulated oocytes in C57BL/6J female mice at 10–14 wk of age (Table 1). In all, 85% (23/27) and 96% (24/25) of them ovulated after eCG-hCG and AIS-hCG injections, respectively. When mice at defined estrous stages were injected with eCG-hCG, the total numbers of collected oocytes were 11–30 per mouse with the greatest numbers in the estrus group. By contrast, after treatment with AIS-hCG, 16–59 oocytes per mouse were obtained with the metestrus group showing the best result. We undertook two-way ANOVA with a 4×2 factorial design to evaluate the stage of the estrous cycle ($\times$ 4) and the superovulation protocol ($\times$ 2) (Supplemental Table S1; available online at www.biolreprod.org). We found that both the stage and the superovulation protocol had significant effects on the total numbers of oocytes collected. There was no significant interaction between these two factors, indicating that they affected the superovulation efficiency independently and that the AIS-hCG treatment was more efficient than the eCG-hCG treatment. The proportions of oocytes with normal morphology were affected both by the estrous cycle stage and by the superovulation protocol without any interaction between these two factors. Thus, the AIS-hCG protocol resulted in higher rates of production of normal oocytes than did eCG-hCG (Table 1 and Supplemental Table S1).

Synchronization of Estrous Cycles

We then examined whether the estrous cycles of female mice at different stages could be synchronized by sequential injections of progesterone on Days 1 and 2. We found that 93% of the mice were in metestrus by Day 4 (Table 2). Two injections of progesterone were necessary for efficient synchronization because in our preliminary experiments, a single injection of progesterone on Day 1 failed to synchronize the stage of females on Days 3 or 4 (data not shown).

Results of Superovulation after Synchronization of the Estrous Cycle

For final optimization of the protocol for superovulation, we examined the combined effects of estrous cycle synchronization (with or without progesterone treatment) and the superovulation treatment (eCG-hCG or AIS-hCG) on the number of collected oocytes. The experimental design is shown in Figure 1. Among the four groups examined, the AIS-hCG protocol following estrous cycle synchronization (2P4-AIS-hCG) gave the best results in terms of the percentage of female mice that ovulated (100%) and the numbers of collected oocytes (mean 50 oocytes per mouse; Table 3). ANOVA revealed that the superovulation treatment had a significant effect on the numbers of collected oocytes, but the synchronization treatment did not (Supplemental Table S1). There was no significant interaction between the two factors, indicating that AIS-hCG induced better superovulation than did eCG-hCG (Supplemental Table S1). We then examined whether an additional injection of AIS on Day 3 (2P4-2AIS-hCG) would further increase the numbers of collected oocytes. All the treated mice ovulated successfully, and the mean number of collected oocytes reached 62 per animal (Table 3). This number was significantly higher than following the AIS-hCG protocol (Student t-test, $P = 0.004$). To determine how these new superovulation protocols would affect the numbers of oocytes collected, we observed the proportions of female mice divided

TABLE 2. Estrous stage 4 days after injection of progesterone on Days 1 and 2 in the C57BL/6J strain.

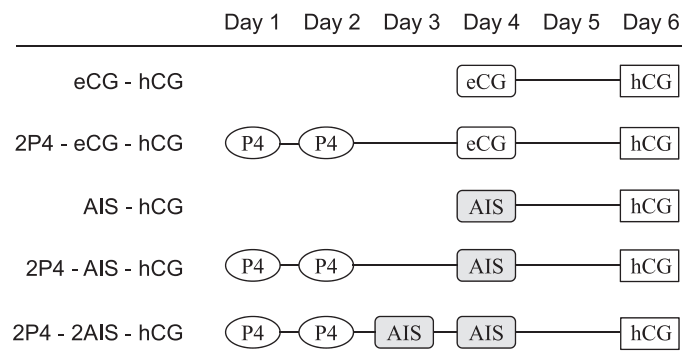| Estrous stage	No. of females (%)			
	Day 1	Day 2	Day 3	Day 4
Proestrus	10 (25)	3 (8)	0 (0)	2 (5)
Estrus	10 (25)	23 (58)	21 (53)	0 (0)
Metestrus	10 (25)	12 (30)	18 (45)	37 (93)
Diestrus	10 (25)	2 (5)	1 (3)	1 (3)

FIG. 1. Schematic representation of treatments for synchronization of the estrous cycle and the induction of superovulation in each experimental group. Progesterone (P4) was injected subcutaneously, and the other reagents were injected intraperitoneally in the evening (1800–2000 h) on each day.

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TABLE 3. Results of superovulation and in vitro fertilization rates by each treatment in the C57BL/6J strain.

Experimental group[a]	No. of females ovulated/tested (%)	Mean ± SEM no. of oocytes				In vitro fertilization	
		Total	Normal	Abnormal	Normality (%)	No. of replicates	Fertilization rate (%)
eCG-hCG[b]	25/29 (86.2)	21 ± 2	17 ± 2	5 ± 1	80 ± 3	5	95.3 ± 1.1
2P4-eCG-hCG[b]	14/15 (93.3)	23 ± 3	18 ± 2	5 ± 1	82 ± 4	5	97.0 ± 2.1
AIS-hCG[b]	24/25 (96.0)	41 ± 5[c]	40 ± 5	1 ± 0	94 ± 3	3	87.3 ± 5.8
2P4-AIS-hCG[b]	21/21 (100)	50 ± 5[d]	49 ± 5	1 ± 0	97 ± 1	6	84.2 ± 4.2
2P4-2AIS-hCG	23/23 (100)	62 ± 5[e]	59 ± 5	3 ± 1	96 ± 1	7	85.2 ± 4.0
AIS-eCG-hCG[f]	6/6 (100)	43 ± 6	37 ± 6	6 ± 1	85 ± 6	2	83.9

[a] For the details of experimental groups, see Figure 1.
[b] Two-way ANOVA was performed to analyze the total numbers of oocytes and the fertilization rates within the top four rows. Results of post hoc tests: total no. oocytes, eCG < AIS; fertilization rate, AIS < eCG (see also Supplemental Table S1).
[c–e] Student t-test was performed to analyze the mean numbers of oocytes among three AIS groups ([c,e]$P < 0.05$, [c,d;d,e]$P > 0.05$: not significant).
[f] Coinjection of AIS and eCG [31].

into five groups according to the numbers of oocytes collected (Fig. 2). While mice with >75 oocytes were not observed in the two eCG injection groups, they accounted for 39% in the 2P4-2AIS-hCG group. Conversely, the proportion of females with ≤25 oocytes decreased from 53%–59% in the eCG groups to ≤10% in the 2P4-AIS-hCG and 2P4-2AIS-hCG groups. In an additional experiment, we coinjected AIS and eCG before hCG injection according to a previous study using immature female mice (4 wk of age) [31]. The mean number of collected oocytes per mouse was very similar to that of the AIS-hCG group (43 vs. 41), indicating that the protocol devised for immature female mice is not always applicable to the adult female mice used in this study (Table 3).

Plasma FSH Levels

To gain insight into the mechanism of these high superovulation yields, we measured the circulating FSH levels in female mice treated to induce superovulation. The plasma FSH levels on Days 5 and 6 in mice of the 2P4-2AIS and 2P4-AIS groups were significantly higher than those in 2P4-NGS (control) animals (Table 4). This indicated that high FSH levels were maintained for at least 2 days after AIS injection(s) following estrous synchronization.

IVF Using Superovulated Oocytes

We then performed IVF to examine whether oocytes obtained by the AIS treatment had normal ability for fertilization in vitro using freshly prepared epididymal spermatozoa. Although the fertilization rates were high at 84.2%–97.0% in all the experimental groups (Table 3), they were higher with eCG-hCG than with AIS-hCG according to ANOVA (Supplemental Table S1). To determine how lower fertilization rates might be associated with AIS-hCG treatment, we first performed a chymotrypsin digestion test to assess any hardening of the zona pellucida. There was no difference in the digestion time between the AIS-hCG, eCG-hCG, and control (hCG alone) groups, indicating that zona pellucida hardening was not involved in the reduced fertilization rate in the AIS-hCG group (Fig. 3A and Supplemental Table S1). We then performed IVF experiments after adjusting the numbers of oocytes in the insemination droplets (13–22 per droplet). There were no differences in the fertilization rates between the AIS-hCG, eCG-hCG, and control (hCG alone) groups, indicating that the lower fertilization rates after AIS treatment were most likely attributable to the increased numbers of oocytes per drop (max. 98 oocytes) (Fig. 3B and Supplemental Table S1). Indeed, when the fertilization rates and the numbers of oocytes per insemination droplet were plotted against each other, there was an inverse correlation between the fertilization rate and the number of oocytes per droplet, with the lower fertilization rates after AIS stimulation being associated with an increased number of oocytes per droplet (Fig. 4).

Development of Embryos

To assess the developmental ability of 2-cell embryos generated by IVF following the 2P4-2AIS-hCG treatment, we transferred a total of 45 embryos into the oviducts of three pseudopregnant recipients. A high percentage (84%, 38/45) of transferred embryos implanted in the uteri successfully and 60% (27/45) developed into normal-looking offspring. These
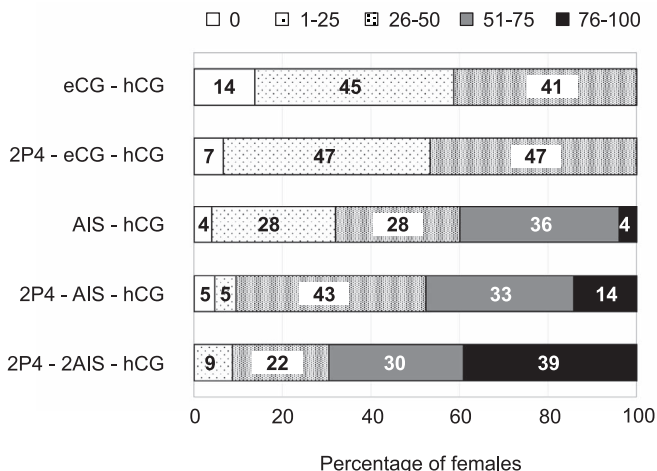

FIG. 2. Distribution of the numbers of ovulated oocytes by each treatment in the C57BL/6J strain. The oocyte numbers per mouse were divided into five ranges (0, 1–25, 26–50, 51–75, and 76–100).

TABLE 4. Plasma FSH levels in female mice treated with anti-inhibin serum (AIS) or control serum.

Treatment[a]	FSH (pg/ml)[b]	
	Day 5 (n)	Day 6 (n)
2P4-AIS	467 ± 28 (5)*	610 ± 48 (5)*
2P4-2AIS	465 ± 62 (5)*	560 ± 50 (5)*
2P4-NGS	34 ± 13 (4)	Not tested

[a] For the details of treatments, see Figure 1. NGS, nonimmune goat serum.
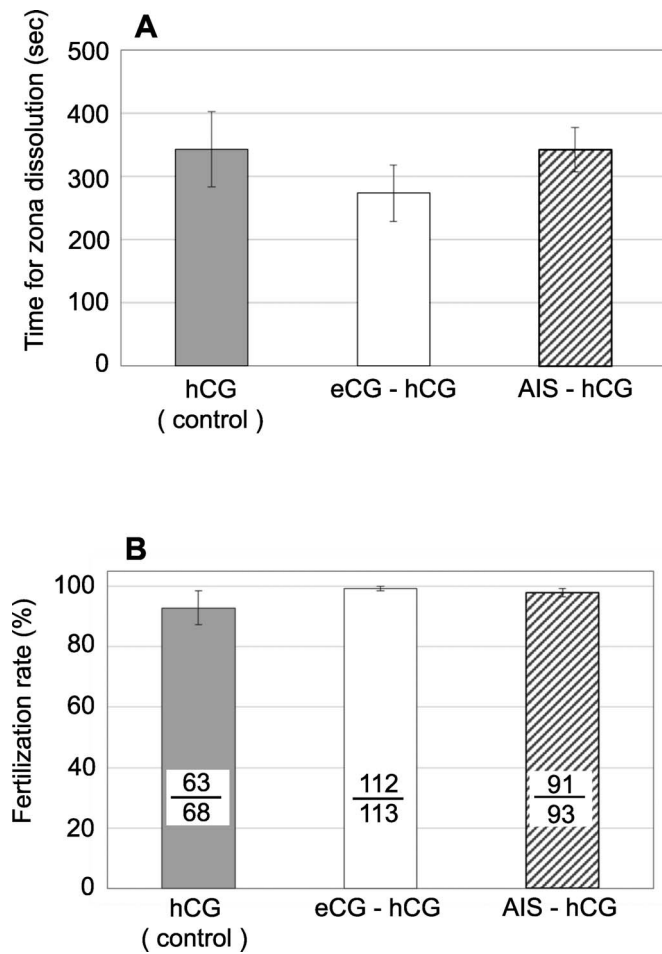[b] Analyzed by Student t-test; *$P < 0.01$ compared with 2P4-NGS.

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FIG. 3. **A**) Time taken for dissolution of the zona pellucida of MII oocytes collected after each treatment. Groups of seven oocytes were placed in a 1 μl droplet of 1% α-chymotrypsin in PB1 medium at room temperature to assess the level of zona pellucida hardening. Six replicates were done for each. **B**) Fertilization in vitro with controlled number of oocytes obtained by each treatment (means ± SEM). IVF was performed in droplets of insemination medium containing similar numbers of oocytes (13–22 per droplet). The values within the bars show the numbers of 2-cell embryos based on the numbers of normal oocytes inseminated.
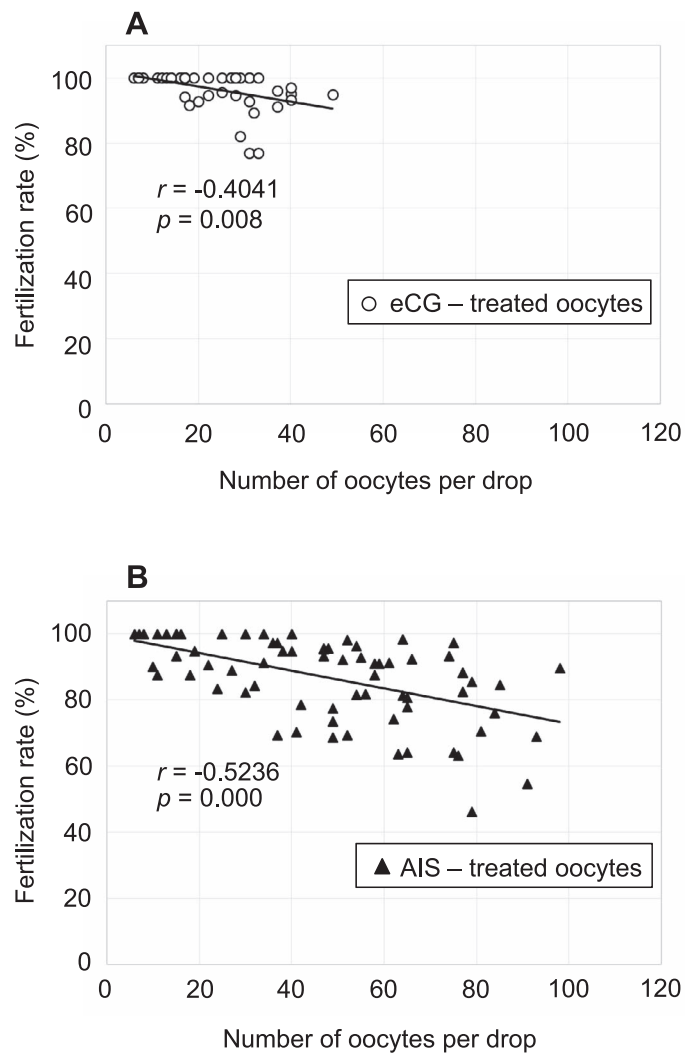

FIG. 4. **A**, **B**) IVF rates plotted according to the number of oocytes per insemination droplet. There was an inverse correlation between the fertilization rate and the number of oocytes per droplet.

results were not significantly different from those of our conventional IVF experiments following eCG-hCG injection with 80% (32/40) implantation and 58% (23/40) birth rates. The overall reproductive efficiency per female was calculated as the number of normal oocytes $\times$ fertilization rate (2-cell embryos/normal oocytes) $\times$ birth rate (offspring/transferred embryos). The resulting efficiency estimates were 9 ($17 \times 95\% \times 58\%$) and 30 ($59 \times 85\% \times 60\%$) pups per female in the eCG-hCG and 2P4-2AIS-hCG groups, respectively. Thus, a 3.3-fold higher efficiency was achieved by the combined synchronization of the estrous cycle and 2AIS-hCG treatment compared with the conventional eCG-hCG protocol in the C57BL/6J strain.

Application of the New Superovulation Protocol to Other Strains of Mice

We examined how the best protocol (2P4-2AIS-hCG) might improve the superovulation efficiencies in BALB/c, ICR, and B6D2F1 strains of female mice. BALB/c is one of the major inbred strains known to be a poor responder to eCG-hCG stimulation; ICR is a major outbred strain; and B6D2F1 is an

F1 hybrid strain often used for oocyte collection. Mice from these three strains responded better to 2P4-2AIS-hCG than to eCG-hCG, reaching 3.0, 2.2, and 2.8 times higher numbers of oocytes, respectively (Table 5). Thus, the effect of our new superovulation protocol was highly consistent among different strains of mice.

Superovulation and Production of Offspring from Aged Mice

It is known that the reproductive performance of female mammals decreases with age [32, 33]. Therefore, we examined whether more oocytes could be recovered after applying our new superovulation protocol to aged mice. At 46 wk of age, means of 5 ± 2 and 13 ± 2 normal-looking oocytes per female were obtained after treatment with eCG-hCG and 2P4-2AIS-hCG, respectively (Table 6). At 62–63 wk of age, the mean numbers were 5 and 10 normal-looking oocytes per mouse, respectively (Table 6). The total numbers of oocytes were 39 and 82, respectively, from eight aged mice. Finally, 7 and 21 offspring, respectively, were born after IVF using these oocytes followed by embryo transfer. Thus, the final yields of offspring

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TABLE 5. Results of superovulation and in vitro fertilization rates in other inbred, outbred, and hybrid strains.

Strain	Experimental group[a]	No. of females ovulated/tested (%)	Mean ± SEM no. of oocytes				In vitro fertilization	
			Total	Normal	Abnormal	Normality (%)	No. of replicates	Fertilization rate (%)
BALB/cA	eCG-hCG	21/23 (91)	18 ± 2	13 ± 1	6 ± 1	77 ± 4	7	88.4 ± 4.5
	2P4-2AIS-hCG	9/10 (90)	55 ± 10	55 ± 9	0 ± 0	99 ± 0	4	91.3 ± 2.8
ICR	eCG-hCG	16/18 (89)	34 ± 5	27 ± 4	7 ± 2	81 ± 4	3	90.1 ± 4.1
	2P4-2AIS-hCG	6/6 (100)	74 ± 15	70 ± 13	4 ± 2	96 ± 2	2	93.3
B6D2F1	eCG-hCG	5/5 (100)	28 ± 10	23 ± 7	5 ± 2	88 ± 6	1	99.1
	2P4-2AIS-hCG	5/5 (100)	79 ± 8	78 ± 8	1 ± 1	99 ± 1	1	95.1

[a] For the details of experimental groups, see Figure 1.

were three times higher in aged female mice using these new superovulation protocols.

DISCUSSION

This study was undertaken to establish high-yield superovulation protocols that could be applied to female mice of different strains and ages. We confirmed that the protocols we have developed significantly increased the numbers of oocytes collected from C57BL/6, BALB/cA, ICR, and B6D2F1 strains of mice aged 10–20 wk and from C57BL/6 mice at around 1 yr of age or older. Because it is desirable to collect as many oocytes as possible from one animal, many efforts have been made to improve superovulation regimens in mice. Consequently, it is generally accepted that animals before puberty (at 3–5 wk of age) tend to ovulate a larger number of oocytes than do adults following conventional eCG-hCG treatment [10, 33, 34]. Takeo and Nakagata [31] have extended this protocol by cotreatment with AIS and eCG followed by hCG, and finally obtained as many as 100 oocytes per animal using prepubertal C57BL/6 female mice at 4 wk of age. However, this extraordinary superovulation efficiency was specific for females at this immature age (T. Takeo, personal communication). Indeed, as far as we could ascertain, this protocol was not superior to the AIS-hCG treatment for adult C57BL/6 mice (15 wk of age; Table 3). This cotreatment protocol might be best suited for female mice before the onset of their estrous cycles. Because prepubertal females are not always used in mouse ART experiments, devising a robust superovulation protocol using adult females at different ages should have practical significance. We also showed that our protocol had broad applicability among different strains of mice. These advantages are important not only for biomedical research but also for the operation of mouse repository centers, including ours [35–37]. In these centers, ART plays a central role in the collection, storage, and distribution of mouse strains; therefore, improving the efficiency of superovulation should reduce the total costs, effort, and rearing spaces.

Using AIS instead of eCG was one of the key points for improving the superovulation regimens used in this study. We have reported that some wild-derived strains of mice,

especially the subspecies *M. musculus molossinus*, are poor responders to eCG and ovulated a mean of only <10 oocytes per animal [13, 14]. This has long made them ART resistant [38]; however, we found that such wild-derived strains could be induced to superovulate successfully by AIS-hCG treatment. In this study, the BALB/cA strain ovulated the smallest mean number of oocytes (18 ± 2) among the strains tested using the eCG-hCG protocol. We confirmed that this number could be increased to 44 ± 5 by AIS-hCG stimulation (data not shown) and further increased to 55 ± 10 with 2P4-2AIS-hCG (Table 5). Because laboratory mice in general carry large genomic regions from *M. musculus molossinus*, it is probable that there might be poor responder strains other than BALB/cA that could be superovulated successfully by AIS-hCG or 2P4-2AIS-hCG.

The AIS treatment also enhanced the eCG-hCG protocol in terms of the rates of normal oocytes per those ovulated with or without estrous synchronization (Tables 1, 3, 5, and 6, and Supplemental Table S1). We confirmed that these oocytes derived from AIS-hCG treatment had the same quality as those from eCG-hCG, as assessed by the chymotrypsin digestion test and by IVF under defined conditions of oocyte density in the insemination droplets. After embryo transfer, the resultant 2-cell embryos developed into offspring successfully at a rate of 60% (27/45), which was not significantly different from that following eCG-hCG superovulation (58%, 23/40). Therefore, the improvement of the overall efficiency of producing offspring by AIS treatment was attributable to the increased numbers of normal oocytes ovulated as well as to the consistency of their developmental potential.

Another technical improvement in this study was estrous cycle synchronization by progesterone treatment. In animals classified as having a complete estrous cycle, such as goats, horses, cows, and guinea pigs, silicone tubes containing crystallized progesterone are embedded subcutaneously for a few weeks to synchronize the estrous cycle [16, 39]. These animals are maintained at the luteal phase during exposure to progesterone. By contrast, there has been no reliable method for synchronization of the estrous cycle in animals having a short, nonfunctional luteal phase (incomplete estrous cycle) such as mice and rats, except for the method of caging with

TABLE 6. Results of superovulation protocol and in vitro fertilization rates in aged female mice of the C57BL/6N strain.

Age (wk)	Experimental group[a]	No. of females ovulated/tested (%)	Mean ± SEM no. of oocytes				In vitro fertilization rate (%)
			Total	Normal	Abnormal	Normality (%)	
46	eCG-hCG	5/5 (100)	10 ± 3	5 ± 2	5 ± 1	46 ± 10	18/24 (75)
	2P4-2AIS-hCG	4/4 (100)	13 ± 2	13 ± 2	1 ± 1	95 ± 5	37/50 (74)
62–63	eCG-hCG	7/8 (88)	7 ± 2	5 ± 2	2 ± 1	71 ± 8	18/39 (46)
	2P4-2AIS-hCG	8/8 (100)	12 ± 3	10 ± 3	2 ± 1	89 ± 5	50/82 (61)

[a] For the details of experimental groups, see Figure 1.

male mice, that is, exposure to their urinary pheromones (the so-called Whitten effect [40]). In this study, we first demonstrated that the estrous cycle in mice could be synchronized simply by injecting progesterone twice. Most (93%) C57BL/6 females were at the metestrus stage on Day 4 after injections of progesterone on Days 1 and 2, and the mean number of collected oocytes increased from 41 (AIS-hCG) to 50 (2P4-AIS-hCG). This increase seems to be a minor improvement compared with the doubling of mean oocyte numbers by the use of AIS instead of eCG (21 vs. 41; Table 3). However, it should be noted that the advantage of estrous cycle synchronization is to minimize the number of nonresponsive mice that ovulate small numbers of oocytes. By the administration of progesterone, most (>90%) of the mice ovulated more than 25 oocytes (Fig. 2). This could be important when only limited numbers of females are available for experiments.

Finally, we measured the plasma FSH levels to gain insight into the mechanisms of our superovulation regimens. Female mice from both the 2P4-AIS-hCG and 2P4-2AIS-hCG groups showed significantly higher FSH levels than did control mice administered with nonimmune goat serum. Although there was no significant difference between the FSH levels of the 2P4-AIS-hCG (single AIS on Day 4) and 2P4-2AIS-hCG regimens (sequential AIS on Days 3 and 4), it is very likely that the latter treatment resulted in a 1-day longer exposure (Days 4–6) of follicles to high concentrations of FSH than the former treatment (Days 5 and 6). This might explain the higher yields of ovulated oocytes in the latter than in the former groups. Interestingly, it was reported that continuously high levels of FSH could rescue follicles that would otherwise undergo spontaneous apoptotic degeneration [41]. Therefore, although still speculative, it is possible that the estrous synchronization brought a consistent number of follicles that were responsive to FSH (pre- and early antral follicles [42]), and then AIS increased the number of follicles reaching the full antral stage by inhibiting follicular degeneration.

In conclusion, we have successfully devised a high-yield superovulation protocol by combining estrous cycle synchronization and AIS treatment. This protocol might be applicable to a broad range of mouse strains of different ages. It would promote the effective use of genetically invaluable female mice and reduce the need to euthanize animals in ART.

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