Studies on Cryopreservation of Embryos and Gametes in Mice

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I. Introduction

Recently, successful cryopreservation of mouse embryos and unfertilized oocytes at -196°C by a simple method called vitrification or ultrarapid freezing has been reported [2–4, 7, 20–21, 24–25]. On the other hand, the freezing of mouse spermatozoa has been reported by several groups recently [15, 18–19, 22–23, 26]. Herein I describe a simple method which I have established for the cryopreservation of embryos and gametes (unfertilized oocytes and spermatozoa) in mice, and discuss the system for producing young from cryopreserved embryos and gametes.

II. Ultrarapid freezing of embryos and unfertilized oocytes

1. Methods of ultrarapid freezing [6]

1) Cooling method: Between 15 and 30 embryos/oocytes in a small volume of medium were pipetted into a glass capillary and were transferred into 20–30 μl of a cryoprotective solution (2 M-dimethyl sulphoxide + 1 M-acetamide + 3 M-propylene glycol) [6] in a freezing tube (volume: 0.5 ml) at room temperature. After 5–10 seconds, the freezing tube was capped and plunged directly into liquid nitrogen.

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2) Warming method: The sample was removed from the liquid nitrogen and warmed by directly transferring it to a 37 °C waterbath and was immediately diluted by a single addition of 0.3 ml of a 0.3 M-sucrose solution when the samples began to liquify. The contents of each tube were pipetted into a culture dish and the embryos/oocytes were recovered. The embryos/oocytes recovered were washed by transfer through four changes of medium and then their morphology was examined.

2. Viability of embryos and unfertilized oocytes after thawing

1) Embryos at various stages [4–6, 12]: Table 1 shows the morphology and *in vitro* development of frozen-thawed mouse embryos at various stages from the hybrid strain (B6C3F₁ × Jcl: ICR). The rates of morphologically normal embryos on thawing were high for 1-cell, 2-cell, 4-cell and morula (over 90%), but the viability of the blastocyst was very low and only 13% of recovered embryos were morphologically normal. The proportions of the frozen-thawed embryos that developed from 1-cell, 2-cell, 4-cell and morula stage to blastocyst ranged from 51% to 80% and the morula was the highest of all stages.

2) Two-cell embryos from inbred strains [8]: Table 2 summarizes the survival of frozen-thawed 2-cell embryos from inbred strains (BALB/c, C3H/He, C57BL/6N and DBA/2N). The percentages of morphologically normal embryos ranged from 81% to 92% on thawing. After transferring the morphologically normal embryos into the oviducts of pseudopregnant recipients, 70–60% of them developed into normal young.

3) Unfertilized oocytes from inbred strains [9]: Tables

3 and 4 summarize the viability of frozen-thawed unfertilized oocytes from inbred strains (BALB/c, C3H/He, C57BL/6N and DBA/2N) after thawing. On thawing, 60–91% of the recovered oocytes were morphologically normal (Table 3). Following fertilization *in vitro* of frozen-thawed oocytes, the proportions of the oocytes which developed to the 2-cell stage 24 h after insemination ranging from 34% to 83% (Table 4). Nearly all 2-cell embryos obtained from frozen-thawed oocytes were transferred and 18–43% of them developed into normal young.

3. Production of transgenic mice from cryopreserved pronuclear oocytes [1]

Table 5 summarizes the results for the production of transgenic mice by microinjection of chicken β -actin promoter-driven firefly luciferase cDNA into the cryopreserved pronuclear oocytes. The DNA construction was microinjected into a total of 185 frozen-thawed oocytes, 131 (71%) of which survived the microinjection and were transferred into recipients. Of 7 recipients, 5 became pregnant and littered a total of 17 offspring. As a control, the DNA construction was also microinjected into 221 unfrozen (fresh) oocytes, 159 (72%) of which survived and were transferred. Of 6 recipients, all became pregnant and littered a total of 21 offspring. The production rates for transgenic mice from frozen-thawed oocytes and fresh oocytes were 1.6% (2/125) and 1.3% (2/149), respectively. These findings indicate that the efficiency of production of transgenic mice from frozen-thawed oocytes is close to that from unfrozen oocytes. This suggests that frozen-thawed oocytes can be used instead of fresh oocytes to produce transgenic mice.

Table 1. The morphology and *in vitro* development of frozen-thawed mouse embryos at various stages from hybrid strain^{a)}

Stage of embryo at freezing	No. of embryos frozen	No. of embryos recovered (%)	No. of embryos morphologically normal on thawing (%)	No. of embryos cultured	No. of embryos developed to blastocyst (%)
1-cell ^{b)}	142	135 (95)	122 (90)	120	61 (51)
2-cell	148	141 (95)	131 (93)	131	75 (57)
4-cell	120	113 (94)	107 (95)	107	76 (71)
Morula	125	119 (95)	110 (92)	110	88 (80)
Blastocyst	100	90 (90)	12 (13)	—	—

^{a)} B6C3F₁ (♀) × Jcl:ICR (♂) ^{b)} Pronuclear oocyte.

Table 2. The survival of frozen-thawed 2-cell mouse embryos from inbred strains

Strain	No. of embryos frozen	No. of embryos recovered (%)	No. of embryos morphologically normal on thawing (%)	No. of embryos transferred	No. of pregnant/ No. of recipients	No. of live young (%)
BABL/c	128	120 (94)	105 (88)	105	6/8	36 (34)
C3H/He	133	127 (95)	115 (91)	111	6/8	34 (31)
C57BL/6N	123	117 (95)	108 (92)	105	6/7	63 (60)
DBA/2N	141	135 (96)	109 (81)	109	3/8	8 (7)

Table 3. The morphology of frozen-thawed unfertilized mouse oocytes from inbred strains on thawing

Strain	No. of embryos frozen	No. of embryos recovered (%)	No. of embryos morphologically normal on thawing (%)
BALB/c	117	168 (95)	129 (77)
C3H/He	153	146 (95)	123 (84)
C57BL/6N	160	154 (96)	140 (91)
DBA/2N	198	186 (94)	112 (60)

Table 4. Development of 2-cell embryos from frozen-thawed oocytes fertilized by fresh spermatozoa and production of young following embryo transfer

Strain	No. of oocytes inseminated	No. of oocytes developed to 2-cell embryo (%)	No. of 2-cell embryos transferred	No. of pregnant/No. of recipients	No. of live young (%)
BABL/c	129	102 (79)	102	7/7	34 (33)
C3H/He	123	87 (71)	87	5/6	27 (31)
C57BL/6N	140	119 (83)	114	7/8	49 (43)
DBA/2N	112	38 (34)	38	2/3	7 (18)

Table 5. Production of transgenic mice from frozen-thawed and unfrozen (fresh) oocytes microinjected chicken β -actin promoter-driven firefly luciferase cDNA

Oocyte	No. of oocytes		No. of oocytes transferred	No. of pregnant/No. of recipients	No. of live young	
	Injected	Survived (%)			Total (%) ^{a)}	Transgenic (%) ^{a)}
Frozen-thawed	185	131 (70.8)	125	5/7	17 (13.6)	2 (1.6)
Unfrozen (fresh)	221	159 (71.9)	149	6/6	21 (14.0)	2 (1.3)

^{a)} live young as percentage of oocytes transferred.

III. Cryopreservation of spermatozoa

1. Cryopreservation method [16]

Two tails of the epididymides of mouse were minced

in 400 μ l of cryopreservation solution (18% raffinose and 3% skim milk in distilled water) and the spermatozoa were dispersed from the organs. The sperm suspension was put into a plastic straw (volume 0.25 ml) and the straws were heat-sealed. The specimens were

then cooled by putting them into a liquid nitrogen gas layer for 10 min and were plunged directly into liquid nitrogen, where they were stored for 1 to 9 months before thawing. At that time, the specimens were thawed in a waterbath maintained at room temperature (20 to 24 °C), for 5 min. After removing the cryopreservation solution from the sperm suspension by filtration [23], the sperm suspension was used for insemination.

2. Fertilizing ability *in vitro* of frozen-thawed spermatozoa

Table 6 summarizes the cryopreservation results for spermatozoa from inbred (BALB/c, C3H/He, C57BL/6N, CBA/JN and DBA/2N) and F₁ hybrid strains (B6C3F₁, BDF₁ and CDF₁) [16]. Spermatozoa from all of the strains were successfully frozen, although the fertilization rates for frozen-thawed spermatozoa varied among strains (26% for C57BL/6N to 89% for DBA/2N). Nearly all 2-cell embryos fertilized by frozen-thawed spermatozoa were transferred to the oviducts of pseudopregnant recipients and 35–62% of the 2-cell embryos developed into normal young. In addition, the spermatozoa of transgenic mice were also frozen suc-

cessfully (Table 7 and Fig. 1) [17].

3. Artificial insemination with frozen-thawed spermatozoa [11]

The method for injecting the thawed sperm suspension into the Fallopian tube of ovulated pseudopregnant females was essentially the same as that described previously [10]. As shown in Fig. 2, about 3–5 μ l of the thawed sperm suspension (10,000–15,000 spermatozoa) was sucked into a glass capillary and injected with the glass capillary into the Fallopian tube ampullas containing ovulated oocytes in pseudopregnant females through the wall of the tube on day 1 of pseudopregnancy. Table 8 summarizes the results for conception and litter size. Five out of 12 females inseminated became pregnant and gave birth to 28 young (mean litter size: 5.6 ± 1.50).

IV. Production of normal young following transfer of embryos obtained by *in vitro* fertilization between cryopreserved gametes [13]

Table 9 summarizes the rates of fertilization between frozen-thawed gametes and the production of young

Table 6. Development of 2-cell embryos from oocytes fertilized by frozen-thawed mouse spermatozoa and production of young following embryo transfer

Strains of frozen-thawed spermatozoa	No. of oocytes examined	No. of oocytes fertilized (%)	No. of oocytes developed to 2-cell embryos (%)	No. of 2-cell embryos transferred	No. of pregnant/No. of recipients	No. of live young (%)
BALB/c	141	67 (48)	66 (47)	57	3/4	20 (35)
C3H/He	131	96 (73)	96 (73)	96	6/6	49 (51)
C57BL/6N	157	40 (26)	40 (26)	40	2/3	14 (35)
CBA/JN	119	91 (77)	89 (75)	89	4/5	43 (48)
DBA/2N	138	121 (89)	121 (89)	96	5/6	59 (62)
B6C3F ₁ ^{a)}	118	70 (59)	70 (59)	70	5/5	39 (56)
BDF ₁ ^{b)}	126	94 (75)	94 (75)	94	5/5	52 (55)
CDF ₁ ^{c)}	144	110 (76)	110 (76)	108	6/6	59 (55)

^{a)} C57BL/6N (♀) × C3H/He (♂), ^{b)} C57BL/6N (♀) × DBA/2N (♂), ^{c)} BALB/c (♀) × DBA/2N (♂).

Table 7. Development of 2-cell embryos from oocytes fertilized by frozen-thawed luciferase transgenic mouse spermatozoa and production of young following embryo transfer

No. of oocytes examined	No. of oocytes fertilized (%)	No. of oocytes developed to 2-cell embryos (%)	No. of 2-cell embryos transferred	No. of pregnant/No. of recipients	No. of live young (%)
65	36 (55)	36 (55)	36	2/2	23 (64)

Fig. 1. Southern blot analysis of young derived from frozen-thawed sperm of a transgenic mouse. Tail DNA (10 μ g) was digested with Bam H I, and the fragments were blot hybridized with a probe that was a Bam H I–Bam H I fragment (1.9Kbp) from the transgene. The hybridizing bands (1.9Kbp; arrow) indicate the integration of injected fragments. Numbers of mice are right.

following embryo transfer. When unfertilized oocytes (C57BL/6N and C3H/He) and spermatozoa (DBA/2N and Jcl: ICR) were frozen separately and then *in vitro* fertilization was performed with DBA/2N spermatozoa and C57BL/6N oocytes, DBA/2N spermatozoa and C3H/He oocytes, Jcl: ICR spermatozoa and C57BL/6N oocytes, and Jcl: ICR spermatozoa and C3H/He oocytes after thawing, the rate of oocyte fertilization and development to 2-cell embryos after insemination *in vitro* was in the range of 22–45% (Fig. 3). All 2-cell embryos were transferred to pseudopregnant recipients and 23–34% of the 2-cell embryos developed to normal young (Fig. 4). The reproductive ability of these young was tested by pairing 8-week-old offspring from the same group. Five females were paired with five males in each group, and all females became pregnant and delivered live young.

V. Conclusion

In this study a system for producing young from cryopreserved gametes and embryos has been established (Fig. 5) [14]. Especially in gametes, the cryopreserved oocytes could be fertilized *in vitro* by means of fresh spermatozoa and the cryopreserved spermatozoa could fertilize fresh oocytes and could be used for artificial insemination. Moreover, the cryopreserved oocytes could be fertilized by the cryopreserved spermatozoa and the embryos obtained by *in vitro* fertilization between cryopreserved gametes could develop into

Fig. 2. Method of injection of frozen-thawed mouse spermatozoa into the Fallopian tube containing ovulated oocytes of a pseudopregnant female.

Table 8. Conception and litter size following insemination of frozen-thawed mouse spermatozoa into Fallopian tube of pseudopregnant females

Sperm ^{a)}	No. of pseudopregnant females used	No. of animals which delivered live young (%)	No. of live young (Litter size)
Frozen-thawed	12	5 (41)	28 (5.6 ± 1.50) ^{b)}
Unfrozen (fresh)	6	4 (67)	30 (7.5 ± 2.69) ^{b)}

^{a)} About 3–5 μ l of sperm suspension (10,000–15,000 spermatozoa) was injected into each Fallopian tube. ^{b)} Mean $\pm$ S.D.

Table 9. Fertility of frozen-thawed mouse gametes assessed by *in vitro* fertilization and production of young following transfer of embryos obtained between cryopreserved gametes

Gametes		No. of oocytes inseminated	No. of oocytes fertilized (%)	No. of oocytes developed to 2-cell embryo (%)	No. of 2-cell embryos transferred	No. of pregnant/No. of recipients	No. of live young (%)
Spermatozoa	Oocytes						
DBA/2N	C3H/He	179	65 (36)	64 (36)	64	3/3	15 (23)
DBA/2N	C57BL/6N	218	98 (45)	98 (45)	98	4/4	31 (32)
Jcl:ICR	C3H/He	245	54 (22)	54 (22)	54	3/3	15 (28)
Jcl:ICR	C57BL/6N	246	67 (27)	66 (27)	66	3/3	23 (35)

Fig. 3. Appearance of 2-cell embryos obtained from frozen-thawed mouse gametes after insemination *in vitro*.

Fig. 4. Twenty-one-day-old young developed from 2-cell embryos fertilized by *in vitro* frozen-thawed mouse gametes.

Fig. 5. System for producing young from frozen-thawed gametes and embryos.

normal, live young after embryo transfer. In the future, if the oocytes and spermatozoa of many mouse strains are frozen separately, the desired embryos and young will be able to be produced from various combinations of cryopreserved gametes.

The freezing methods for gametes and embryos pre-

sented here are very simple, are not time-consuming and reduce freezing equipment costs. I therefore expect that these methods will become routine techniques for the frozen storage of mouse gametes and embryos in various laboratories and contribute to the preservation of genetic resources.

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