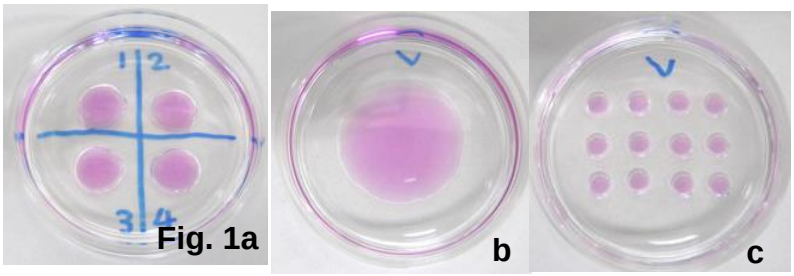
IVF with frozen sperm

1. Preparation of culture dishes

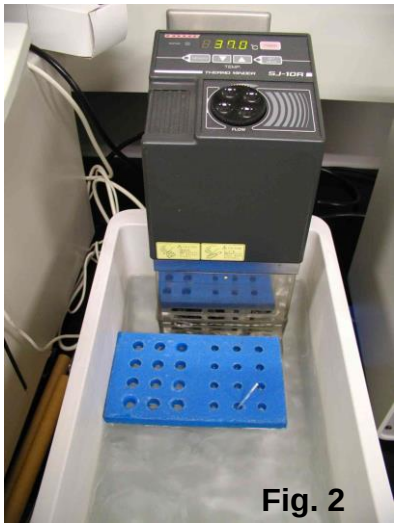
On the day of experiment, drops of medium are prepared as follows, and kept in a CO_2 incubator (37 °C, 5% CO_2 in air) to equilibrate for at least 20 min.

1) For oocyte collection and fertilization: a 35 mm plastic dish (per four females) containing four 80 μL drops of 1.25 mM reduced glutathione (GSH) -mHTF medium* overlaid with oil (Fig. 1a).
2) For sperm preincubation: a 35 mm plastic dish containing a 300 μL drop of 0.4mM MBCD -PVA-HTF medium** (about 15 mm in diameter, with the edge widened with the tip of a pipette) overlaid with mineral oil (Fig. 1b).
3) For embryo culture: a 35 mm plastic dish containing twelve 10 μL drops of CZB medium containing glucose (or KSOM a/a medium) overlaid with mineral oil (Fig. 1c).



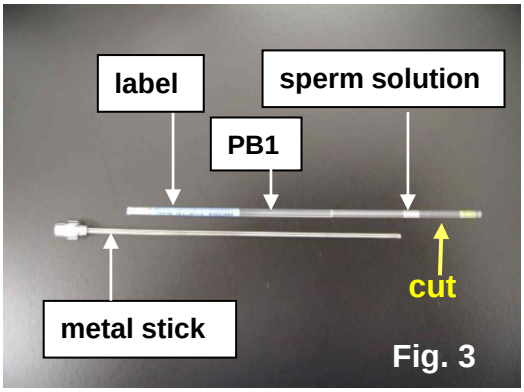
2. Collection of oocytes

1) Collect mature MII oocytes from females who have been induced to superovulate, and place them into the 80 μL drops of GSH-mHTF medium (Fig. 1a).



3. Thawing of frozen sperm

1) Retrieve a straw containing the frozen sperm from the liquid nitrogen with cryogloves and a face mask to avoid accidental injury.
2) After holding the straw in the air for 10 s, immerse it in a water bath at 37 °C for 15 min (Fig. 2).



4. Preincubation of the recovered sperm

1) Wipe the surface of the straw with a paper towel and expel the sperm solution onto the bottom of a plastic dish. The sperm solution takes up about 5 mm of the length of the straw and is enclosed in the end opposite the label. It is more opaque than the PB1 solution (Fig. 3).
2) Cut the straw with scissors between the edge and the sperm solution, and retrieve only the sperm solution by pushing it out with a metal stick (plunger) inserted from the opposite end or with a micropipette. Care must be taken not to mix the sperm solution with the PB1 solution, which is also enclosed in the straw as a weight balancer.

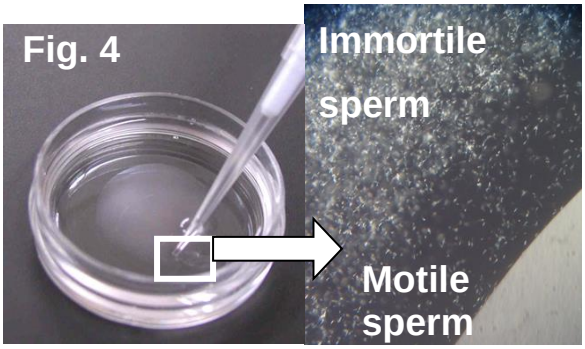
3) Transfer the entire volume (~10 μL) of sperm solution to the drop of 0.4mM MBCD-PVA-HTF medium in the culture dish (Fig. 1b).

4) Incubate it (37 °C, 5% CO_2) for 40-50 min.

4. Insemination

1) After preincubation, check the condition of sperm, for example, hyperactivated movement, concentration of sperm and percentage of motile sperm under the dissecting microscope.

2) Add about 15 μL of the thawed sperm suspension to a drop of GSH-mHTF medium containing the oocytes. It is important to collect motile sperm from the periphery of a drop with a thin pipette (Fig. 4). When the motility of sperm is less, or the number of oocytes are more than 40, add about 10-20 μL of the sperm suspension.



3) Check the condition of the oocytes enclosed by cumulus cells after about 15 min from insemination. If the amount of sperm solution in the drop of fertilization dish is not enough, add 10 - 20 μl of sperm solution from sperm preincubation dish.

5. Transfer the oocytes to the culture medium

1) At 3–4 h after insemination, pick up the morphologically normal oocytes and wash them in two drops of PB1 or M2 medium.

2) Transfer these oocytes into culture drops of fresh CZB or KSOM a/a medium (Fig. 1c).

6. Assessment of fertilization and embryo transfer

1) On the day after IVF, count the numbers of two-cell embryos and the unfertilized oocytes, respectively. We usually calculate the fertilization rate as: no. two-cell embryos/(no. two-cell embryos + morphologically normal unfertilized oocytes).

2) The two-cell embryos are transferred into the oviducts (usually about 6-10 embryos/oviduct) of pseudopregnant females on the day 0.5 (the day following sterile mating).

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				mHTF		PVA−HTF*	mCZB	KSOM**
Reagents	Campanies	Cat #	Size	mM	mg /100 ml	mg /100 ml	mg /100 ml	mg /100 ml
NaCl	FUJIFILM Wako Chemicals	191-01665	500 g	101.6	581.7	581.7 mg	476.5 mg	555 mg
KCl	FUJIFILM Wako Chemicals	163-03545	500 g	4.7	35	35	35.8	18.6
KH_2PO_4	FUJIFILM Wako Chemicals	169-04245	500 g	0.4	5	5	16.3	4.8
$MgSO_4 \cdot 7H_2O$	FUJIFILM Wako Chemicals	131-00405	500 g	0.2	5	5	29.6	5
$NaHCO_3$	FUJIFILM Wako Chemicals	191-01305	500 g	25	210	210	211	210
$CaCl_2 \cdot 2H_2O$	FUJIFILM Wako Chemicals	033-25035	500 g	2	30	30	25	25.1
EDTA・2Na	FUJIFILM Wako Chemicals	343-01861	50 g	-	-	-	3.7	0.4
Polyvinyl alcohol	Sigma-Aldrich	P8136	250 g	-	-	10	10	-
Phenol red sodium salt	Sigma-Aldrich	P4758	5 g	-	0.2	0.2	0.2	0.2
Glucose	FUJIFILM Wako Chemicals	049-31165	500 g	2.8	50	50	100	3.6
Na pyruvate	Sigma-Aldrich	P8574	5 g	0.3	3.6	3.6	3.5	2.2
Sodium DL-lactate (60% sylup)	Sigma-Aldrich	L7900	100 mL	23.3	400 µl	400 µl	370 µl	174 µl
L-Glutamine reduced	Sigma-Aldrich	G6013	5 g	-	-	-	14.6	14.6
Hypotaurine	Sigma-Aldrich	H1384	250 mg	-	11	11	-	-
Penicillin G・K salt	Sigma-Aldrich	P7794	1 MU	-	10	10	6	6.3
Streptmycin sulfate	FUJIFILM Wako Chemicals	196-08511	5 g	-	-	-	5	5
Albumin, Bovine Serum,	Merck Millipore	12657	25 g	-	300	-	300	100

* : Add 0.4 mM Methyl-β−cyclodextrin (MBCD) before use.

** : Add the essential and non essential amino acids before use.

Preparation methods of sperm preincubation and fertilization media

1. Fertilization medium: 1.25 mM GSH-mHTF medium*

(1) Prepare 25 mM GSH (stock solution)

Weigh about 3 mg of GSH, and add 130 µL mHTF/ mg of GSH

[Glutathione; Sigma G6013-5G), 25 mM GSH：7.683 mg/mL＝1 mg/130 µL]

(2) Prepare 1.25 mM GSH-mHTF medium

On the morning of experiment, add 25 µL of 25 mM GSH to 475µL of mHTF

2. Sperm preincuvation medium: 0.4mM MBCD-PVA-HTF medium**

(1) Prepare 50 mM MBCD (stock solution)

Add 66.7 mg of MBCD (Methyl-β-cyclodextrin; Sigma C-4555-1g) to 1 mL of PVA-mHTF

(2) Prepare 0.4mM MBCD-PVA-HTF medium

On the morning of experiment, add 8 µL of 50 mM MBCD to 992 µL of PVA-mHTF

References

Hasegawa et al. J. Reprod. Dev., 58:156-161, 2012; Takeo et al. Biol. Reprod., 85:1066-1072, 2011.