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Rescue *In Vitro* Fertilization Method for Legacy Stock of Frozen Mouse Sperm

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Abstract. Sperm cryopreservation has been widely adopted for maintenance of the genetically engineered mouse (GEM). The cryopreserved sperm are being exchanged among many institutes worldwide. However, the recipients are not always able to obtain high fertilization rates with the frozen sperm shipped from senders. In this study, we cryopreserved mouse sperm *via* various methods and performed *in vitro* fertilization (IVF) in which the combination of methyl-beta-cyclodextrin for sperm preincubation and reduced glutathione for insemination was used (the MBCD-GSH IVF). In addition, frozen sperm sent from the Jackson Laboratory (USA) were thawed and used for IVF in the same manner. The fertilization rates of both the sperm cryopreserved *via* the methods applied in some countries and the cryopreserved GEM sperm improved when used with the MBCD-GSH IVF method. Therefore, we strongly believe that the MBCD-GSH IVF method brings about relatively high fertilization rates with any strain of frozen mouse sperm.

Key words: C57BL/6, Cryopreserved sperm, *In vitro* fertilization, Mouse, Transport

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Over the past 20 years, a large number of mice with induced mutations have been produced worldwide [1–5]. Accordingly, many mouse repositories have been established in each country, while the exponential increase in mouse strains has necessitated the enlargement of existing mouse repositories and the establishment of new ones [6, 7]. The increased centralized repository capacity and major advancements in sperm cryopreservation have made archiving strains relatively economical and reliable. Therefore, sperm freezing has become widely adopted for the maintenance of mice with induced mutations. As a result, frozen sperm samples are now exchanged among many institutes and universities worldwide [8, 9]. However, recipients are not always able to obtain high fertilization rates with frozen mouse sperm shipped from senders. This is obviously a major problem.

Recently, we established an *in vitro* fertilization (IVF) method using preincubation medium containing methyl-beta-cyclodextrin (MBCD) [10] and fertilization medium with reduced glutathione (GSH) [11] that yields high fertilizing ability for frozen sperm (MBCD-GSH IVF method).

In this study, we froze mouse sperm *via* the methods applied to maintain genetically engineered mice (GEM) even now in some countries and then used the MBCD-GSH IVF method to perform IVF using these frozen sperm. Mouse sperm were frozen *via* two different methods: one was a method using cryotubes developed by Sztein *et al.* [12] (cryotube freezing method), and the other was a method using straws and cryoprotectant solution containing monothioglycerol (MTG) developed by Ostermeier *et al.* [13] (MTG-straw freezing method). After thawing, IVF was performed using the cryopreserved sperm. We carried out IVF using the method stipulated for each freezing method (cryotube IVF method and MTG-straw IVF method respectively), as well as using the MBCD-GSH IVF method. We then compared the fertilization rates for the cryopreserved sperm. In addition, GEM (C57BL/6 background) sperm samples frozen using the MTG-straw freezing method, which were sent to our center from the Jackson Laboratory (USA), were thawed, and the MBCD-GSH IVF method was performed using these sperm.

Table 1 and Table 2 show a comparison of fertilization rates and *in vitro* development of 2-cell embryos between the cryotube IVF method and the MBCD-GSH IVF method and between the MTG-straw IVF method and the MBCD-GSH IVF method respectively using the wild mouse strains. As shown in Tables 1 and 2, fertilization rates improved when the MBCD-GSH IVF method was used on sperm frozen *via* the cryotube freezing method and the MTG-straw freezing method (3.9% and 33.0% for the cryotube IVF and MBCD-GSH IVF methods respectively; 37.4% and 88.3% for the MTG straw IVF and MBCD-GSH IVF methods respectively). Subsequently, fertilization rates using the MBCD-GSH IVF method for frozen GEM sperm were very high (94.1% and 95.8% respectively), while half of the transferred 2-cell embryos developed into live young for each strain (Table 3).

In our previous report, we demonstrated that the combination of MBCD for cryopreserved sperm preincubation and GSH for IVF (the MBCD-GSH IVF method) using fresh and cryopreserved oocytes produced very high fertilization rates [11, 14]. In the present

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Table 1. Comparison of the rate of fertilization and *in vitro* development between the cryotube IVF method and the MBCD-GSH IVF method using sperm frozen by the cryotube freezing method

IVF method	Male No.	No. of inseminated oocytes	No. of 2-cell embryos (%)	No. of cultured 2-cell embryos	No. that developed to blastocysts (%)
Cryotube	1	39	0 (0.0)	0	0 (0.0)
	2	74	2 (2.7)	2	2 (100.0)
	3	35	2 (5.7)	2	1 (50.0)
	4	44	1 (2.3)	1	1 (100.0)
	5	91	6 (6.6)	6	4 (66.7)
	Total	283	11 (3.9)	11	8 (72.7)
MBCD-GSH	1	40	14 (35.0)	14	10 (71.4)
	2	33	7 (21.2)	7	5 (71.4)
	3	44	20 (45.5)	20	16 (80.0)
	4	50	15 (30.0)	15	11 (73.3)
	5	66	21 (31.8)	21	17 (81.0)
	Total	233	77 (33.0)*	77	59 (76.6)

Fertilization rate was calculated as the no. of 2-cell embryos divided by the no. of inseminated oocytes × 100. Two-cell embryos were cultured to the blastocyst stage *in vitro,* and developmental rate was calculated as the no. of blastocysts divided by the no. of cultured 2-cell embryos × 100. *P<0.05 compared with the cryotube IVF method.

Table 2. Comparison of the rate of fertilization and *in vitro* development between the MTG straw IVF method and the MBCD-GSH IVF method using sperm frozen by the MTG-straw freezing method

IVF method	Male No.	No. of inseminated oocytes	No. of 2-cell embryos (%)	No. of cultured 2-cell embryos	No. that developed to blastocysts (%)
MTG straw	6	27	13 (48.1)	13	13 (100.0)
	7	63	18 (28.6)	18	17 (94.4)
	8	39	22 (56.4)	22	22 (100.0)
	9	57	18 (31.6)	18	17 (94.4)
	10	36	12 (33.3)	12	10 (83.3)
	Total	222	83 (37.4)	83	79 (95.2)
MBCD-GSH	6	24	23 (95.8)	23	21 (91.3)
	7	41	30 (73.2)	30	26 (86.7)
	8	39	35 (89.7)	35	29 (82.9)
	9	29	28 (96.6)	28	23 (82.1)
	10	46	42 (91.3)	42	38 (90.5)
	Total	179	158 (88.3)*	158	137 (86.7)

Fertilization rate was calculated as the no. of 2-cell embryos divided by the no. of inseminated oocytes × 100. Two-cell embryos were cultured to the blastocyst stage *in vitro,* and developmental rate was calculated as the no. that developed to blastocysts divided by the no. of cultured 2-cell embryos × 100. *P<0.05 compared with the MTG straw IVF method.

Table 3. Production of normal young following transfer of embryos obtained by the MBCD-GSH IVF method using sperm frozen by the MTG-straw freezing method

Strain of frozen GEM sperm	No. of inseminated oocytes	No. of 2-cell embryos (%)	No. of transferrd 2-cell embryos	No. of live young (%)
1	185	174 (94.1)	60	30 (50)
2	165	158 (95.8)	60	30 (50)

Fertilization rate was calculated as the no. of 2-cell embryos divided by the no. of inseminated oocytes × 100. Two-cell embryos were transferred to recipients, and birth rate was calculated as the no. of 2-cell embryos divided by the no. of transferred 2-cell embryos × 100.

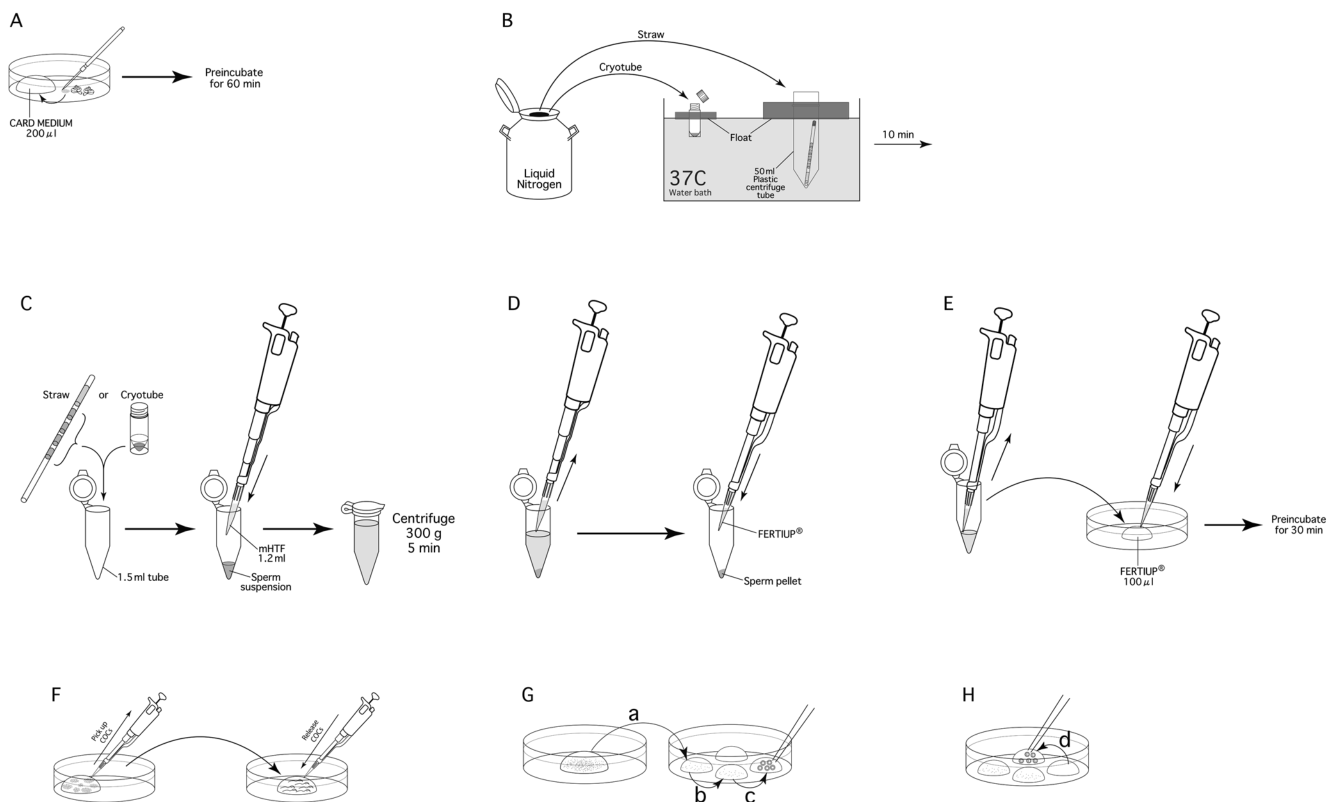

Fig. 1. Rescue IVF protocol using cryopreserved mouse sperm. A: Transfer cumulus oocytes complexes (COCs) into a drop of CARD MEDIUM. B: Remove a frozen sperm sample and immerse it in a water bath maintained at 37 C. C: Transfer the sperm suspension into a tube, and add mHTF to the tube containing the sperm suspension. Thereafter, immediately centrifuge the tube. D: Remove the supernatant and add FERTIUP® mouse sperm preincubation medium into the tube. E: After pipetting gently, transfer the entire sperm suspension into the drop of FERTIUP® preincubation medium, and incubate the sperm in a CO_2 incubator. F: Introduce the preincubated COCs into the drop of sperm suspension, and incubate them in the CO_2 incubator. G: After 3 h, wash the oocytes three times in drops of mHTF, and then culture them in the CO_2 incubator. H: Twenty-four hours after insemination, transfer just the obtained 2-cell stage embryos to a fourth drop of mHTF. (See http://www.kyudo.co.jp/Fertiup/methods.htm).

study, we also showed that fertilization rates for sperm frozen by the methods adopted even now in some countries [12, 13] can be improved using the MBCD-GSH IVF method [11]. Therefore, we believe strongly that the MBCD-GSH IVF method achieves relatively high fertilization rates with any frozen mouse sperm, and especially with legacy stocks of frozen sperm.

Methods

Animals

C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan) and Charles River Laboratories Japan (Kanagawa, Japan) for use as oocyte and sperm donors. ICR mice (CLEA Japan) were used as recipients for the transfer of 2-cell embryos. All animals were kept under a 12 h dark-light cycle (lights on: 0700–1900 h) at a constant temperature of 22 ± 1 C with free access to food and water. All animal experiments were approved by the Animal Care and Use Committee at Kumamoto University.

Sperm freezing and thawing

The sperm were collected from the vasa deferentia and caudae epididymides of three-month-old male mice and were frozen *via* the cryotube freezing method [12] and the MTG-straw freezing method [13]. Frozen samples were rapidly thawed by transferring them from liquid nitrogen into a 37 C water bath. Subsequently, the frozen-thawed sperm were used for IVF. Half of the sperm were used for either the cryotube IVF method or the MTG-straw IVF method [12, 13], and half of the remaining samples were used for the MBCD-GSH IVF method [11].

IVF

The procedures for the cryotube IVF method and the MTG-straw IVF method were as described by Sztein *et al.* [12] and by Ostermeier *et al.* [13] respectively. The MBCD-GSH IVF method is shown in Fig. 1. The oocytes used were obtained from females at 2–3 months of age. Females were induced to superovulate *via* injections of 7.5 IU PMSG (ASKA Pharmaceutical, Tokyo, Japan) and 7.5 IU human chorionic gonadotropin (ASKA Pharmaceutical) administered 48 h apart. Intact cumulus oocyte complexes (COCs) were released from the excised oviducts of superovulated females into a drop (200 μl) of CARD MEDIUM (Kyudo, Saga, Japan) covered with paraffin oil in a plastic dish (Cat.#No.#430588, Corning, Corning, NY, USA) 15–17

h after hCG and were preincubated for 60 min (Fig. 1-A). Sperm samples frozen *via* the cryotube freezing method and the MTG-straw freezing method were removed from the liquid nitrogen and immersed in a 50 ml plastic centrifuge tube (in a water bath maintained at 37 C) for 10 min (Fig. 1-B). After thawing, the sperm suspension in a cryotube or a straw was transferred into a 1.5 ml tube (Cat. No. 509-GRD, BM Equipment, Tokyo, Japan), and 1.2 ml of mHTF [15] was added slowly to the tube containing the sperm suspension. Thereafter, the tube was immediately centrifuged at 300g for 5 min (Fig. 1-C). After centrifugation, as much supernatant was removed as possible, and an appropriate amount of FERTIUP® mouse sperm preincubation medium (Kyudo) was added to the tube (the final volume was adjusted to 100 µl) (Fig. 1-D). After pipetting gently, the entire sperm suspension (100 µl) was transferred into a drop of FERTIUP® mouse sperm preincubation medium (100 µl) covered with paraffin oil in a dish. The dish was placed in an incubator (37 C, 5% CO_2 in air) for 30 min (Fig. 1-E). Then, the preincubated COCs were sucked up from the drop of CARD MEDIUM using a tip (Cat. No.#17021-1, NIPPON Genetics, Tokyo, Japan) and introduced into the drop of sperm suspension (insemination) (Fig. 1-F). The COCs were incubated in an incubator. After 3 h, the inseminated oocytes were washed three times in drops (80 µl) of mHTF covered with paraffin oil in a dish (Fig. 1-G (a–c)), and cultured at 37 C with 5% CO_2. Seven to eight hours after insemination, the formation of pronuclei was observed under a phase contrast microscopy. Twenty-four hours after insemination, just the obtained 2-cell stage embryos were transferred to a 4th drop of mHTF (Fig. 1-H (d)). Fertilization rates were calculated as the total number of 2-cell embryos divided by the total number of inseminated oocytes, multiplied by 100. All 2-cell embryos were cultured to the blastocyst stage in KSOM [16]. In addition, GEM (C57BL/6 background) sperm frozen *via* the MTG-straw freezing method, which were sent to our center from the Jackson Laboratory (USA), were thawed and used for IVF in the same manner. A portion of the 2-cell embryos were transferred into the oviducts of ICR females (10 embryos/oviduct) on the day a vaginal plug was found (day 1 of pseudopregnancy) using the method of embryo transfer through the wall of the oviduct [17]. After 19 days, the number of offspring was recorded.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 5.0 software (GraphPad, San Diego, CA, USA). Group results were compared using the Student's *t*-test after arcsine transformation of the percentage data; $P<0.05$ was considered significant.

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