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Cryopreservation of Gene Disrupted Mouse Spermatozoa

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Abstract: *Cryopreservation of mouse spermatozoa has recently become available for use. In this study, we attempted to apply this technique to the maintenance of gene disrupted mouse lines. Cauda epididymides that were taken from Et1^{tm1Csk} and Gk^{tm1Csk} mice were minced in 100 μl of a cryopreservation solution which consisted of 18% raffinose and 3% skim milk. Sperm suspensions in plastic straws were cooled rather rapidly by being placed in the gas phase above liquid nitrogen, and then were stored at −196°C. The frozen straws were thawed rapidly being by immersing in a water bath at 30°C. For in vitro fertilization, 1 μl of thawed sperm suspensions from each mouse strain was added directly to oocytes contained in 200 μl of medium. Following pre-incubation of the frozen-thawed spermatozoa, ICR oocytes were introduced into the medium containing the frozen-thawed spermatozoa. The fertilization rates of the oocytes inseminated with Et1^{tm1Csk} and Gk^{tm1Csk} frozen-thawed spermatozoa were 71% and 77%, respectively. The development rates into young after embryo transfer were in a range of 32–65%. Progeny tests revealed that the mutated locus/loci were transmitted to the next generation according to the Mendelian law of the inheritance of characteristics. These results indicate that cryopreservation of spermatozoa provides an effective alternative to embryo freezing for maintenance of gene disrupted mouse strains.*

Key words: *Mouse, Spermatozoa, Cryopreservation, In vitro fertilization, Gene targeting.*

Recently, a large number of various strains of gene disrupted mice have been produced in different laboratories [1]. An important issue associated with gene targeting technology is maintenance of the established gene disrupted mouse lines. Embryo freezing is generally used for this purpose but requires the obtaining of a number of early-pregnant gene disrupted mice prior to the freezing procedures. A paradoxical operation is thus required, so that a breeding colony must be expanded at considerable effort and cost in order to subsequently reduce or abolish it. The survival of mouse oocytes and embryos preserved by freezing or vitrification ranges between 16% and 54%, even when they are derived from random-bred or F1 hybrids [2]. Furthermore, as the embryo freezing consists of several procedural steps such as the collection of embryos, freezing (vitrification), thawing, culture and embryo transfer, even a 10% loss of embryos at each step substantially influences overall productivity. It has been estimated that only 6.5% to 45% of frozen embryos successfully develop to term after thawing and subsequent embryo transfer [3].

Although cryopreservation of spermatozoa has been applied to the improvement of breeding in domestic animals and treatment of infertility in humans, there was no practical method of cryopreservation for mouse spermatozoa until just a few years ago. Recently, it has become possible to freeze a large number of mouse spermatozoa immediately after collection from the epididymides of a small number of males [4–12]. This has encouraged attempts to apply this technique to the maintenance of gene disrupted mice, since the cryopreservation of spermatozoa appeared to be more simple, less time-consuming and less costly than that of embryos in maintaining gene disrupted mouse strains.

In this study, we report an improved method of sperm cryopreservation based on our previous version [8] for maintenance of gene disrupted mice, with examination of the transmission of the disrupted gene in mice derived from cryopreserved spermatozoa by progeny test.

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Materials and Methods

Freezing of mouse spermatozoa

Spermatozoa were obtained from 12 week-old *Et1*^{tm1Csk} and *Gk*^{tm1Csk} heterozygous male mice of the 129/SvJ × ICR genetic background. These two strains, which were derived in our laboratory, lack the endothelin-1 [13] and glucokinase genes [14], respectively. The procedures for spermatozoa cryopreservation were essentially the same as described previously [8]. Eighteen percent raffinose (Wako, Tokyo, Japan) and 3% (w/v) skim milk (Difco, Mich., USA) were dissolved in distilled water at 60°C. The solution was centrifuged at 10,000 g for 15 min, and the supernatant was filtered and used as the cryopreservation solution (pH: 6.2 to 6.3, osmolality: 480 to 500 m-osmoles). Two tails of the epididymides that were taken from one male mouse were minced in 100 μl of the cryopreservation solution in a 4-well multidish (No. 176740, Nunc, Roskilde, Denmark). The small volume of 100 μl was used so as to concentrate the sperm suspension as it was collected. The spermatozoa were dispersed from the organs at room temperature by shaking the dish for about 2 min. The sperm suspension at a concentration of 10 to 14 × 10^7 cells/ml was then divided into 10 samples, and 10 μl of each specimen was put into a plastic straw (volume 0.25 ml, IMV, l'Aigle, France) and the straws heat-sealed. The straws were then cooled by putting them into the neck (liquid nitrogen gas layer) of the container (volume 3 l: SC/3, IMV, l'Aigle, France) for 10 min, and were plunged directly into liquid nitrogen, where they were stored for 1 to 3 months before thawing. Ultimately, each straw contained 10 to 14 × 10^5 spermatozoa.

Preparation of oocytes and in vitro fertilization

ICR (CLEA, Tokyo, Japan) females at 8 weeks of age were each injected with 5 i.u. of equine chorionic gonadotrophin (eCG; Serotropin, Teikokuzoki Co. Ltd., Tokyo, Japan) and human chorionic gonadotrophin (hCG; Puberogen, Sankyo Co. Ltd., Tokyo, Japan) at an interval of 48 h, and the oocytes surrounded by cumulus cells were collected from the excised oviducts 14 to 15 h after the hCG injection.

By immersing the frozen plastic straws in a water bath at 30°C, the sperm in the plastic straw were thawed and left immersed for 15 min, and 1 μl of thawed sperm suspension was added to the 200 μl of the human tubal fluid (HTF) medium [15] for *in vitro* fertilization at a sperm concentration of 500 to 700 cells/μl. Following incubation of the frozen-thawed spermatozoa for 1.5 h in 5% CO_2 in air at 37°C, the ICR oocytes were introduced into the HTF medium containing the frozen-thawed spermatozoa. At 24 h after insemination, fertilization was defined as development to a 2-cell stage embryo.

As a positive control, ICR oocytes were inseminated with freshly collected and subsequently preincubated *Et1*^{tm1Csk} and *Gk*^{tm1Csk} heterozygous spermatozoa at a final concentration of 150 cells/μl according to the method of Toyoda *et al.* [16].

Embryo transfer and genotyping of the progeny

The fertilized eggs which had developed to the 2-cell stage were transferred to the oviducts of ICR pseudopregnant recipients on Day 0.5 of pseudopregnancy as previously described [17]. The presence of heterozygous mice was determined by Southern blot analysis of genomic DNA from the tip of the tail in each strain of mice after the weaning of the progeny [13, 14]. Mature heterozygotes in each strain of gene disrupted mice were bred with each other to produce a homozygous mutant. Homozygous mutant animals were identified by the apearance of a specific phenotype, i.e., a craniofacial anomaly in the case of endothelin-1 [13] or a marked glucosuria in glucokinase [14].

All the mice were maintained in light-controlled (lights on from 0400 to 1800) and air-conditioned rooms. Animal room temperature and humidity controls were set at 21 to 24°C and 45 to 65%, respectively.

Statistical Analysis

The data in Tables 1 and 2 were analyzed statistically by Tukey's test for nonparametric multiple comparisons and the chi-square test, respectively. In all statistical tests, a difference was considered significant if $p<0.05$.

Results and Discussion

The percentage of motile cryopreserved spermatozoa from gene disrupted mice in the fertilization medium at insemination ranged from 40% to 50%. The motility of freshly collected spermatozoa (control) was over 80%. In our previous version of this sperm cryopreservation and *in vitro* fertilization, the thawed sperm suspension was diluted and subsequently the cryopreservation solution was removed by filtration from the diluted sperm suspention [8]. In the present study, to overcome the reduction of sperm motility brought about by the filtration as well as to reduce the time required to prepare the sperm suspension, 1 μl of thawed suspension was

Table 1. *In vitro* fertilization with frozen-thawed gene disrupted mice spermatozoa and subsequent embryo transfer

Gene disrupted mouse strain	Spermatozoa	No. of oocytes inseminated	No. (%) of oocytes fertilized and developed to 2-cell stage	No. of parturition/no. of recipients	No. of live young/no. of embryos transferred (%)
Et1^{tm1Csk}	Frozen-thawed	136	90 (71)[a]	3/5	29/90 (32)[a]
	Fresh	130	98 (75)[a]	—	—
Gk^{tm1Csk}	Frozen-thawed	137	106 (77)[a, c]	5/5	66/102 (65)[b]
	Fresh	440	380 (86)[b, c]	—	—

ICR oocytes were inseminated with spermatozoa from heterozygous gene disrupted mice. Values with the same superscripts are not significantly different in the same column at P>0.05.

Table 2. Results of Genotyping of young derived from frozen-thawed spermatozoa and their progeny test

Gene distrupted mouse strain	No. of heterozygotes/ no. of examined (%)	Progeny test			
		No. of mating pairs	No. of parturition	No. of homozygotes/ no. of young (%)	P=
Et1^{tm1Csk}	16/28 (57)	7	11	32/141 (23)	0.650
Gk^{tm1Csk}	17/36 (47)	5	12	44/135 (33)	0.148

directly added to the 200 μl of the HTF medium and allowed to *in vitro* fertilize without removal of the cryopreservation solution, as described in Materials and Methods. The negative effect of cryoprotectant for *in vitro* fertilization might be negligible in an approximately × 200 dilution of sperm suspension. In our preliminary experiments, when 2 or more μl of the thawed sperm suspension was added to the 200 μl of the fertilization medium, the fertilization rates were significantly lower than for 1 μl of the thawed sperm suspension (data not shown).

As shown in Table 1, when the oocytes were inseminated with freshly collected *Et1*^{tm1Csk} and *Gk*^{tm1Csk} spermatozoa at a final concentration of 150 cells/μl, 75% (98/130) and 86% (380/440) 'respectively' were fertilized and developed to the 2-cell stage at 24 h after insemination. In frozen-thawed *Et1*^{tm1Csk} and *Gk*^{tm1Csk} spermatozoa, the proportions of 2-cell stage embryos 24 h after insemination were 71% (90/136) and 77% (106/137), respectively, which was not significantly different from that of each unfrozen control (P>0.05). These results indicate that cryopreservation does not influence the fertilization capacity in spermatozoa from gene disrupted mice. When the 2-cell stage embryos which were fertilized with *Gk*^{tm1Csk} frozen-thawed spermatozoa were transferred to the recipients, 65% of them developed into young. In *Et1*^{tm1Csk} spermatozoa, however, only 32% of the transferred embryos developed to term. It was not clear whether this difference between *Gk*^{tm1Csk} and *Et1*^{tm1Csk} was caused by the difference in the targeted gene in this study. Two of 5 recipients did not reach delivery after term in *Et1*^{tm1Csk}. In the remaining 3 recipients, 54% (29/54) of the transferred embryos developed into young, so that the low efficiency (32%) of embryo transfer which was observed in *Et1*^{tm1Csk} might be caused by individual differences in the recipients rather than the targeted gene.

After weaning of these young, 57% (16/28) and 47% (17/36) of which were *Et1*^{tm1Csk} and *Gk*^{tm1Csk}, respectively, they were confirmed to be heterozygous mutant mice by means of Southern blot analysis (Table 2). Seven pairs of heterozygotes yielded 141 offspring in *Et1*^{tm1Csk}. Twenty-three percent of these were confirmed as homozygotes. In *Gk*^{tm1Csk}, 33% (44/135) of offspring derived from the mating of heterozygotes were homozygous animals. Results of progeny tests indicate that the mutated locus/loci were transmitted to the progeny in each gene disrupted mouse strain according to the Mendelian law of the inheritance of characteristics (Table 2).

The cryopreservation and the *in vitro* fertilization system described in this study affords a fast, relatively simple procedure, and a high fertilization rate. It has been reported that the combination of *in vitro* fertilization and embryo transfer techniques to control disease and breeding in mice is advantageous because many eggs can be fertilized from a single male at once and the day of birth can be easily controlled [18]. Furthermore the cryopreservation of spermatozoa may make the trans-

port of genetic material to the farthest parts of the world feasible. Therefore, cryopreservation of spermatozoa provides a useful alternative to embryo freezing for the maintenance and transport of gene disrupted mouse strains.

In conclusion, cryopreservation of spermatozoa in combination with *in vitro* fertilization is a promising technique for the maintenance and transport of gene disrupted mice colonies.

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