Comparison of Intrabursal Transfer of Spermatozoa, A New Method for Artificial Insemination in Mice, with Intraoviductal Transfer of Spermatozoa

Masahiro Sato,^{1,3} Ayako Nagashima,² Toshiteru Watanabe,¹ and Minoru Kimura²

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Purpose: The objective of this paper was to compare the *in vivo* fertilizing abilities of fresh epididymal spermatozoa with a new method of artificial insemination in mice, so-called “intrabursal transfer of spermatozoa (ITS),” which requires transfer of spermatozoa into a space near the infundibulum between the ovary and ovarian bursa of superovulated females, and the previous method, so-called “intraoviductal transfer of spermatozoa (IOTS),” especially as regards sperm number and capacitation.

Methods: Spermatozoa freshly isolated from B6C3F1 males were injected into superovulated B6C3F1 females on E 0.4 (10:00 AM) by the IOTS or ITS method. Embryos at two-cell stage were collected from the females 1 day after injection and their morphology was scored. Some females were allowed to survive at midgestational stages and inspected for development of normal fetuses.

Results: When 1 μ L of a sperm suspension containing uncapacitated 1×10^5 spermatozoa freshly isolated from B6C3F1 males was injected by the IOTS or ITS method, normal two-cell embryos were recovered from the females at rates ranging from 14 to 23% with each method. This rate was much lower than that (93% on average) for embryos obtained by natural mating. Neither injection of 1×10^3 or 1×10^4 spermatozoa nor induction of capacitation improved *in vivo* fertilization rate. In both cases, females given spermatozoa exogenously yielded midgestational fetuses (E 12.5–13.5) with average litter sizes between 2.5 and 2.8.

Conclusion: ITS was comparable to IOTS with the conditions used. These two methods will be valuable for artificial insemination in mice for propagation of offspring from particular transgenic or mutant lines that are difficult to breed, although further attempts to improve *in vivo* fertilization ability are required.

KEY WORDS: Artificial insemination; epididymis; intrabursal transfer; ovary; oviduct; spermatozoa.

INTRODUCTION

In vitro fertilization (IVF) of oocytes and artificial insemination (AI) can be used to propagate offspring from particular transgenic or mutant lines that are

difficult to breed because of occasional defect in spermatogenesis. IVF requires a large quantity of spermatozoa, a large number of oocytes, and subsequent retransfer of fertilized embryos to a pseudopregnant female mouse. On the other hand, AI of spermatozoa into the reproductive tract of pseudopregnant females containing ovulated oocytes is more convenient and simpler than IVF for obtaining living mice.

AI in mice has been practical for many years. The traditional methods used were either by insemination into the cervix via the vagina or injection of the

¹ The Institute of Medical Sciences, Tokai University, Bohseidai, Isehara, Kanagawa 259-1193, Japan.

² Department of Molecular Life Science, Tokai University, Bohseidai, Isehara, Kanagawa, Japan.

³ To whom correspondence should be addressed; e-mail: masasato@is.icc.u-tokai.ac.jp.

sperm into the uterus laparotomy (1). In this case, a large number of spermatozoa is required to obtain pups: for example, 50 μL of sperm suspension containing more than 3×10^6 spermatozoa is needed for AI of a female (2). Nakagata (3) first developed a novel method for AI in mice by injecting epididymal spermatozoa into the ampullary region of the oviduct [hereafter termed "intraoviductal transfer of spermatozoa (IOTS)"]. Soon after the technique was also described by De Repentigny and Kothary (4). In these two cases, a small number of spermatozoa (1×10^4 to 5×10^4) is sufficient to perform AI by transferring spermatozoa directly into the ampulla of a pseudopregnant female mouse. De Repentigny and Kothary (4) reported using IOTS that 68% of mice were fertilized successfully by transferring spermatozoa (2×10^4 to $5 \times 10^4/2\text{--}5 \mu\text{L}$) into the oviducts of females. The yields was 49 fetuses with an average litter size of 3.3. Nakagata (3) found that 67% of mice yielded 30 live young with an average litter size of 7.5 ± 2.69 after oviduct transfer of fresh spermatozoa (1×10^4 to $1.5 \times 10^4/3\text{--}5 \mu\text{L}$) into pseudopregnant females. We had an interest in the IOTS method, since it may be sufficient for obtaining pups from a low number of spermatozoa of genetically engineered mice such as transgenic and knock-out mice with AI through the oviductal route. In a preliminary experiment, we performed AI into the ampulla of superovulated females by injecting 2 μL of sperm suspension containing 2×10^4 spermatozoa, and obtained a low rate of in vivo fertilization (14 of 126 [11%] oocytes developed to normal two-cell embryos after recovery from oviducts) (unpublished data). This low rate of fertilization may have been due in part to alteration of the microenvironment within the ampulla, since injection of 2 μL sperm suspension caused great expansion of the ampulla itself. We therefore developed another route for AI of mice, so-called "intrabursal transfer of spermatozoa (ITS)," and found that with it spermatozoa can be introduced via the ovarian bursa into a space near the infundibulum of ovulated females (5). This procedure appears not to directly affect the microenvironment of the ampulla, and thus results in increase in the in vivo fertilization rate after AI. An additional advantage of this procedure is that a large amount of sperm solution (up to about 10 μL) can be introduced, which will be particularly beneficial for injection of frozen-thawed spermatozoa, since the population of actively motile spermatozoa is reportedly greatly reduced after freezing and thawing (6–9). However, the ITS method using fresh epididymal spermatozoa resulted in a low in vivo fertilizing ability (4–21%) with an average litter size of 3.5, which

was much lower than that of females with an average litter size of 12.5 (5).

In this study, we compared the in vivo fertilizing abilities of fresh epididymal spermatozoa with IOTS and ITS, especially as regards sperm number and capacitation, and found that the two methods yielded similar in vivo fertilization rates ranging from 14 to 23%, when sperm suspension containing 1×10^5 uncapacitated spermatozoa per μL was introduced. These rates were not significantly improved by changing the sperm number introduced or by induction of capacitation.

MATERIALS AND METHODS

Mice

Spermatozoa from male B6C3F1 mouse (12–16-weeks old; purchased from CLEA Japan, Tokyo, Japan) was used for AI. Female B6C3F1 mouse (12–20-weeks old, CLEA Japan, Tokyo, Japan) was used as recipients for AI. The B6C3F1 females, which had been induced to superovulate by eCG and hCG treatment, but not given any spermatozoa, did not yield any normal two-cell embryos or pathenotes (5). These points are also mentioned in Table I. ICR males (13–20-weeks old, CLEA Japan) were used as vasectomized mice for obtaining pseudopregnant females. These mice were kept on a 12 h light/12 h dark schedule (lights on from 0700 h to 1900 h) and allowed food and water ad libitum. Experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* of Tokai University.

Isolation of Epididymal Spermatozoa

Spermatozoa were isolated from four cauda epididymides of two male B6C3F1 mice. The organs were minced in 200 μL of TYH medium (10) under paraffin oil on a 4-well multidish (No. 17640, Nunc, Naperville, IL), and the spermatozoa were allowed to disperse at room temperature for 10 min and immediately used for AI (within 1 h after sperm isolation) after counting sperm number with a hemocytometer. These spermatozoa were designated "uncapacitated spermatozoa." In some cases, spermatozoa were incubated at 37°C for 1.5 h to induce capacitation after dispersal at room temperature for 10 min and then subjected to sperm counting and subsequent AI (within 2.5 h after sperm isolation). These spermatozoa were designated "capacitated spermatozoa." The sperm suspension was diluted with TYH medium until

Table I. In Vivo Fertilizing Abilities^a of Fresh Epididymal Spermatozoa Introduced by ITS and IOTS Methods

Experiments (no. of spermatozoa introduced)	Sperm condition ^b	No. of pregnant recipients/total no. of recipients used ^c	Observation 4 h after culture				
			No. of normal two-cell embryos (%)	No. of abnormal two-cell embryos ^d	No. of unfertilized one-cell eggs ^e	No. of degenerated embryos ^f	No. of normal blastocysts (%) ^g
ITS ^h							
1 × 10 ⁵ /μL	A	4/7	15 (23)	4	32	14	14 (93)
1 × 10 ⁴ /μL	A	4/6	20 (18)	10	40	39	17 (85)
1 × 10 ³ /μL	A	4/7	14 (30)	8	25	9	12 (86)
1 × 10 ⁵ /μL	B	4/7	7 (10)	4	62	32	6 (86)
1 × 10 ⁴ /μL	B	3/5	7 (18)	10	22	4	7 (100)
1 × 10 ³ /μL	B	4/7	16 (27)	4	18	22	14 (88)
IOTS ^h							
1 × 10 ⁵ /μL	A	3/6	7 (14)	8	34	10	7 (100)
1 × 10 ⁴ /μL	A	4/7	6 (13)	20	22	30	6 (100)
1 × 10 ³ /μL	A	4/6	7 (19)	12	17	11	5 (71)
1 × 10 ⁵ /μL	B	5/7	12 (18)	8	48	26	9 (75)
1 × 10 ⁴ /μL	B	3/5	7 (14)	4	18	20	7 (100)
1 × 10 ³ /μL	B	2/5	3 (17)	4	11	4	3 (100)
ITS with TYH medium (negative control)		0/5	0 (0)	1	30	32	0 (0)
Normal mating (positive control)		4/5	74 (93)	0	6	4	74 (100)

^a *In vivo* fertilizing abilities of spermatozoa introduced by ITS and IOTS methods were determined by inspection of recovered two-cell stage embryos. As a negative control, 1 μL of TYH medium was introduced into superovulated females by the ITS method. As a positive control, superovulated females were naturally mated with normal males and 2 days later inspection of the two-cell stage embryos was performed. Fertilizing ability was expressed as a percentage (number of normal two-cell embryos/number of normal two-cell embryos + number of abnormal two-cell embryos + number of unfertilized one-cell eggs).

^b A indicates spermatozoa that have not been capacitated; B indicates spermatozoa that have been capacitated.

^c Injection of spermatozoa into recipients was performed twice on separate days. The number of recipients used for ITS and IOTS ranged from 5 to 7 per experiment.

^d Abnormal two-cell embryos were those with a different size for each blastomere, generation of multipolar bodies between blastomeres, or very loose contact between blastomeres.

^e Unfertilized one-cell eggs were embryos exhibiting the first polar body on their surface and a clear cytoplasmic outline.

^f Degenerated embryos were embryos exhibiting irregular shape, cytoplasmic fragmentation, multiple polar bodies released onto the surface of an embryo or low-density cytoplasm.

^g *In vitro* developmental rate was expressed as a percentage (number of normal blastocysts/number of normal two-cell embryos) after *in vitro* culture of two-cell stage embryos for 4 days.

^h One microliter of sperm suspension containing 1 × 10⁵ spermatozoa was injected into superovulated females by the ITS or IOTS method.

it contained 1 × 10⁵, 1 × 10⁴, or 1 × 10³ sperms per μL. The suspension was then used for IVF and *in vivo* insemination, as described below.

ITS and IOTS

ITS was performed as previously described (5) and is schematically shown in Fig. 1. Briefly, B6C3F1 females were given 5 IU each of eCG and hCG intraperitoneally at 18.00 h 48 h apart. In the morning (10:00 AM), after injection of hCG, the female mouse was anesthetized with pentobarbital and the ovary/oviduct was exposed. Using a transfer pipette, 1 μL of the sperm suspension (containing 1 × 10⁵, 1 × 10⁴, or 1 × 10³ spermatozoa) was taken up and transferred directly into a space between the ovary and

ovarian bursa near the infundibulum of an oviduct. The same process was repeated for the opposite ovarian bursa. As a negative control, 1 μL of TYH medium (without sperm) was given intrabursally.

The procedure used for IOTS, schematically shown in Fig. 1, was essentially the same as that for ITS, except that 1 μL of the sperm suspension (containing 1 × 10⁵, 1 × 10⁴, or 1 × 10³ spermatozoa) was transferred directly into an ampulla of an oviduct.

As a positive control, superovulated B6C3F1 females were mated with normal B6C3F1 males (12–20-weeks old) at a ratio of 1:1, and the following morning these females with a vaginal plug were designated E (embryonic day) 0.4 (10:00 AM).

Injection of spermatozoa into recipients was performed twice on separate days. The number of

Fig. 1. Schematic representation of ITS and IOTS. For ITS, a micropipette containing spermatozoa was inserted into a space between ovary and ovarian bursa. For IOTS, a micropipette containing spermatozoa was inserted into the ampulla region through the oviductal wall. Arrow and arrowhead indicate ovarian bursa and ampulla, respectively.

recipients used for ITS and IOTS ranged from 5 to 7 per experiment.

IVF for Assessment of the Ability of Spermatozoa to Fertilize Oocytes In Vitro

Immature (3–4-weeks old) B6C3F1 females were induced to superovulate by intraperitoneal injection of 5 IU each of eCG and hCG given 48 h apart. IVF was performed in the morning (10:00–12:00 AM) after injection of hCG, using methods modified from that described by Tada *et al.* (6). Epididymal spermatozoa, which had been freshly collected or preincubated in TYH medium at 37°C for 1.5 h, were used for the control of ITS and IOTS. Spermatozoa were diluted directly into the fertilization drops of medium containing oocytes surrounded by cumulus cells. The final sperm concentration was 1×10^6 to 2×10^6 spermatozoa per ml in all groups. After incubation at 37°C for 6 h, only oocytes exhibiting normal morphology were picked up and washed through three changes of M2 medium (11) to remove attached spermatozoa and

cumulus cells, and incubated in M16 medium (11) on a well of a Terasaki microtest plate (No. 5260, Nunc, Naperville, IL) covered with paraffin oil for an additional 20 h. The number of oocytes that developed to the two-cell stage was recorded.

Assessment of In Vivo Fertilizing Ability of Spermatozoa Introduced by ITS or IOTS and In Vitro Developmental Rate of Fertilized Eggs

One day after ITS or IOTS, two-cell stage embryos were collected at E 1.4 from the oviducts. The embryos were scored under a dissecting microscope with an attached digital camera (FUJIX HC-300/OL, Fuji Film, Tokyo, Japan) and cultured with M16 medium using a Terasaki microtest plate under paraffin oil in an atmosphere of 5% CO₂ and 95% air at 37°C for 4 days up to the late morula/early blastocyst stage. In vivo fertilizing ability was expressed as a percentage (number of normal two-cell embryos/number of normal two-cell embryos + number of abnormal two-cell embryos + number of unfertilized one-cell eggs)

according to previously reported criteria (5). The in vitro developmental rate of normal two-cell embryos recovered was expressed as a percentage (number of normal late morulae/early blastocysts/number of normal two-cell embryos).

Assessment of In Vivo Developmental Rate of Fertilized Eggs

In some cases, females given eCG-hCG were mated with vasectomized ICR males. The following morning, females with a vaginal plug were given 1 μ L of sperm suspension containing 1×10^5 spermatozoa by IOTS or ITS. After treatment, these females were inspected on E 12.5–13.5 to determine whether ITS- or IOTS-derived fetuses had developed. In the positive control, the superovulated females were mated with normal fertile ICR males (10–20-week old, CLEA Japan). In the negative control, the superovulated females with a vaginal plug were given 1 μ L of TYH medium without spermatozoa by ITS.

Statistical Analysis

All data were analyzed by χ^2 analysis. Values of $P < 0.05$ were considered to indicate significant difference.

RESULTS

Spermatozoa Given by The ITS or IOTS Method Could Fertilize Oocytes In Vivo, Although at a Low Fertilization Rate

Different numbers of uncapacitated epididymal spermatozoa ranging from 1×10^5 to 1×10^3 spermatozoa per μ L were transferred to a space between the ovarian bursa and ovary of ovulated females or to an ampulla of an oviduct via the oviductal wall immediately after sperm isolation. One day after sperm transfer, females were sacrificed to determine whether fertilization had occurred successfully. When 1×10^5 uncapacitated spermatozoa were given on E 0.4 by the ITS method, four of the seven mice (57%) were successfully inseminated by this technique and yielded normal two-cell embryos with rates of efficiency ranging from 15 to 50% (23% on average) (Table I; Fig. 2(A)). Similarly, insemination by 1×10^4 or 1×10^3 spermatozoa resulted in production of normal 2-cell embryos with rates of efficiency ranging from 11 to 23% (18% on average) and from 14 to 60% (30% on average), respectively (Table I). The in vivo fertilizing abilities did not differ significantly among the three groups. In the positive control group,

in which females had been normally mated with fertile males, four of the five mice (80%) possessed normal two-cell embryos with rates of efficiency ranging from 75 to 100% (93% on average) (Table I; Fig. 2(A)). In the negative control group, in which solution (TYH medium) alone was given, no normal two-cell embryos were observed (Table I; Fig. 2(A)). Introduction of 1×10^5 uncapacitated spermatozoa by the IOTS yielded normal two-cell embryos with rates of efficiency ranging from 10 to 25% (14% on average) (Table I; Fig. 2(A)). Similar efficiencies were also obtained when 1×10^4 or 1×10^3 spermatozoa were inseminated [9–21% (13% on average) and 12–28% (19% on average), respectively] (Table I). There is no significant difference in the in vivo fertilizing efficiency between the ITS and IOTS methods for each sperm group. These findings suggest that the number of sperm inseminated is not related to improvement of in vivo fertilizing ability of inseminated spermatozoa.

Induction of Capacitation Did Not Improve In Vivo Fertilizing Ability of Spermatozoa Given by the ITS or IOTS Method

To test whether induction of capacitation improves the in vivo fertilizing ability of spermatozoa given by the ITS or IOTS method, freshly isolated epididymal spermatozoa were incubated at 37°C for 1.5 h and then administered by ITS or IOTS. Concomitantly, these incubated spermatozoa were subjected to IVF to confirm that incubation of sperm at 37°C for 1.5 h in fact causes capacitation. The in vivo fertilization rates ranged from 14 to 18% for IOTS and from 10 to 27% for ITS when various numbers of spermatozoa (1×10^3 to 1×10^5 spermatozoa per μ L) were used (Table I). These rates were not significantly different from those obtained with uncapacitated spermatozoa. On the other hand, the fertilization rate of oocytes fertilized by spermatozoa that had been incubated at 37°C for 1.5 h was 88–70%, while those of oocytes fertilized by freshly isolated spermatozoa was 12–25%, indicating that incubation at 37°C for 1.5 h did cause capacitation in isolated spermatozoa. These findings indicate that (i) capacitation did not result in improvement of AI in our system, and (ii) sperm number did not play a critical role in influencing in vivo fertilization rate.

In Vivo Development of Oocytes Fertilized by Spermatozoa Introduced by ITS or IOTS Method

When 1 μ L of sperm suspension containing 1×10^5 uncapacitated spermatozoa was administered to

Fig. 2. (A) Embryos cultured for 4 h and 4 days after ITS or IOTS and subsequent recovery at two-cell stage. Positive control, embryos obtained from females that had been naturally mated with normal males; negative control, embryos obtained after intrabursal transfer of TYH medium; ITS, embryos obtained after injection of 1×10^5 spermatozoa at E 0.4 by the ITS method; IOTS, embryos obtained after injection of 1×10^5 spermatozoa at E 0.4 by the IOTS method. A certain proportion of cultured embryos was found to develop blastocysts (indicated by arrows). (B) Uteri recovered from a recipient at E 12.5–13.5 after ITS or IOTS. Note that a few fetuses had developed in the group of females treated by ITS or IOTS, whereas more than 10 fetuses were found to have developed in the females naturally mated.

superovulated pseudopregnant females mated with vasectomized males by the ITS method, 8 of 10 mice (80%) yielded 22 normal midgestational fetuses (Table II; Fig. 2(B)). Similarly, injection of sperm suspension containing 1×10^5 uncapacitated spermatozoa into superovulated pseudopregnant females by the IOTS method resulted in production of 15 normal

fetuses from six of nine females (67%) (Table II; Fig. 2(B)). In the positive control, in which females had been mated normally with fertile males, all (six of six) mice yielded a total of 87 normal midgestational fetuses with an average litter size of 14.5 (Table II). In the negative control, no fetus was obtained among a total of seven recipients examined

Table II. In Vivo Developmental Abilities^a of Oocytes Fertilized by Fresh Epididymal Spermatozoa Introduced by the ITS and IOTS Methods

Experiments	No. of pregnant recipients /total no. of recipients used ^b	Observation on E 12.5–13.5	
		No. of normal fetuses	No. of fetuses exhibiting developmental retardation or shrinkage
ITS ^c	8/10	22	6
ITS ^d (negative control)	0/7	0	0
IOTS ^c	6/9	15	5
Normal mating ^e (positive control)	6/6	87	15

^a In vivo developmental abilities of oocytes fertilized by spermatozoa introduced by the ITS or IOTS method into superovulated pseudopregnant females were determined by inspection of E 12.5–13.5 embryos.

^b Injection of spermatozoa into recipients was performed twice on separate days.

^c One microliter of sperm suspension containing 1×10^5 spermatozoa was injected into superovulated pseudopregnant females by the ITS or IOTS method.

^d One microliter of TYH medium (without spermatozoa) was injected into superovulated pseudopregnant females by the ITS method.

^e Superovulated females were mated to males. In the following day, females with vaginal plugs were selected and inspected for pregnancy at E 12.5–13.5.

(Table II), indicating that anesthesia using pentobarbital does not induce pathenogenetic activation of ovulated oocytes in this system, although anesthesia-mediated pathenogenesis has been reported to occur in some strains (12). Assuming that superovulated females usually deliver 14.5 ($\pm 87/6$) pups, the average litter sizes of the females receiving ITS or IOTS were estimated to be 2.8 and 2.5, respectively.

DISCUSSION

The objectives of the work reported here is 1) to compare the efficiencies of the two routes (ITS and IOTS) of injection into the upper end of the genital tract, 2) examine the need for capacitation, and 3) examine whether the number of sperm inseminated is critical. ITS and IOTS can result in successful fertilization of oocytes, although at low fertilization efficiency. In this study, neither variation in sperm number from 1×10^3 to 1×10^5 spermatozoa/ μL nor capacitation significantly affected efficiency.

In a previous study using ITS, in which 1×10^4 or 1×10^3 spermatozoa per μL were introduced, almost

all female mice yielded two-cell embryos with rates of efficiency ranging from 4 to 40% (4–21% for injection of 1×10^4 spermatozoa per μL and 21–40% for injection of 1×10^3 spermatozoa per μL). When ITS was performed by injection of 1 μL of sperm suspension containing 1×10^4 spermatozoa, 6 of 10 mice (60%) yielded a total of 19 normal midgestational fetuses with an average litter size of 3.2. These ITS results, based on inspection at the two-cell and E 12.5 stages, approximated those reported by De Repentigny and Kothary (4).

Why was the ITS-mediated in vivo fertilization rate low? It is highly probable that insufficient spermatozoa have reached the site of fertilization, so-called “ampulla.” On Day 1 after ITS, we examined whether spermatozoa were associated with unfertilized oocytes or attached to the zona pellucida, and confirmed the presence of spermatozoa attached to the zona pellucida in some of unfertilized oocytes and those in the flushed fluid (data not shown), indicating that the exogenous spermatozoa did reach the site of fertilization and fertilize oocytes. This event was also found in the group of IOTS. Notably, sufficient number of spermatozoa might have been provided in the latter case, because the IOTS was performed by direct introduction of sperm into ampulla region. In this context, the number of spermatozoa introduced in the gonadal route appears not to be important. Certain other factors might affect in vivo fertilization rates. First, control of the timing of introduction of spermatozoa appears to be an important factor, since fertilization is thought to occur in vivo at midnight to early morning of E 0 (13). For IVF, however, oocytes isolated from females in the morning (10:00 AM) 17 h after hCG administration can be efficiently fertilized (with efficiencies ranging 80–90%) by fresh spermatozoa (6). This slight difference in timing of fertilization may suggest that fertilization and subsequent development of fertilized eggs proceeds slightly slower in vitro than in in vivo conditions. Current experiments aim to determine at what time spermatozoa should be given intrabursally to achieve maximal in vivo fertilization rate. Second, capacitation is thought to be another factor affecting the in vivo fertilization rate of spermatozoa exogenously injected, since it is reported that IVF using uncapacitated spermatozoa resulted in great reduction of fertilization rate (13). In a previous study (5) using uncapacitated spermatozoa, fertilization rates ranging from 4 to 21% were achieved when 1×10^4 spermatozoa were given by the ITS method. In the present study, we obtained similar fertilization rates (14–18%) using capacitated spermatozoa

(at 1×10^4 spermatozoa per μL) regardless of use of IOTS or ITS (see Table I). It is possible that capacitation would have occurred rapidly during the time when the introduced spermatozoa migrate into an oviduct or reach the ovulated oocytes in the ampulla. Thus, neither ITS nor IOTS requires preincubation (which normally requires at least 1.5 h at 37°C) of epididymal spermatozoa to induce capacitation.

This work would have been much more valuable if it had been done on the strains of mice where rescue techniques are likely to be required, such as C57BL/6 and 129 strains, both of which are often subfertile and frequently used for experiments using transgenic and knock-out mice. If the AI techniques described here are inefficient with B6C3F1 hybrid strains, they are very likely to be almost useless with strains of most interest. Thus, attempts to improve ITS (for example, by performing insemination earlier) are strictly required and now in progress.

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