Microinsemination (Intracytoplasmic Sperm Injection)

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Microinsemination schedule

Day 1 (19:00) Administration of 300 IU/kg pregnant mare's serum gonadotropin (PMSG)
Day 3 (19:00) Administration of 300 IU/kg human chorionic gonadotropin (hCG)
Day 4 (9:00~) Collection of ova and ICSI 14-17 hours after hCG administration
Day 5 (10:00~) Oviduct transfer

1. Preparation of mediums

Materials

Ultrapure water, BSA, sterilization filter (0.20 μm)

Preparation of Stocks A and B (Tables 1 and 2)

1. All reagents shown in Table 1 are dissolved with distilled water, and the volume is adjusted to 100 ml. The solution is sterilized by filtration through a 0.20-μm filter, and stored at 4°C as Stock A.
2. All reagents shown in Table 2 are dissolved with distilled water, and the volume is adjusted to 100 ml. The solution is sterilized by filtration through a 0.20-μm filter, and stored at 4°C as Stock B.

Preparation of mR1ECM (Table 3)

1. All reagents shown in Table 3 are dissolved with distilled water, and the volume is adjusted to 100 ml. The osmotic pressure is adjusted to about 310 mOsm.
2. The solution is sterilized by filtration through a 0.20-μm filter, and stored at 4°C.

Table 1 Composition of Stock A

Reagent	100ml
NaCl	6.428g
KCl	0.239g
Glucose	1.352g
Penicillin G	0.075g
Streptomycin	0.05g
Na-lactate (60%)	1.9ml

Table 2 Composition of Stock B

Reagent	100ml
$CaCl_2 \cdot 2H_2O$	0.294g
$MgCl_2 \cdot 6H_2O$	0.102g

Table 3 Composition of mR1ECM

Reagent	100ml
Stock A	10ml
Stock B	10ml
$NaHCO_3$	0.210g
Na-pyruvate	0.0055g
MEM AA (x50)	2ml
MEM NEAA (x100)	1ml
L-Glutamine	0.0146g

3. At the time of use, an appropriate volume (about 10 ml) is transferred to a tube, combined with BSA at 4 mg/ml, and sterilized by filtration through a 0.20-μm filter.

Preparation of Hepes-R1ECM (Table 4)

1. All reagents shown in Table 4 are dissolved with distilled water. Since polyvinyl alcohol (PVA) is not readily dissolved, the solution is kept in a refrigerator for several days for complete dissolution, and the volume is adjusted to 100 ml.
2. The solution is sterilized by filtration through a 0.20-μm filter, and stored at 4°C.

Table 4 Composition of Hepes-R1ECM

Reagent	100ml
Stock A	10ml
Stock B	10ml
$NaHCO_3$	0.0337g
Na-pyruvate	0.0055g
MEM AA(x50)	2ml
MEM NEAA(x100)	1ml
L-Glutamine	0.0146g
Hepes buffer (1M)	2.2mL
PVA	0.0100g

Preparation of Hepes-R1ECM/PVP

Polyvinyl pyrrolidone (PVP) is added to Hepes-R1ECM at 12% (w/v). After filter sterilization, the solution is aliquoted, and stored at -20°C until use.

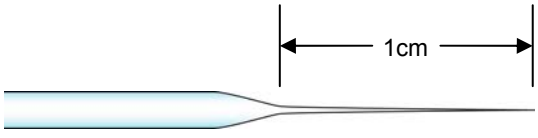
2. Preparation of injection and holding pipette

Materials

Microcapillary, mechanical puller (PN-3, NARISHIGE), microforge (MF-900, NARISHIGE)

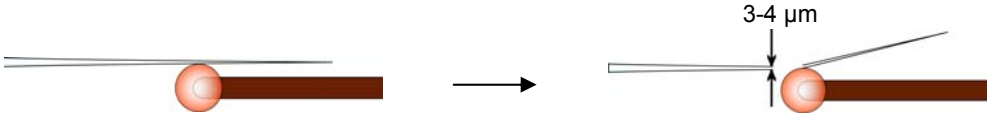
Preparation of injection pipettes

1. The puller's platinum filament in the adjustable region of the heater is molded using forceps and precision tools.
2. The middle of the capillary is set, to be enclosed by the filament of the heater.
3. The heater and magnet scales are arbitrarily set. Since the pulling condition varies depending on the puller model, temperature, and humidity, settings are adjusted to prepare an about 1 cm shank region (Figure below).
4. The capillary extended by the puller is horizontally attached to the microforge.



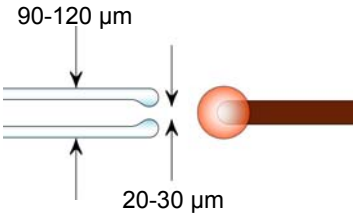
5. A glass bead equipped at the tip of the platinum heater is contacted to the capillary at a site with an outer diameter of about 3-4 μm, (macroscopically determined).

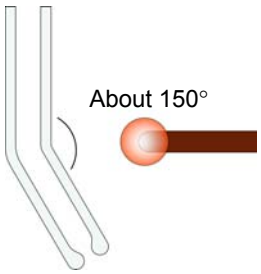
6. The tube is appropriately heated, and the power-supply of the heater is turned off when fusion of the capillary and glass bead starts, by which the tip is vertically cut off.



Preparation of holding pipettes

1. A capillary extended by a puller is scratched with an ampoule cutter at a site with an outer diameter of about 90-120 μm to vertically cut the tip.
2. The capillary is horizontally attached to a microforge, and the tip is rounded (until the inner diameter of the tip becomes 20-30 μm) by heating with the glass bead equipped at the tip of the platinum heater.





3. After attaching the capillary to the microforge, the glass bead is appropriately heated, and the capillary is bent at about 150° using the heated bead.

3. Microinsemination

1) Preparation of oocytes

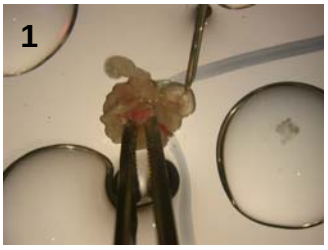
Materials

mR1ECM, mineral oil, hyaluronidase, 26G x 1/2 syringe needle, 1 ml-syringe, plastic dish (35 mm), capillary for ovum manipulation, mouthpiece, sterile filter paper, sterilization filter (0.20 μm), forceps with hooks, forceps without hooks, iris forceps, surgical scissors, ophthalmologic scissors, stereoscopic microscope, CO_2 incubator

Method

1. Hyaluronidase is dissolved with mR1ECM at 1 mg/ml, and sterilized by filtration.
2. Three 100 μl-drops of mR1ECM and one drop of hyaluronidase solution are formed and covered with mineral oil in a 35-mm plastic dish, and equilibrated in an incubator at 37°C in 5% CO_2 and 95% air together with the remaining culture medium.
3. The donor female rat is sacrificed by cervical dislocation 14 hours after hCG administration, and the oviducts are excised and placed on sterile filter paper.

4. The excised oviducts are placed near the hyaluronidase drop, and the ampullae of the oviduct are opened using a 26G syringe needle under a stereoscopic microscope. Released cumulus oocyte complexes are pulled into the hyaluronidase drop (Photos 1 and 2).
5. After complete removal of cumulus cells from the collected cumulus oocyte complexes, the oocytes are washed 3 times by moving the mR1ECM drops, and stored in a CO_2 incubator until ICSI.



2) Preparation of spermatozoa

Materials

mR1ECM, 5-ml test tube, cryotubes, sterile filter paper, forceps with hooks, forceps without hooks, iris forceps, surgical scissors, ophthalmologic scissors, razor

Method

1. The caudal epididymides are excised from a mature male rat (3-6 months of age) and placed on a sterile filter paper.
2. The seminiferous tubes in the caudal epididymides are cut with a razor. Sperm masses are squeezed out and suspended with 2 ml of mR1ECM.
3. The sperms are incubated for 30 minutes in a CO_2 incubator, and sperms that swim up (1.0 ml of culture supernatant) are transferred into a 5-ml test tube, and homogenized using an ultrasonic homogenizer (10 seconds at the lowest output), by which the tails of the sperms are cut off.
4. The sperm suspension is aliquoted into cryotubes (100 μl each), and cryopreserved in liquid nitrogen until use.
5. A cryotube is taken out of a liquid nitrogen tank, and inverted with the lid open to remove liquid nitrogen in the tube.
6. The lid is then closed, and the cryotube is immersed in a 20-25°C water to defrost sperms.

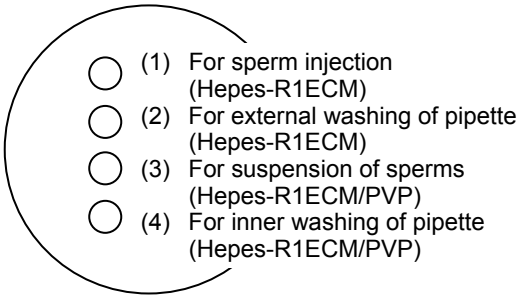
3) Microinsemination procedure

Materials

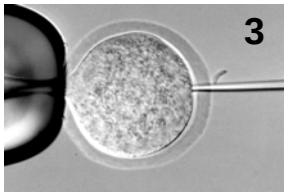
Hepes-R1ECM, Hepes-R1ECM/PVP, plastic dish (35 mm), injection pipette, holding pipette, oocyte manipulation capillary, mouthpiece, stereoscopic microscope

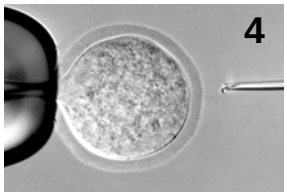
Method

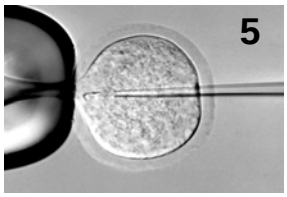
1. One drop (6 μl) each of solutions (1) - (4) are formed in the lid of a plastic dish (35 mm), as shown on the right.
2. The inside of an injection pipette is washed by repeated blowing off mercury and aspirating Hepes-R1ECM/PVP in Drop (4).
3. Sperms are placed in Drop (3), and oocytes are placed in Drop (1).
4. A sperm is aspirated into the injection pipette and transferred into Drop (2) and then Drop (1) for sperm injection.



5. An ovum is retained, placing the first polar body at 12:00 or 6:00 position, using the holding pipette.
6. The sperm is released, and only the zona pellucida is penetrated by several piezopulses (intensity: 2, speed: 1) (Photo 3).
7. The sperm is re-aspirated into the pipette (Photo 4). The pipette is then deeply inserted into the egg cytoplasm, and a low positive pressure is loaded on the injector.
8. A single piezopulse (intensity: 1, speed: 1) is loaded to make the pipette penetrate the egg cell membrane, and the sperm is simultaneously injected into the egg cytoplasm (Photo. 5), immediately followed by rapidly pulling the pipette out of the oocyte.
9. The ovum containing the injected sperm is recovered into Hepes-R1ECM, and kept at room temperature for 10 minutes.







4) Culture of ICSI oocytes

Materials

mR1ECM, plastic dish (35 mm), capillary for egg-manipulation, mouthpiece, stereoscopic microscope, CO_2 incubator

Method

1. After washing 3 times with mR1ECM drops, oocytes are cultured in a CO_2 incubator.

2. The viability and fertility are determined 6 hours after sperm injection. Oocytes with both the male and female pronuclei and the 2nd polar body are regarded as normal fertilized zygotes.
3. The cleavage rate of fertilized oocytes is determined 24 hours after sperm injection, and embryos with normal morphology are subjected to oviduct transfer.

4. List of instruments and reagents

List of instruments (Maker, Cat. No.)		
Mechanical puller	Narishige	PN-3
Microforge	Narishige	MF900
Ultrasonic homogenizer	BRANSON·SONIFIER250	
Osmometer	VOGEL	OM802-D
Cryotube	Nunc	363401
Glass capillary	Sutter	B100-75-10
List of reagents (Maker, Cat. No.)		
$CaCl_2 \cdot 2H_2O$	Wako	031-00435
$MgCl_2 \cdot 6H_2O$	SIGMA	M0250
NaCl	Wako	191-01665
KCl	Wako	163-03545
$NaHCO_3$	Wako	191-01305
Glucose	Wako	041-00595
Na-pyruvate	SIGMA	P2256
Na-lactate	Nacalai Tesque	31605-72
Penicillin G	Meiji Seika	20×10^4 units/vial
Streptomycin	Meiji Seika	1g (potency)/vial
Bovine serum albumin	SIGMA	A6003
Hyaluronidase	SIGMA	H3506
Mineral oil	SIGMA	M8410
MEM Amino Acid Solution	GIBCO	11130-051
MEM Non-Essential Amino Acids Solution	GIBCO	11140-050
L-Glutamine	SIGMA	G3126
Hepes Buffer Solution	GIBCO	15630-080
Polyvinylalcohol	SIGMA	P8136