—Review—

Recent technical breakthroughs for ARTs in mice

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Abstract: Assisted reproductive technologies (ARTs) in mice were recently advanced when two long-existing technical barriers were overcome. The first barrier was the limited number of mature oocytes after conventional superovulation, especially in inbred strains of mice. A combination of estrous cycle synchronization and anti-inhibin serum treatments increased the number of collected oocytes from female mice by approximately 3–4 times in many strains. The second barrier was the low fertilization rate after *in vitro* fertilization (IVF) using frozen-thawed spermatozoa. The addition of reduced glutathione in the fertilization medium dramatically increased the IVF yields, even in cryopreserved/warmed spermatozoa from the C57BL/6J strain, which is one of the strains most sensitive to cryoinjury. This result encouraged the use of cryopreserved spermatozoa in mouse strains worldwide for the preservation and transportation of their genetic characteristics. The final yield to produce offspring from one female was increased from 9 to 30. In IVF with cryopreserved spermatozoa from the C57BL/6J strain, the final yield using these technological innovations was estimated to be ninefold higher than previously. Following this improvement, the efficiency of ARTs in mice was increased dramatically and the decrease in the number of euthanized animals contributes to animal welfare and reduces labor and expense.

Key words: ART, Mouse, Superovulation, IVF, Frozen sperm

Introduction

The laboratory mouse is one of the most important species for biomedical research. Because of their defined genetic background and relatively easier genetic modifications, including transgenesis, gene targeting, and gene editing, many kinds of mouse strain have been developed as models for human diseases and functional analysis of mouse genes. These mouse strains have been collected and preserved at repository facilities worldwide and are distributed to researchers upon request [1–6]. All of these processes and assisted reproductive technologies (ARTs) are used in popular background strains of mice, especially the C57BL/6 strain.

ARTs using mice are thought to be well developed, but there are still several problems to resolve. On the one hand, the results of ARTs in mice have shown that the efficiency of ARTs highly depends on the mouse's genetic background in the number of ovulated oocytes [7–10], fertility rates [7, 9, 11, 12], survivability of frozen/recovered embryos, and developmental rates to term after embryo transfer [9, 13–16]. On the other hand, these various data show the sensitivities of each strain to various agents and conditions, such as exogenous gonadotropin, *in vitro* environmental agents, and the handling of oocytes and embryos outside the incubator. By optimizing each technology, the differences in results between mouse strains will be reduced. In particular, wild-derived mouse strains were thought to be ARTs-resistant for a long time [17] until the technologies were improved by Hasegawa *et al.* [18] and subsequent research [16]. Two kinds of innovative technologies were used in these studies: the first was the improvement of the superovulation method, by increasing the secretion of endogenous follicle-stimulating hormone (FSH) by anti-inhibin serum (AIS) injection; and the second was to use two media for IVF, which include the reduced glutathione (GSH) and methyl-*β*-cyclodextrin (MBCD), which are known to be effective for the preincubation of oocytes and spermatozoa, respectively. These innovations widely expanded the range of efficient strains of mice and promoted the use of cryopreserved spermatozoa. Thus, the effective technologies for most strains are innovatively progressive with less strain specificity. This review introduces the ARTs developed in recent years that are effective across strain differences.

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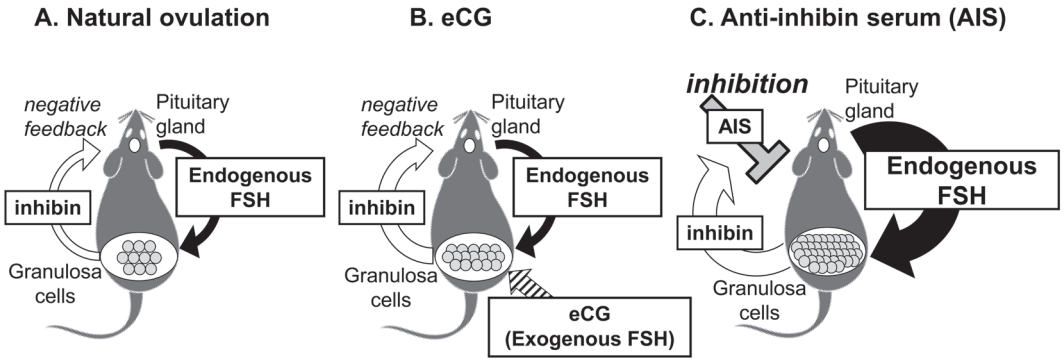

Fig. 1. Schema of mechanisms for developing follicles using endogenous or exogenous follicle-stimulating hormone (FSH). The number of oocytes in natural ovulation is controlled by the negative feedback action of inhibin secretion from granulosa cells (A). In a popular superovulation treatment, the number of oocytes increased by exogenous injection of eCG (B). By injection of anti-inhibin serum (AIS), endogenous inhibin secretion is neutralized and endogenous FSH secretion is maintained at high levels from the pituitary gland, inducing much larger number of developing follicles (C).

Superovulation

Superovulation with anti-inhibin serum

The number of collected oocytes from females is an important factor to determine the final yield of the reproductive experiment. For example, if 40 oocytes per female are obtained, the total yield increases twofold compared to 20 oocytes. In the standard superovulation method, the injection of equine chorionic gonadotropin (eCG) is used to stimulate follicular development in most rodents, not only in mice [19–21]. About 48 h later, human chorionic gonadotropin (hCG) is injected to stimulate an exogenous luteinizing hormone surge to induce ovulation. Matured MII oocytes are then ovulated around 12–14 h later. Many facilities have reported the number of ovulated oocytes as a value of strain specificity [7–10]. In addition, the number of oocytes is also affected by the age [10, 19, 22–25] and estrous stages of treated females [26–30]. It is probable that exogenous eCG prepared from pregnant mares is not always effective at obtaining the maximum number of oocytes in some strains and cases of mice.

In 1997, a group led by Taya and Watanabe succeeded in inducing superovulation using another strategy: the injection of AIS in various species of laboratory and domestic animals, such as golden hamsters [31], cows [32], horses [33], guinea pigs [34], mice [27], rats [29], and goats [35]. They produced an inhibin α-subunit antiserum from castrated goats immunized against [Tyr30]-inhibin α (1–30) conjugated to rabbit serum albumin. Inhibin secreted from granulosa cells in developing follicles induces a negative feedback action that acts on the anterior pituitary gland and limits the secretion of FSH (Fig. 1). This mechanism regulates the number of ovulated oocytes characteristic to each species and strain/breed of animals. By injection of AIS, the secretion of endogenous FSH continues over their usual limit and much larger numbers of ovulated oocytes can be expected without exogenous eCG injections. Wang *et al.* collected 73.0 ovulated oocytes from one adult (3-month-old) female mouse of ddY strain after treatment by AIS injections on day 1 of diestrus, followed by an injection of hCG 48 h later, and mating with a fertile male, compared to 41.6 oocytes collected after eCG injections instead of AIS [27]. Furthermore, the highest number of oocytes that they collected was 102.8 by AIS–hCG injections and mating at 26 days old.

Application to wild-derived mouse strains

Wild-captured (derived) mouse strains were newly introduced as a genetic resource with the expectation that they would demonstrate some disease-resistant or disease-prone characteristics in their unlimited polymorphisms as opposed to the limited genetic diversity of classical laboratory mice, which were derived from a relatively small pool of mice [36–39]. However, these wild-derived mice were thought to be ARTs-resistant for a long time [17]. In 2012, we succeeded in collecting about 25 oocytes from both the MSM and JF1 strains belonging to *Mus musculus molossinus* using AIS–hCG injections, a fivefold increase over the standard superovulation method of eCG-hCG injections [18]. We also succeeded in the cryopreservation of embryos of 37 strains, including the MSM and JF1 strains that comprise five subspecies of

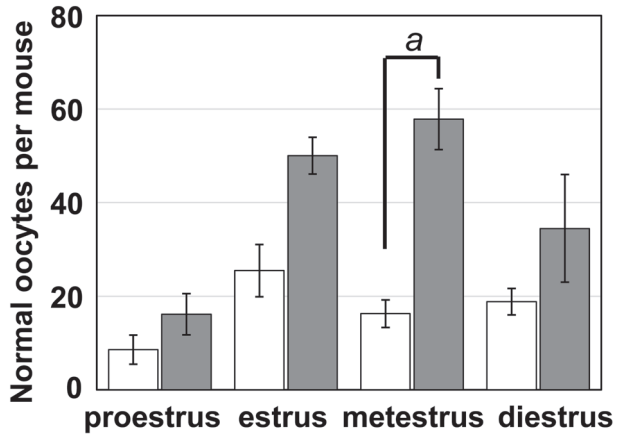

Fig. 2. Results of superovulation induced by eCG or AIS injection at each estrous stage [40]. The number of normal oocytes per female are expressed as the mean ±SEM (n=6–7). There was a significant difference between eCG (white bar) and AIS (gray bar) injections at the metestrus stage according to *post hoc* multiple comparisons using the Tukey-Kramer procedure (a, $p<0.01$).

Mus musculus, which were obtained with optimized superovulation and *in vitro* fertilization (IVF) methods. The viability of the embryos was confirmed by their development to offspring using a combination of improved and standard embryo-transfer methods [16]. Following these results, successful superovulation led to the successful preservation of rare wild-derived mouse strains.

Influence of estrous cycle and synchronization

The results of superovulation in mouse and rat may be affected by their strain [7–10, 14], age [10, 19, 22–25], and estrous stages [26–30]. Therefore, we investigated the relationships between the estrous cycle and the results of superovulation induced by eCG–hCG or AIS–hCG injections (Fig. 2) [40]. Significant differences were not found in the eCG–hCG injection group (11–30 oocytes at each stage), but the highest number of oocytes was obtained at the metestrus stage in the AIS–hCG injection group (16–59 oocytes). Therefore, we considered controlling the estrous cycle by using progesterone (P4) injections, as in guinea pigs and domestic animals [41–43]. In these animals with a complete estrous cycle, a silicon tube filled with P4 was embedded subcutaneously or into the vagina to maintain the luteal phase. After removing this tube, superovulation was induced by several simultaneous hormone treatments. The mouse is a species with an incomplete estrous cycle; therefore, different methods for estrous synchronization might be needed. However, sequential P4 injections in the evenings on days 1 and 2 were found to result in the synchronization of most of the mice (93%) at the metestrus stage [40] as determined by their vaginal cytology [44].

Novel superovulation protocol

We succeeded in collecting 59 morphologically normal oocytes (approximately 3.5-fold) from one inbred female of C57BL/6J strain using a novel superovulation protocol combining estrous cycle synchronization and two AIS injections (2P4–2AIS–hCG). This was approximately 3.5 times the number of 17 normal oocytes obtained by the standard eCG–hCG injection method (Table 1) [40]. We obtained more than 75 normal oocytes from 43% of the treated females. We considered that the reason for this high production of oocytes by two daily AIS injections may be the continuously high level of FSH for 3 days, which might rescue follicles that would otherwise undergo spontaneous apoptotic degeneration [45]. We confirmed that this protocol was effective for several other mouse strains at 10–20 weeks of age using BALB/cA (13 and 55 oocytes by the standard and novel methods, respectively) as the inbred strain, ICR (27 and 70 oocytes) as the outbred strain, and B6D2F1 (23 and 78 oocytes) as

Table 1. Superovulation and IVF rates induced by various treatments in the C57BL/6J strain [40]

Experimental group / Day of injection						No. of females	Mean number of oocytes (±SE)			Fertilization rate (%)
1	2	3	4	5	6		Total	Normal	Abnormal	
			eCG		hCG	29	21 ± 2	17 ± 2	5 ± 1	95.3 ± 1.1
P4	P4		eCG		hCG	15	23 ± 3	18 ± 2	5 ± 1	97.0 ± 2.1
			AIS		hCG	25	41 ± 5[a]	40 ± 5	1 ± 0	87.3 ± 5.8
P4	P4		AIS		hCG	21	50 ± 5	49 ± 5	1 ± 0	84.2 ± 4.2
P4	P4	AIS	AIS		hCG	23	62 ± 5[b]	59 ± 5	3 ± 1	85.2 ± 4.0

*: Two-way ANOVA was performed to analyze the mean numbers of oocytes within the top four rows: total number of oocytes, eCG <AIS. **: *Post hoc* multiple comparisons using the Turkey-Kramer procedure was performed to analyze the mean numbers of oocytes for three AIS groups (a-b: $p<0.05$).

the hybrid strain [40]. Even for aged C57BL/6N females, about twice as many normal oocytes were collected by AIS treatment at both 46 weeks (5 and 13 oocytes) and 62–63 weeks (5 and 10 oocytes) of age.

Ultrasuperovulation method in immature mice

In 1961, Zarrow and Wilson reported the results of superovulation induced by eCG in immature female mice [19]. The highest number (approximately 60) of ovulated oocytes was more clearly shown in 24- and 26-day-old Swiss mice than at other ages. The timing of the peak oocyte number depends on the puberty of each strain, which is peculiar to each strain and is based on their body weight. As noted above, Wang *et al.* collected 102.8 oocytes from one female using by AIS–hCG injections and mating at 26 days old in ddY mice maintained in a closed colony [27]. Recently, Takeo and Nakagata succeeded in obtaining 107.2 ovulated oocytes (approximately four-times more than by eCG–hCG treatments) from 4-week-old inbred C57BL/6 mice using simultaneous injections of both AIS (indicated as IAS in their paper) and eCG followed by hCG 48 h later [46]. They showed that oocytes collected from various mouse strains were capable of fertilization *in vitro* and development to term [47]. Several reports have mentioned the low developmental ability to term of oocytes collected from immature females at around 3 weeks old due to cytoplasmic and nucleic abnormalities [48–50]. It may be necessary to confirm the normal development to offspring when oocytes from females younger than 4 weeks old are used.

In Vitro fertilization

Preincubation medium for oocytes before insemination

In 2010, Bath reported excellent results, achieving *in vitro* fertilization of 89% of oocytes with cryopreserved C57BL/6J sperm in a fertilization medium supplemented by GSH [51]. Bath improved the fertilization rates with cryopreserved sperm in four popular strains: from 7% to 89% in C57BL/6J, from 14% to 69% in 129S1, from 50% to 86% in FVB/NJ, and from 73% to 98% in C3H/HeJ. This was an important result, because the C57BL/6J stain is very often used to produce gene-modified strains which are known to have low fertilization rates with cryopreserved sperm due to cell damage during cooling [52]. GSH is reported to protect cells [53] and the motility and DNA integrity of human sperm [54] against the toxic effects of oxidative damage. GSH is especially thought to promote the reduction of the disulfide bonds in the structure of zona pellucida. Shortly after Bath's breakthrough, improved IVF protocols using cryopreserved sperm were reported by some facilities. We also developed a new, simpler IVF protocol with GSH and increased the fertilization rates from 34.0% to 75.0% using cryopreserved C57BL/6J sperm [55]. IVF protocols using cryopreserved sperm have also been established which involve supplementation of GSH to the fertilization medium and MBCD to the sperm preincubation medium [16, 56–59].

Preincubation medium for sperm

Over the years, researchers have considered using reagents to promote the capacitation of mouse sperm before fertilization. In particular, the loss of cholesterol from the surface membrane of sperm is an important process in establishing the *in vitro* conditions for capacitation [60]. The efflux of cholesterol from cultured cells was reported to be stimulated by the addition of cyclodextrins to the medium [61], and in 1998, Choi and Toyoda showed that MBCD enhanced the capacitation of mouse sperm by stimulating cholesterol efflux from plasma membrane. These treated sperm increased the fertility rate after co-culture with cumulus-free mouse oocytes [62]. Several months later, Visconti *et al.* demonstrated that the capacitation of mouse and bovine sperm was associated with an increase in protein tyrosine phosphorylation, which was induced by the addition of *β*-cyclodextrin instead of bovine serum albumin (BSA) to the medium, and this resulted in the induction of the acrosome reaction and successful fertilization [63]. In 2008, Takeo *et al.* found that MBCD supplementation of the sperm preincubation medium was effective at capacitating cryopreserved C57BL/6J sperm [64], as the fertilization rate increased to 67% compared to 19% in a control group supplemented with BSA.

Increased transportation of cryopreserved sperm

The discovery of efficiencies gained by the addition of the reagents, GSH and MBCD, to the preincubation media has dramatically changed the results of IVF using cryopreserved sperm in the mouse (Fig. 3). We have also reported that the optimization of IVF conditions, including using a microdroplet system and preincubation supplemented with GSH and MBCD in media, which resulted in oocytes being fertilized by only five cryopreserved sperm [65]. This study showed that the optimization of the relationships between sperm motility, volume of fertilization medium, and the number of oocytes in a drop is important for the production of fertilized oocytes. The IVF protocols using cryopreserved mouse sperm have spread widely and they have become popular techniques in workshops at each repository facility, e.g. Kumamoto University, Jackson Laboratory, and RIKEN BRC. Cryo-

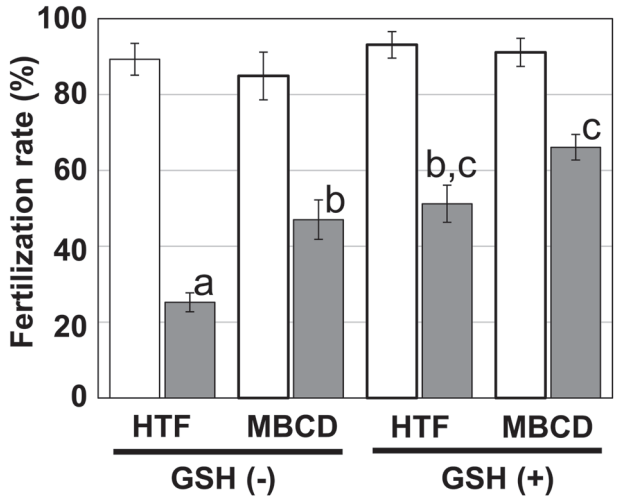

Fig. 3. Effects of preincubation media on the rates of fertilization *in vitro* using fresh and frozen C57BL/6J sperm. The fertilization rates are expressed as the mean ±SEM (n=5) for each group of fresh (white bar) and frozen (gray bar) sperm. The results were improved by the addition of GSH to HTF as a fertilization medium, and by the addition of MBCD instead of BSA as a sperm preincubation medium in the frozen sperm group. There were significant differences in each group according to the Tukey-Kramer test (a-b, a-c, b-c, $p<0.05$).

preserved sperm is now being transported between facilities more often than before, via mouse repository centers such as our center. Our center has cryopreserved sperm from more than 3,700 strains, and in 2015, about 200 strains were transported to researchers as sperm or pups recovered from these sperm. These innovations in ARTs have increased the number of occasions when frozen sperm has been used for preservation and transportation over recent years.

Final Yield of pups Using Innovative ARTs

Effective production of pups

It is important to calculate the number of pups from one female as the total yield by ART. In the literature, the total yield of each strain has been expressed using multiple factors, including the number of normal oocytes collected from one female after superovulation treatment, fertilization rate *in vitro* with fresh sperm, survival rate of embryos after cryopreservation, and developmental rate to term after embryo transfer into oviducts of pseudopregnant females [16]. For example, 9–12 pups were produced by one female in the popular C57BL/6J strain (Table 2) [9, 16, 46, 66, 67]. After using AIS as a superovulation reagent, the final yield numbers have dramatically changed to 30 pups at BRC and to 42 pups by estimation at CARD. If the ARTs efficiencies were increased by 3–4-fold, each facility would be able to reduce the number of animals euthanized by one third or one fourth.

In addition, when the final yield is estimated using the fertilization rate *in vitro* using cryopreserved sperm from the C57BL/6J strain, the efficiency has increased ninefold compared to the numbers obtained without these technological innovations (17 × 30% × 57.5% = 3 vs. 59 × 75% × 60% = 27).

Future issues regarding reproductive technologies

Following these technical developments, the rates of fertilization *in vitro*, survival after embryo cryopreservation, and development into blastocysts in culture have been refined by up to 80–90%. However, the developmental rates into offspring after embryo transfer are still 40–70% of the number of transferred embryos. In other words, 30–60% of the produced embryos do not survive and fail to develop to term in the oviducts and

Table 2. Total yields of offspring produced by one female of the C57BL/6J strain via ARTs at various repository facilities

Facility abbreviation* [Reference no.]	Age of females (weeks)	Superovulation method	Nomal oocytes per female	IVF (%)	ET (%)	Total yield per female
NIRS [66]	8–12	eCG	18.8	91.1	52.0	8.9
JAX [9]	3–4	eCG	25.0	66.3	53.1	8.8
CIE [67]	8–16	eCG	20.0	83.2	60.0	10.0
CARD [46]	4	eCG	27.7	96.4	43.6	11.6
	4	IASe**	107.2	89.8	NT	42.0***
RBRC [16, 40]	10–20	eCG	17.0	95.3	57.5	9.3
	10–20	AIS****	59.0	85.2	60	30.2

* NIRS: National Institute of Radiological Sciences; JAX: The Jackson Laboratory; CIEA: Central Institute for Experimental Animals; CARD: Center for Animal Resources and Development; RBRC: RIKEN BioResouce Center. ** Mixture of inhibin anti-serum and eCG. *** Total yield was provisionally calculated using the rates of embryo transfer after eCG treatment. **** 2P4 (estrous cycle synchronization) and two shots of anti-inhibin serum. NT: not tested.

uteri of pseudopregnant females. This problem seems to be common in other species, including humans and domestic animals. Embryo qualities have been analyzed for, chromosome abnormalities [25, 68–70], epigenetic modification [48, 71–73], and gene expression [74, 75]. In addition, the establishment of criteria for the assessment and quality of the management system [76] for each species will be important in the near future.

Conclusion

In recent years, innovations in ARTs using mice have progressively increased the productive yield of embryos from one female, such as from 9 to 30 (more than threefold) in the C57BL/6J mouse strain by using AIS in the superovulation method. In addition, the protocols for IVF with cryopreserved sperm have been refined at each facility and the use of cryopreserved sperm for the transportation of mouse strains has increased in recent years. The final yield for producing offspring with cryopreserved sperm has increased from 3 to 27 per female, a ninefold increase over that of before. Thus, the developments of ARTs has made it possible to decrease the number of animals used in life science studies.

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