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—Original Article—

Microdroplet *In Vitro* Fertilization Can Reduce the Number of Spermatozoa Necessary for Fertilizing Oocytes

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Abstract. Successful *in vitro* fertilization (IVF) in mice has been achieved using spermatozoa at concentrations specifically optimized for the experimental conditions, such as species and source of spermatozoa. Although IVF in mice is mostly performed using about 80–500 μl drops, it is expected that the number of spermatozoa used for insemination can be reduced by decreasing the size of the IVF drops. The present study was undertaken to examine the extent to which the number of spermatozoa used for IVF could be reduced by using small droplets (1 μl). We devised the experimental parameters using frozen–thawed spermatozoa from C57BL/6 mice in anticipation of broader applications to other mouse facilities. We found that as few as 5 spermatozoa per droplet could fertilize oocytes (1 or 3 oocytes per droplet), although the fertilization rates were low (13–15%). Practical fertilization rates (> 40%) could be achieved with frozen-thawed C57BL/6J spermatozoa, which are sensitive to cryopreservation, when 20 sperm per droplet were used to inseminate 3 oocytes. Even with spermatozoa from a very poor quality suspension (10% motility), about 25% of oocytes were fertilized. Our calculations indicate that the number of inseminated spermatozoa per oocyte can be reduced to 1/96–1/240 by this method. In two separate embryo transfer experiments, 60% and 47%, respectively, of embryos developed to term. Our microdroplet IVF method may be particularly advantageous when only a limited number of motile spermatozoa are available because of inadequate freezing-thawing or genetic reasons.

Key words: Cryopreservation, *In vitro* fertilization, Mouse, Spermatozoa

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The laboratory mouse has long been the most widely used mammalian species for studies of human diseases, mammalian genetics and biomedical research. Therefore, archiving mouse genetic resources by safe and cost-effective ways is mandatory as an alternative to maintaining live colonies. Development of techniques of cryopreserving embryos of mouse strains began as early as the 1970s, particularly for inbred strains and their hybrids [1]. Currently, as the number of genetically modified mice is increasing at an overwhelming rate, there is a large amount of work focusing on the cryopreservation of spermatozoa, because this strategy enables resurrection of a large cohort of genetically modified mice from a single male [2, 3]. However, mouse spermatozoa are inherently sensitive to cryoinjury and are easily damaged by freeze–thawing under suboptimal conditions [4, 5]. The spermatozoa of the C57BL/6J strain, the standard background for mouse genetic engineering, are known to be particularly sensitive to freeze–thawing [6, 7]. Therefore, *in vitro* fertilization (IVF) using frozen–thawed spermatozoa is technically very difficult, and this has been a considerable technical obstacle for archiving mouse genetic resources.

Recently, significant and successive improvements for IVF using frozen–thawed spermatozoa have been made. The addition of methyl-β-cyclodextrin (MBCD) to the sperm preincubation medium greatly increased the fertilizing ability of frozen-thawed spermatozoa [8], and the addition of reduced glutathione (GSH) [9] to the IVF medium significantly promoted penetration of fertilizing spermatozoa through the zona pellucida of oocytes. Later, it was found that these two techniques synergistically improved the fertilization rates of IVF using frozen-thawed spermatozoa, and therefore, this combination is now routinely used in many laboratories and mouse archive centers [10–12].

Despite these technical advancements in IVF technology in mice, there are still some circumstances under which very few fertilized oocytes are obtained *in vitro*. One of the technical drawbacks of conventional IVF is that it needs a number of motile spermatozoa to achieve a practical fertilization rate. For example, it is recommended that the sperm concentration is adjusted to about 10^2–10^3 sperm/μl in about 80–500 μl IVF medium [3, 10]. Therefore, we sometimes have to abandon IVF when the donor male produces a very small number of sperm or sperm are severely damaged by freezing or thawing procedures. Intracytoplasmic sperm injection may assist in these cases, but it requires a high level of skill and an expensive micromanipulator set [13]. It is known that within the oviducts of female animals, only a few spermatozoa reach the cumulus-oocyte

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complex after a long trip through the female genital tract [14]. Therefore, we may expect that, if we are able to mimic *in vivo* fertilization conditions, we may reduce the number of spermatozoa needed for fertilizing oocytes *in vitro*. Although studies have reported 600 [15] and 100–840 [16] to be the minimal number of spermatozoa necessary for fertilizing mouse oocytes *in vitro*, these studies achieved a fertilization rate of only 13%. In this study, we examined the extent to which we could minimize the number of spermatozoa for IVF by reducing the volume of the inseminating droplets. We adopted the optimized procedure for IVF using spermatozoa that had been inadequately frozen in cryotubes.

Materials and Methods

Animals

All mice used in this study were purchased from CLEA Japan (Tokyo, Japan). C57BL/6JJcl (B6J) and C57BL/6NJcl (B6N) were used to collect mature oocytes and epididymal spermatozoa. Combinations of oocytes and spermatozoa from the same strains of mice were used for IVF. For embryo transfer experiments, female ICR mice were used as pseudopregnant recipients after being mated with vasectomized ICR males. All mice were maintained under a specific-pathogen-free condition. They were provided with water and commercial laboratory mouse chow *ad libitum* and housed under controlled lighting conditions (light: 0700–2100 h). All 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.

Conventional IVF

Spermatozoa from the epididymal caudae of male mice (> 12 weeks old) were preincubated in 200 µl of human tubal fluid (HTF) medium [17] containing 0.11 mg/ml hypotaurine (Sigma-Aldrich, St Louis, MO, USA) covered with mineral oil at 37 C under 5% CO_2 in air for 0.5–1 h. In some experiments, HTF medium containing 1 mg/ml polyvinyl alcohol instead of bovine serum albumin, 0.11 mg/ml hypotaurine and 0.2–0.4 mM MBCD (no. C4555; Sigma-Aldrich) (MBCD-HTF) was used for sperm preincubation. Superovulation was induced in females by an injection of 7.5 IU equine chorionic gonadotropin (eCG; Peamex, Sankyo, Tokyo, Japan), followed 48–50 h later by 7.5 IU human chorionic gonadotropin (hCG; Puberogen, Sankyo). Cumulus-oocyte complexes were collected from the oviducts and placed into 80 µl drops of fertilization medium (20–25 per drop). The basic fertilization medium was HTF, which was supplemented with 1.5 mM GSH (no. G6013; Sigma-Aldrich) when frozen–thawed sperm was used for IVF. Insemination was carried out by adding preincubated spermatozoa to the fertilization medium containing oocytes. The final sperm concentration was adjusted to 2.5×10^5 and 5.0×10^5 cells/ml for fresh and frozen–thawed spermatozoa, respectively. At 4–6 h after insemination, the oocytes were removed from the fertilization medium, washed in CZB medium [18] supplemented with glucose (mCZB) and cultured overnight in 7 µl drops of fresh mCZB. Embryos that reached the 2-cell stage on the next day were counted as fertilized oocytes and used for further *in vitro* culture or embryo transfer experiments.

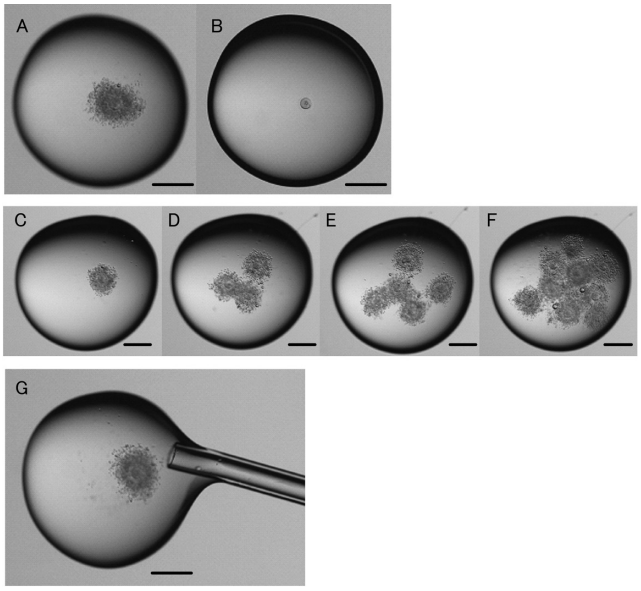

Fig. 1. Microdroplets containing MII oocytes for IVF. A and B show a single cumulus-enclosed and denuded oocyte in a droplet, respectively. One (C), 3 (D), 5 (E) and 10 (F) oocytes can be placed in a droplet without a discernible increase in droplet volume. A defined number of spermatozoa are transferred into each droplet with a glass capillary (G).

Microdroplet IVF

About half of cumulus-oocyte complexes were placed in HTF medium containing 0.1% hyaluronidase (no. 385931; Merck Millipore, Darmstadt, Germany) to remove the cumulus cells and washed three times with fresh HTF. These denuded oocytes and nontreated cumulus-enclosed oocytes were moved to HTF medium with or without 1.5 mM GSH and incubated for 0.5–1 h to allow GSH to elicit its effect, if any (Fig. 1A and B). Then, 1, 3, 5 or 10 oocytes were transferred with a glass capillary into a 1 µl droplet (about 1 mm diameter) (Fig. 1C–F). Motile, fresh or frozen–thawed sperm that had been preincubated in HTF or MBCD-HTF for 0.5 h (Experiments 3 and 4) or 1 h (Experiments 1 and 2) were collected from the periphery of a preincubation drop with a thin pipette. Five, 10, 20 or 50 sperm were aspirated using a capillary (100 µm diameter) with a small volume of medium (< 0.2 µl) and transferred to droplets containing oocytes (Fig. 1G). Each experimental group consisted of at least 2 droplets, and experiments were repeated at least four times using different males (except for the second experiment in Experiment 4). At 4–6 h after insemination, oocytes were transferred to mCZB medium for culture overnight. On the following day, the oocytes that developed into 2 cells with normal morphology and a second polar body were considered to be fertilized.

Cryopreservation of sperm

Spermatozoa were frozen according to the method developed by Nakagata and Takeshima [19], with slight modifications. The cryoprotective additives (CPA) consisted of 18% raffinose (Difco, Voigt Global Distribution LLC, Kansas City, MO, USA) and 3%

Table 1. Probabilities (P-values) of main effects on the fertilization rates and their interactions

Experiment	Effect and interaction		Factor(s)	P-value
Experiment 1 (Fig. 2)	Main effect		Oocyte (cumulus-intact or denuded)	0.003
			Medium (with or without GSH)	0.018
			Sperm (number)	0.015
	Interaction	Two factors	Oocyte × medium	0.049
			Medium × sperm	0.875
			Oocyte × sperm	0.888
		Three factors	Oocyte × medium × sperm	0.704
Experiment 2 (B6J in Table 2)	Main effect		Sperm condition (fresh or frozen)	0.001
			Oocyte (number)	0.000
			Sperm number	0.000
	Interaction	Two factors	Sperm condition × oocyte	0.501
			Oocyte × sperm no.	0.800
			Sperm cond. × sperm no.	0.489
		Three factors	Sperm cond. × oocyte × sperm no.	0.896
Experiment 2 (B6N in Table 2)	Main effect		Sperm condition (fresh or frozen)	0.000
			Oocyte (number)	0.047
			Sperm no.	0.000
	Interaction	Two factors	Sperm condition × oocyte	0.970
			Oocyte × sperm no.	0.993
			Sperm cond. × sperm no.	0.478
		Three factors	Sperm cond. × oocyte × sperm no.	0.696
Experiment 3 (Fig. 3)	Main effect		MBCD (conc.)	0.345
			Time (preincubation)	0.400
			Sperm (number)	0.006
	Interaction	Two factors	MBCD × time	0.948
			Time × sperm	0.482
			MBCD × sperm	0.797
		Three factors	MBCD × time × sperm	0.784

Results were obtained by three-way or two-way ANOVA. A probability of $P < 0.05$ was considered significant (underlined).

dehydrated skim milk (Difco). Fat and blood were removed from the cauda epididymis using filter paper. About 10 epididymal incisions were made with fine scissors under 100 µl of CPA in a 4-well plastic dish (no. 176740; Nunc, Roskilde, Denmark). The spermatozoa were dispersed by gentle shaking of the dish for 1 min at room temperature, and the sperm suspension was divided into eight aliquots (10 µl each). Each aliquot was placed inside a 0.25 ml plastic straw (Cassou straw; IMV Technologies, L'Aigle, France). The straw ends were sealed with straw powder (FHK straw powder; Fujihira Industry, Tokyo, Japan). The sealed straws were cooled in a freezing canister (50 ml plastic syringe) floating on liquid nitrogen for 10–60 min and were then immersed directly in liquid nitrogen. In Experiment 4, 50 or 100 µl of the sperm suspension with CPA was placed into a cryotube (no. 366656, Nalge Nunc International, Rochester, NY, USA), and the cryotube was directly immersed 1 cm under the surface of liquid nitrogen [14].

Thawing of cryopreserved sperm

On the day of the IVF experiments, the straws or cryotubes containing frozen spermatozoa were removed from the liquid nitrogen and immersed in a water bath at 37 C for 15 min. Five or ten microliters of frozen/thawed sperm suspension retrieved from straws or cryotubes, respectively, was transferred into 200 µl of HTF or MBCD-HTF medium and incubated under 5% CO_2 in air at 37 C for 0.5–1 h, as described above.

Embryo transfer

Embryos that had reached the 2-cell stage at 24 h in culture after insemination were transferred into the oviduct of pseudopregnant ICR females (9–17 weeks old) on day 0.5. On day 19.5, the recipient females were examined for the presence of fetuses, and live pups were nursed by lactating ICR females.

Statistical analysis

Each experiment was replicated at least four times using spermatozoa from different males, except for the second experiment of Experiment 4. The rates of fertilization *in vitro* were transformed using arcsine transformation and then analyzed by three-way analysis of variance (ANOVA) using the SPSS software (SPSS, Chicago, IL, USA). The Tukey–Kramer and Dunnett's procedures were used for multiple comparisons in Experiments 1 and 2 and Experiments 3 and 4 (except for the second experiment in Experiment 4), respectively.

For the second experiment in Experiment 4, a chi-square test was employed. A P-value < 0.05 was considered statistically significant.

Results

This study consisted of five series of experiments. The first three experiments were undertaken to examine which factors might affect the fertilization rates in microdroplet IVF. A list of factors examined in each experiment is shown in Table 1.

Effect of the presence of cumulus cells, GSH and sperm number on microdroplet IVF (Experiment 1)

To determine whether the presence of cumulus cells could affect the fertilization efficiency, one denuded oocyte or one cumulus-enclosed oocyte was placed in a 1 µl droplet of HTF medium with or without GSH (Fig. 2) and then inseminated with preincubated spermatozoa (B6J). The fertilization rates varied from 0% to 73% according to the experimental condition. As shown in Table 1, three-way ANOVA revealed that the fertilization rate was affected by all three factors (cumulus cells, GSH and sperm number). There was an interaction between the presence of cumulus cells and GSH (Table 1). This indicates that the fertilization rate was determined by the combinations of these factors, and cumulus-enclosed oocytes with GSH-HTF medium resulted in a significantly higher fertilization rate than other combinations (P < 0.05; Fig. 2). The combination of cumulus-enclosed oocytes and GSH-HTF was therefore used for the subsequent experiments. For the number of sperm inseminated, there was a significant difference between 5 and 20 sperm/droplet (Fig. 2).

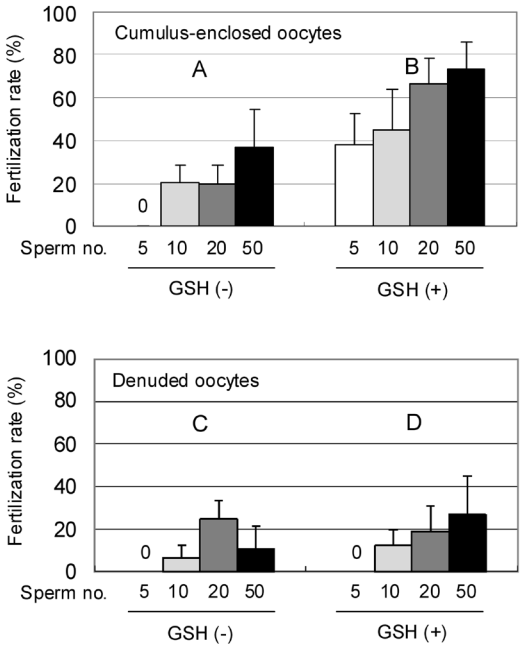

Fig. 2. Effects of the presence of cumulus cells and GSH (Experiment 1). A cumulus-enclosed or denuded oocyte in 1 µl HTF medium with or without GSH was inseminated with a defined number of fresh B6J spermatozoa (5–50). Statistical analysis revealed that there was an interaction between the presence/absence of cumulus cells and GSH and that the group of cumulus-enclosed oocytes with GSH (B) had a significantly better (P < 0.05) fertilization rate than the other groups (A, C, and D). Four or five replicates with 14 to 21 oocytes in each experimental group. Mean ± SEM.

Effect of the combinations of different numbers of oocytes and spermatozoa on microdroplet IVF (Experiment 2)

To determine whether the numbers of oocytes and spermatozoa could affect the fertilization rates in microdroplet IVF, 1, 3, 5 or 10 oocytes per droplet were inseminated with 5, 10, 20 or 50 spermatozoa. We also examined the effects of the mouse strain (B6J or B6N) and condition of spermatozoa (fresh or frozen–thawed). Therefore, four factors were involved in this experiment, as shown in Table 1. For the statistical analysis, we performed three-way ANOVA by analyzing the B6J and B6N groups separately because of the unavailability of four-way ANOVA in the software we used. In the B6 strain, all three factors (sperm freezing, number of oocytes and number of sperm) had significant effects on the fertilization rates, but there were no interactions between them (Table 1). This indicates that all these factors affected the efficiency of microdroplet IVF independently and that the sperm freezing procedure significantly decreased the fertilization rates in B6J strain. This pattern was also seen with the B6N strain, although the effect of sperm freezing on the fertilization rate was milder than for the B6J strain (Table 1, P = 0.001 *vs.* P = 0.000). The overall results are summarized in Table 2.

Effect of MBCD in the sperm preincubation medium on microdroplet IVF using cryopreserved sperm (Experiment 3)

As shown in Experiment 2, B6J spermatozoa were more sensitive to the freezing–thawing procedure than B6N spermatozoa. To further refine the conditions for microdroplet IVF using frozen–thawed B6J sperm, we examined the effects of the sperm preincubation time (0.5 and 1 h) and the presence of MBCD (0, 0.2 and 0.4 mM) on fertilization rates. In this experiment, we used droplets containing 3 oocytes throughout because those containing 5 or 10 oocytes showed relatively low fertilization rates, as shown in Experiment 2. The best result (60% fertilization rate) was obtained when oocytes were inseminated with 50 sperm after preincubation with 0.4 mM MBCD for 0.5 h (Fig. 3). This rate was comparable to that of the conventional IVF using 80 µl drops (63% and 71%; Fig. 3). Of the three factors analyzed, only the sperm number had a significant effect on the fertilization rate (20 sperm < 50 sperm; Table 1).

Microdroplet IVF using spermatozoa cryopreserved by a suboptimal condition (Experiment 4)

The aim of this experiment was to determine whether our microdroplet IVF could be applied to B6J spermatozoa that had been cryopreserved under a suboptimal condition. Recently, we used cryotubes as containers and found that the volume of the sperm suspension may critically affect the survivability of spermatozoa [11]. In this experiment, we used spermatozoa that had been frozen and stored in a 50 or 100 µl suspension, a volume larger than the optimal volume (10 µl). Using spermatozoa frozen in a 50 µl suspension, a 27% fertilization rate was achieved after conventional IVF (40,000 sperm/80 µl drop). After microdroplet IVF (50 sperm/1 µl

Table 2. Fertilization rates after microdroplet IVF using different numbers of spermatozoa and oocytes (Experiment 2)

Strain	No. of eggs/drop	No. of eggs/group	No. of fresh sperm				No. of frozen sperm			
			5	10	20	50	5	10	20	50
C57BL/6J	1	21–27	38 ± 14	45 ± 19	67 ± 12	73 ± 13	10 ± 6	19 ± 16	40 ± 11	51 ± 20
	3	23–38	21 ± 11	54 ± 19	67 ± 14	79 ± 16	13 ± 4	8 ± 5	26 ± 10	39 ± 4
	5	40–45	n.t.	30 ± 7	68 ± 8	93 ± 3	n.t.	15 ± 5	22 ± 10	29 ± 8
	10	50–60	n.t.	n.t.	36 ± 10	73 ± 9	n.t.	n.t.	8 ± 3	21 ± 8
C57BL/6N	1	16–19	47 ± 17	55 ± 6	67 ± 18	83 ± 13	37 ± 15	50 ± 17	55 ± 13	78 ± 10
	3	30–36	23 ± 9	45 ± 13	71 ± 13	93 ± 4	10 ± 4	27 ± 13	50 ± 12	53 ± 4
	5	48–54	n.t.	31 ± 10	62 ± 4	74 ± 7	n.t.	31 ± 3	36 ± 11	40 ± 8
	10	59–70	n.t.	n.t.	36 ± 8	52 ± 10	n.t.	n.t.	21 ± 6	29 ± 5

Mean ± SEM (%). n.t.: not tested. Data were analyzed by three-way ANOVA for each mouse strain (see Table 1). Data from 4 to 6 replicates.

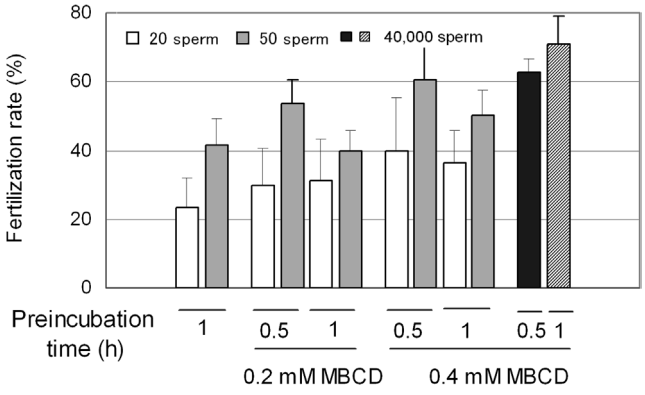

Fig. 3. Effects of the concentration of MBCD in sperm preincubation medium, preincubation time and sperm number for insemination on the fertilization rates using frozen–thawed B6J spermatozoa (Experiment 3). Droplets for microdroplet IVF and conventional IVF contains 3 and 20–25 oocytes, respectively. The best result (60% fertilization rate) was obtained when oocytes were inseminated with 50 sperm after preincubation with 0.4 mM MBCD for 0.5 h (Fig. 3). This rate was comparable to that of conventional IVF using 80 µl drops (63% and 71%, $P > 0.05$, Fig. 3). Only the sperm number had a significant effect on the fertilization rate (20 sperm < 50 sperm; see Table 1). Four or five replicates with 36 to 79 oocytes in each experimental group. Mean ± SEM.

droplet), the fertilization rate was increased to 37%, but this was not a significant difference (Fig. 4A). Using spermatozoa frozen in a 100 µl suspension (< 10% motility), the fertilization rate was 7% with conventional IVF and improved to 25% with microdroplet IVF ($P < 0.05$, chi-square test; Fig. 4B).

Development of embryos produced by microdroplet IVF

We performed embryo transfer to confirm the normality of embryos produced by our microdroplet IVF method using frozen-thawed B6J sperm. After transfer of embryos in the group of 5 oocytes inseminated with 20 spermatozoa, 78% (29/37) were implanted, and 60% (22/37) developed to term. All pups, except for one that had respiration failure, were normal in appearance and showed active movement. Furthermore, we also obtained normal offspring from the group of embryos obtained by microdroplet IVF using inadequately frozen spermatozoa (shown in Fig. 4B; Experiment 4), with the implantation and birth rates being 82% (14/17) and 47% (8/17), respectively.

Discussion

In the present study, we were able to fertilize mouse oocytes using 5 to 50 spermatozoa by microdroplet IVF successfully. Even with frozen–thawed B6J spermatozoa, which are known to be highly sensitive to cryodamage, fertilization rates of more than 40% were achieved by the combination of 3 oocytes and 20 spermatozoa in a droplet. When fresh spermatozoa were used, 10 spermatozoa were enough for a consistent IVF outcome. Such small numbers of spermatozoa used for microdroplet IVF correspond to about 1/800 to 1/2,000 of those necessary for conventional IVF in our laboratory (80 µl drops) (Table 3). The number of inseminated spermatozoa per oocyte was also reduced to about 1/120 to 1/240 and 1/96 to 1/240 in IVF using fresh and frozen-thawed sperm, respectively (Table 3). Thus, it is expected that our microdroplet IVF may increase the chance of fertilizing oocytes *in vitro* when only a small number of motile spermatozoa are collected because of inadequate sperm freezing, ageing of males, or some genetic reasons. Indeed, we were able to increase the fertilization rates from 7% to 25% by using this method when spermatozoa had very poor motility (< 10% motility rate) because of inadequate freezing in cryotubes. We have previously reported that sperm cryopreservation using cryotubes was critically affected by the volume of the sperm suspension in a cryotube [11].

In our preliminary experiments, we also tested smaller droplets (e.g., 0.5 µl) for microdroplet IVF. However, the medium composition in such small droplets may easily be changed by evaporation or by cotransfer of the sperm insemination medium (< 0.2 µl). Finally, we considered 1 µl to be the minimal volume for IVF droplets to achieve consistent results and therefore used this volume throughout this study.

It is widely accepted that the sperm concentration is the major determinant of the efficiency of fertilization *in vitro*. A large number of spermatozoa near the cumulus-oocyte complexes is thought to be necessary for loosening and dispersing the cumulus cell layer by their hyperactivated movement and the activity of acrosomal enzymes [20]. One or a few acrosome-intact spermatozoa then reach the zona pellucida and penetrate the zona while undergoing

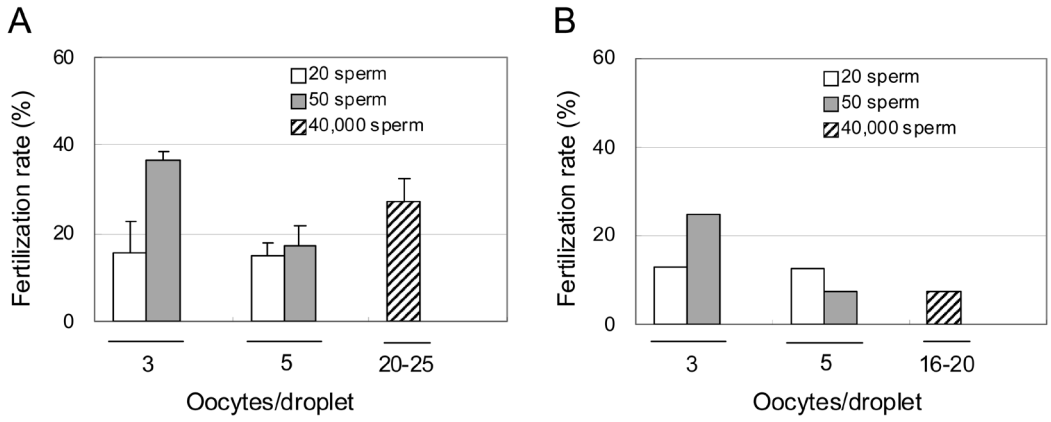

Fig. 4. Microdroplet IVF using spermatozoa with poor motility (Experiment 4). The spermatozoa had been inadequately frozen in extra large volumes of suspension (50 µl in A and 100 µl in B) in cryotubes and were poorly motile after thawing (< 10% motility in B). In A and B, the fertilization rate was increased by microdroplet IVF compared with conventional IVF (80 µl drop; $P < 0.05$ in B). Five replicates for A and a single experiment for B with 23 to 100 oocytes in each experimental group. Mean ± SEM.

Table 3. Comparison of the number of B6J spermatozoa necessary for insemination in each IVF method

Sperm	Microdroplet IVF (sperm/1 µl/droplet)		Conventional IVF (sperm/80 µl/drop)
Fresh	10–20		20,000 (250/µl)
Frozen-thawed	20–50		40,000 (500/µl)
Ratio of sperm number per drop:			
Fresh	1	*vs.*	1,000–2,000
Frozen-thawed	1	*vs.*	800–2,000
Ratio of sperm number per oocyte*:			
Fresh	1	*vs.*	120–240
Frozen-thawed	1	*vs.*	96–240

*Three oocytes are placed in an IVF drop for microdroplet IVF; 20–25 oocytes are used for conventional IVF.

the acrosome reaction. However, contrary to this prevailing idea, a recent time-lapse observation using fluorescent proteins for monitoring the acrosome status revealed that most spermatozoa began the acrosome reaction before reaching the zona and that one of these acrosome-reacted spermatozoa penetrated the zona to fuse with the oocyte [21]. The presence of cumulus cells was also found to be beneficial for the fertilizing ability of spermatozoa [21]. These findings may explain why only a few spermatozoa are present in the oviducts of mammals after mating [14, 20]. It is probable that under natural *in vivo* conditions, fertilizing spermatozoa can penetrate the vestments around the oocyte by their own physical and chemical forces and do not require the aid of other sperm. In relation to this concept, it is interesting to note that under our microdroplet IVF conditions, the sperm to egg ratio can be as low as 1.7:1 to 17:1, which is much lower than in conventional IVF (10^3–10^4:1). It may be that drops routinely used for conventional IVF are too large for the normal interactions between cumulus-oocyte complexes and spermatozoa. Larger drops may allow the factors from cumulus cells and spermatozoa to diffuse freely to the medium around them. Our microdroplet IVF might mimic the natural fertilization condition in terms of its closed microenvironment. Thus, our new IVF method may be useful not only for decreasing the number of spermatozoa required for IVF but also for studying the interactions between cumulus cells and spermatozoa during normal fertilization.

Acknowledgments

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