Video Article

Mouse Sperm Cryopreservation and Recovery using the I·Cryo Kit

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Abstract

Thousands of new genetically modified (GM) strains of mice have been created since the advent of transgenesis and knockout technologies. Many of these valuable animals exist only as live animals, with no backup plan in case of emergency. Cryopreservation of embryos can provide this backup, but is costly, can be a lengthy procedure, and generally requires a large number of animals for success. Since the discovery that mouse sperm can be successfully cryopreserved with a basic cryoprotective agent (CPA) consisting of 18% raffinose and 3% skim milk, sperm cryopreservation has become an acceptable and cost-effective procedure for archiving, distributing and recovery of these valuable strains.

Here we demonstrate a newly developed I•Cryo kit for mouse sperm cryopreservation. Sperm from five commonly-used strains of inbred mice were frozen using this kit and then recovered. Higher protection ratios of sperm motility (> 60%) and rapid progressive motility (> 45%) compared to the control (basic CPA) were seen for sperm frozen with this kit in 5 inbred mouse strains. Two cell stage embryo development after IVF with the recovered sperm was improved consistently in all 5 mouse strains examined. Over a 1.5 year period, 49 GM mouse lines were archived by sperm cryopreservation with the I•Cryo kit and later recovered by IVF.

Video Link

The video component of this article can be found at https://www.jove.com/video/3713/

Protocol

1. Mouse sperm cryopreservation

1. For each line to be cryopreserved, prepare the following before beginning.
    1. 1 well of a 4-well dish with 240 l of kit CPA
    2. 15 sperm cryopreservation straws as follows
        1. Connect the 0.25 ml straws to the 1 ml syringes with the cotton plug closest to the syringe. In each straw, load 8 cm (approximately 160 µl) of IVF medium, then 1 cm of air. Label the straws with the name of the line to be preserved.
2. LN_2 should be 15 cm deep in the foam box. Close the lid.
3. Euthanize 2 male mice from each line to be cryopreserved. Mice should be between 10 and 60 weeks old. Remove the two caudal epididymides along with the vas deferens from each mouse.
4. Place the epididymides into the previously prepared CPA-containing well of a 4-well dish (step 1.1).
5. Make 5-7 longitudinal cuts in each epididymis and gently squeeze each vas deferens to push sperm out.
6. Shake the CPA and epididymis mixture by hand gently at room temperature for 3-5 minutes. This will allow sperm to swim out.
7. Using the previously prepared straw, aspirate 5 mm (approximately 10 µl) of sperm suspension and then 1 cm of air.
8. Repeat this process with up to a total of 15 straws. Load and seal straws within 10 minutes for best sperm survival.
9. Heat seal both sides of the straw carefully. Place the straws in the freezing canister with the sperm column first.
10. Attach the 1 ml pipette provided as part of the kit to the canister to extend the handle. Place the canister in LN_2 in the foam box through the hole in the lid. Allow the canister to float vertically in the LN_2 for 10 minutes.
11. Immerse the canister in the LN2. The sperm is now cryopreserved and can be moved to long-term LN_2 storage.
12. The canister should not be used for the next line until it is warmed up to room temperature.

2. Mouse superovulation

1. Generally, use young female mice of the same background as the sperm donors.
2. Mice should be ~12 g (3-4 weeks of age).
3. Perform an intraperitoneal injection with 5-10 IU of Pregnant mare serum gonadotropin (PMSG).

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4. Wait 46-48 hours.
5. Perform an intraperitoneal injection with 5-10 IU of human chorionic gonadotropin (hCG).
6. Harvest oocytes 14-16 h after hCG injection as described below.

3. Mouse sperm recovery and IVF

1. Before IVF, for each straw to be thawed, prepare the following and equilibrate for at least one hour in a 37°C CO_2 incubator.
    1. Sperm incubation dish: one 35 mm Petri dish, with a 90 µl drop of sperm treatment medium (STM) covered with oil.
    2. Cutting dish: For oocyte collection from a maximum of 10 superovulated females, one 35 mm Petri dish with 3 ml of IVF medium.
    3. IVF well: For each group of 5 superovulated females for IVF, one well of a 4 well dish with 500 µl of IVF medium (if you are using 10 superovulated females, for example, you will need 2 wells. . .)
    4. Washing dish: one 35 mm Petri dish with 3 ml of potassium simplex optimized medium (KSOM) for washing each well of the 4-well IVF dish used
    5. Culture dish: one 35 mm Petri dish with 50 µl drops of KSOM medium covered with oil for embryo culture from each well of the IVF dish.
2. Remove a straw from LN_2 and place in a 37°C water bath for 2-3 minutes.
3. Remove the straw from the water bath and wipe to remove excess water. Make the first cut close to the first air column nearest the cotton plug. Cut the straw in the IVF medium there will still be an air column visible before the sperm column.
4. Then cut the end of the straw in the second air column, being careful to avoid cutting the sperm column. Cut at a 45 angle - this will make it much easier to aspirate the sperm.
5. Aspirate the sperm suspension from the distal end of the straw with a 200 µl pipettor.
6. Place the sperm suspension in the center of the STM drop and incubate the dish for 45-60 minutes in a 37°C 5% CO_2 incubator.
7. During the sperm incubation, dissect the oviducts of the superovulated females into the cutting dish. Use a 30 g needle or forceps to tear each oviduct, releasing the cumulus-oocyte complexes (COCs). Avoid fat and blood in the IVF medium as dirty media makes for extra washing steps.
8. Once all the oviducts have been torn, transfer the COCs from 5 females to each IVF wells. The number of COCs produced by superovulation is very strain dependent.
9. Collect 10-15 µl of sperm from the periphery of the STM drop and fertilize one IVF well approximately 14-16 hours post hCG injection. Repeat fertilization for each well used. Final sperm concentration will be approximately 1 million/ml.
10. Coculture sperm and oocytes for 4-6 hours in a 37°C 5% CO_2 incubator.
11. Wash the embryos with KSOM a minimum of 2 times and culture in KSOM drops for development in a 37°C 5% CO_2 incubator.
12. IVF rate was calculated as two cell stage embryos out of total oocytes used after overnight culture.

4. Representative Results

Sperm from 5 Charles River mouse inbred strains was frozen. Protection ratios are calculated by dividing frozen sperm assessment values by fresh sperm assessment values. Protection ratios of sperm motility were 60.2%, 81.3%, 78.6%, 66.5%, 61.0% for C57BL/6NCrl, 129S2/SvPasCrl, FVB/NCrl, DBA/2NCrl, and BALB/cAnNCrl, respectively . Protection ratios for rapid progressive motility were 47.3%, 69.4%, 57.0%, 52.8%, 54.1% in C57BL/6NCrl, 129S2/SvPasCrl, FVB/NCrl, DBA/2NCrl, and BALB/cAnNCrl, respectively (Fig 1).

The IVF rates with frozen-thawed sperm were 55.7%, 26.4%, 87.3%, 87.8% and 26.2% in C57BL/6NCrl, 129S2/SvPasCrl, FVB/NCrl, DBA/2NCrl, and BALB/cAnNCrl, respectively (Fig 2).

Over a 1.5 year period, 49 GM mouse lines were archived by sperm cryopreservation. These lines were later successfully recovered (as defined by live pups born) by IVF in 4 female strains (C57BL/6, BALB/c, DBA/1 and 129Sv) (Fig 3).

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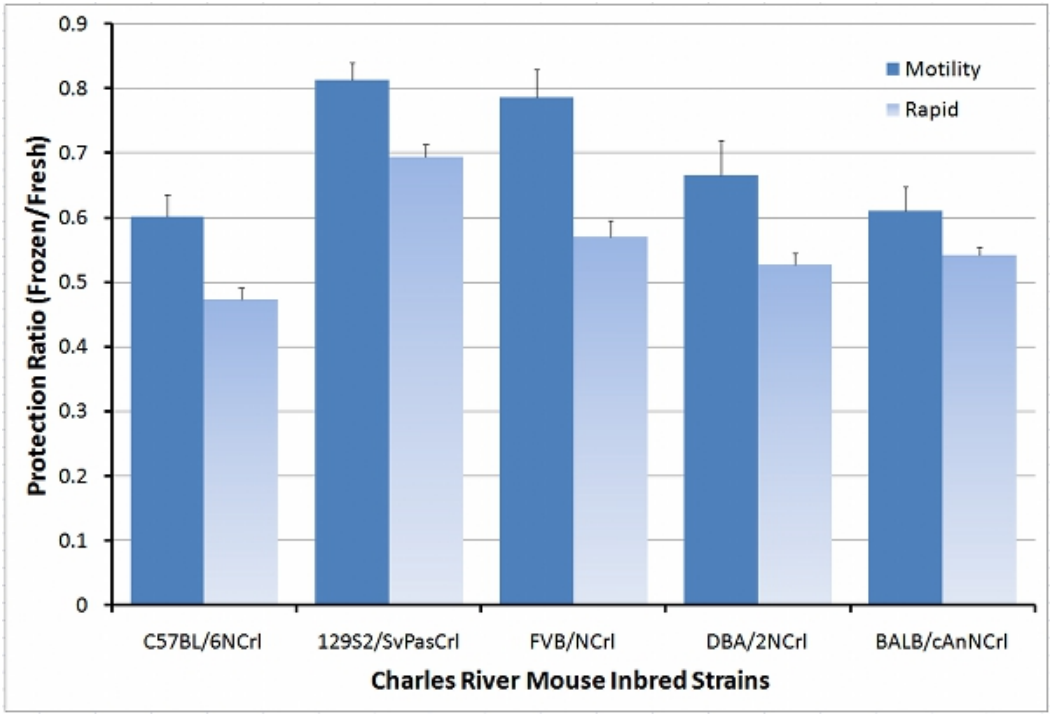

Figure 1. The Protection Ratios of Sperm Motility and Rapid Progressive Motility in Mice

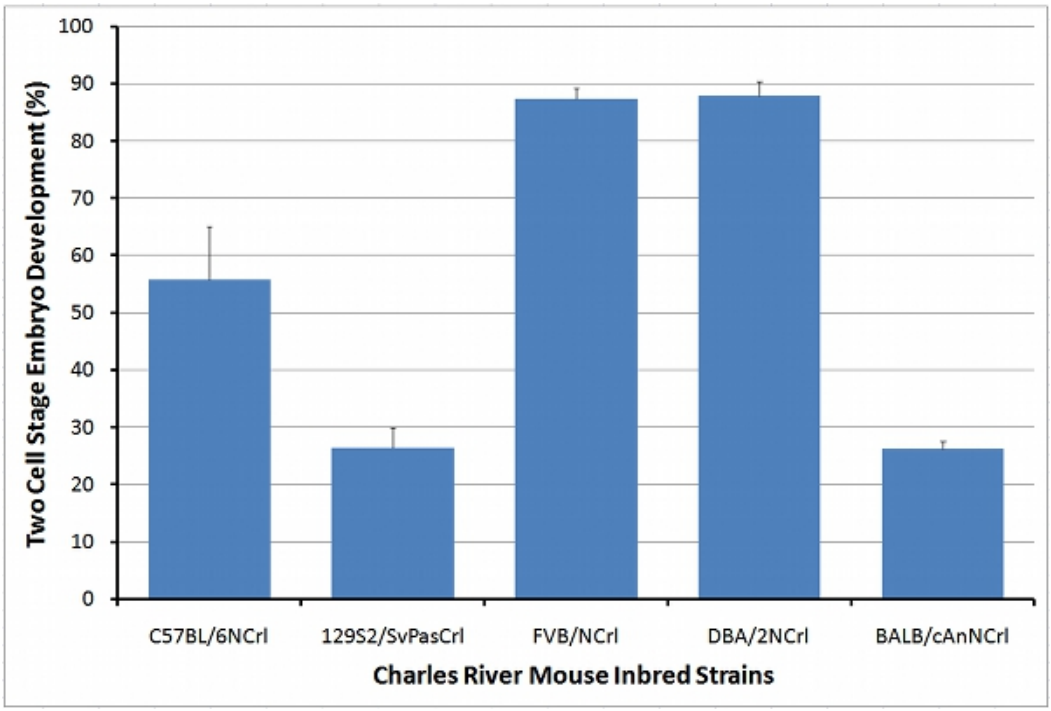

Figure 2. *In vitro* Fertilization with Frozen-thawed Sperm in Mice

Genetic Background of Female Strains	# of Male Donor Lines	# of Females	# of IVF Oocytes
BALB/cAnNCrl	3	99	550
C57BL/6J	5	51	1071
C57BL/6NCrl	37	691	16600
129Sv	1	8	66
DBA/1J	3	76	772

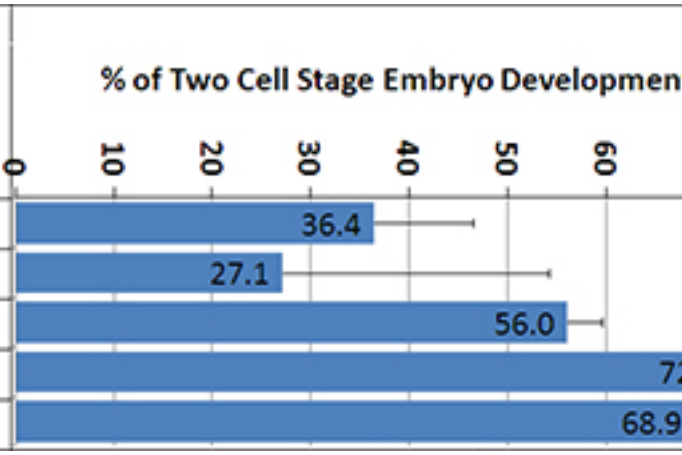

Figure 3. *In vitro* Fertilization with Frozen-thawed Sperm in GM Mice

Discussion

Since 1990, the basic sperm freezing protocol using 18% raffinose and 3% skim milk has been proven reliable in many strains of mice and a large number of mouse lines have been cryopreserved [1,2,3,4,5,6,7]. The major causes of damage to sperm cells during the freezing and thawing process have been associated with ice formation [8,9] and reactive oxygen species [10]. Supplementation with free radical scavengers, such as amino acids, and reducing agents, such as monothioglycerol, in the freezing medium was investigated and proven to substantially increase IVF rate with frozen-thawed sperm in inbred strains including the C57BL/6 [11,6].

In the present protocol, we developed a new I•Cryo kit for mouse sperm cryopreservation by optimizing the basic CPA with commercially available antioxidants and ice blockers and by modifying the sperm freezing and IVF procedure. Protection ratios of sperm motility (>60%) and rapid progressive motility (>45%) were seen for sperm from five inbred mouse strains frozen with the I•Cryo kit. The 2-cell stage embryo development rate with frozen-thawed sperm in 5 mouse inbred strains was markedly improved compared to that with basic CPA [11,6].

In using the kit, the user creates a concentrated sperm suspension by freezing sperm from 2 males for each line. By only freezing 15 straws the process is fast, easy and occupies less storage space for short or long-term storage. Most times, mouse lines can be recovered by thawing only 1 or 2 straws.

Disclosures

The authors are employees of Charles River, the maker of this kit.

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