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Cryopreservation of Mouse Spermatozoa from Inbred and F_1 Hybrid Strains

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Mouse epididymal spermatozoa from inbred (BALB/c, C3H/He, C57BL/6N, CBA/JN and DBA/2N) and F_1 hybrid ($B6C3F_1$, BDF_1 and CDF_1) strains suspended in cryopreservation solution (18% raffinose and 3% skim milk in distilled water) were frozen and stored at -196℃. After thawing at room temperature, sperm motility and fertilizing ability were examined. Spermatozoa from all of the strains were successfully frozen, although the motility and the fertilization rates of frozen-thawed spermatozoa (the proportions of the fresh oocytes from Jcl : ICR strain which developed to pronuclear oocytes and 2-cell embryos after insemination by frozen-thawed spermatozoa) varied among strains (motility : 23% for C57BL/6N to 62% for DBA/2N ; fertilization rates : 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 recepients and 35-62% of 2-cell embryos developed into normal young.——KEY WORDS : cryopreservation, mouse spermatozoa

Recently, the freezing of mouse spermatozoa was reported by some authors [1,8,10,11,13], but the overall fertilizing ability of spermatozoa after thawing was low in these reports. We have previously demonstrated that frozen and thawed spermatozoa of Jcl : ICR mice maintain a high fertilizing ability if the sperm suspension is diluted slowly after thawing [6].

In the present study the fertilizing ability of mouse spermatozoa from five inbred and three F_1 hybrid strains was examined.

Materials and Methods

The sperm freezing and thawing method was essentially the same as that described previously [6,7,11].

Cryopreservation solution : Raffinose (18 %, W/V, WAKO, Tokyo, Japan) and skim milk (3%, W/V, DIFCO, Mich., USA) were dissolved in distilled water at 60℃. The solution was centrifuged at 10,000g for 15 min, and the supernatant was filtered (pore size 0.45 μm : Cat. No. SLHA025OS, Japan Millipore Limited, Tokyo, Japan) and used as the cryopreservation solution.

Freezing and thawing of mouse spermatozoa : Spermatozoa were collected from caudae epididymidis taken from 13-28-week-old BALB/c, C3H/He, C57BL/6N, CBA/JN, and DBA/2N inbred strains, and $B6C3F_1$ (C57BL/6N×C3H/He), BDF_1 (C57BL/6N×DBA/2N), and CDF_1 (BALB/c×DBA/2N) hybrid strains. Four caudae epididymidis were miniced in 400$\mu\ell$ of the cryopreservation solution in an organ tissue culture dish (Falcon 3037, Becton-Dickman Co.), and the spermatozoa were dispersed by shaking the dish at room temperature for about 2 min. The sperm suspension was divided into four samples, and each sample (≒100$\mu\ell$) was transferred into a sampling tube (volume 0.5mℓ : Cat. No. 72.699, Sarstedt, Nümbrecht, Germany). The samples were frozen as in previous reports [6,7,11] and were stored for 3-8 weeks before

thawing. At that time the samples were removed from the liquid nitrogen and thawed at room temperature for 5-7 min.

Dilutions of sperm suspension and removal of cryopreservation solution after thawing: The thawed sperm suspension was transferred gently into the bottom of 0.4mℓ of HTF medium [9] in an organ tissue culture dish (FALCON 3037), and the dish was placed in a 37℃ incubator for 30 min without shaking, so that the sperm suspension was diluted slowly and sperm were allowed to swim into the HTF medium. After dilution, the cryopreservation solution was removed from the sperm suspension by filtration [11], and the samples were incubated for 1.5 h at 37℃ under 5% CO_2 in air, and used for insemination (sperm concentration: 2,000-4,000 cells/$\mu\ell$).

Assessment of sperm motility: After 1.5 h incubation, a sample of the sperm suspension (10$\mu\ell$) was placed on a warm slide (37℃) under a coverslip and examined under a microscope (×100). The motility was expressed as the percentage of actively motile spermatozoa [10].

Assessment of sperm fertilizing and developmental ability: The procedures for fertilization *in vitro* were essentially those described by Toyada *et al.* [12]. Female Jcl: ICR mice (8-10 weeks old) were injected with 5 i.u. each of PMSG and hCG at an interval of 48 h. The oocytes were collected from the excised oviducts 15-17 h after hCG injection, introduced into the HTF medium containing the thawed spermatozoa, and cultured for 24 h. At 6 h and 24 h after insemination, the oocytes were observed under an inverted microscope, and the pronuclear oocytes (the oocytes with two pronuclei and a second polar body) and 2-cell embryos were recorded each time. The oocytes which developed to 2-cell embryos were transferred to the oviducts of pseudopregnant females on the day of finding the vaginal plug (day 1 of pseudopregnancy).

Results

Table 1 compares the motility of thawed spermatozoa among strains. The mean motility of frozen and thawed spermatozoa at insemination ranged from 23% to 62%. The results of the examination of the oocytes 6 h and 24 h after insemination by cryopreseved

Table 1. The motility of thawed mouse spermatozoa at the time of insemination

Strain	Motility (%)*
BALB/c	33 ± 2
C3H/He	41 ± 3
C57BL/6N	23 ± 2
CBA/JN	40 ± 3
DBA/2N	62 ± 3
$B6C3F_1$ [a]	44 ± 2
BDF_1 [b]	44 ± 3
CDF_1 [c]	44 ± 5

a) C57BL/6N(♀)×C3H/He(♂) b) C57BL/6N(♀)×DBA/2N(♂) c) BALB/c(♀)×DBA/2N(♂) * Mean±s.e.m. percentages in 3 experiments for each strain.

Table 2. Fertility of the cryopreserved mouse spermatozoa assessed by *in vitro* fertilization

Strain	No. of oocytes examined	No. of oocytes ferilized (%)	No. of oocytes developed to 2-cell embryos (%)
BALB/c	141	67 (47.5)	66 (46.8)
C3H/He	131	96 (73.3)	96 (73.3)
C57BL/6N	157	40 (25.5)	40 (25.5)
CBA/JN	119	91 (76.5)	89 (74.8)
DBA/2N	136	121 (88.9)	121 (88.9)
$B6C3F_1$	118	70 (59.3)	70 (59.3)
BDF_1	126	94 (74.6)	94 (74.6)
CDF_1	144	110 (76.4)	108 (75.0)

Data of 3 experiments for each strain were combined.

Table 3. Development into live young of 2-cell embryos from oocytes fertilized by frozen-thawed mouse spermatozoa after transfer to pseudopregnant recipients

Strain	No. of recipients used	No. of embryos transferred	No. of recipients which delivered live young	No. of live young		
				♀	♂	total(%)
BALB/c	4	57	3	11	9	20(35.1)
C3H/He	6	96	6	19	30	49(51.4)
C57BL/6N	3	40	2	8	6	14(35.0)
CBA/JN	5	89	4	20	23	43(48.3)
DBA/2N	6	96	5	24	35	59(61.5)
B6C3F$_1$	5	70	5	18	21	39(55.7)
BDF$_1$	5	94	5	25	27	52(55.3)
CDF$_1$	6	108	6	35	24	59(54.6)

Data of 3 experiments for each strain were combined.

spermatozoa are summarized in Table 2. The proportions of the oocytes from Jcl : ICR which developed to pronuclear oocytes and 2-cell embryos 6 h and 24 h after insemination by the cryopreserved spermatozoa was almost the same in each group and was in the range of 26 to 89%. The motility and the fertilizing ability of the cryopreserved C57BL/6N spermatozoa were the lowest, and those of cryopreserved DBA/2N spermatozoa were the highest among the strains used. The development to live young after transfer of 2-cell embryos fertilized *in vitro* by the cryopreserved spermatozoa is shown in Table 3. Nearly all 2-cell embryos were transferred to pseudopregnant recipients and 35 to 62% of the 2-cell embryos developed to normal young.

Discussion

The present study shows that spermatozoa from 5 inbred and 3 F$_1$ hybrid mouse strains can be successfully cryopreserved, and that the 2-cell embryos resulting from fertilization by these frozen and thawed spermatozoa can develop into normal, live young after transfer.

Tada *et al.* [10] reported that spermatozoa from one outbred and 5 inbred strains of mice were successfully frozen in the presence of 18% raffinose and 1.75% glycerol by the pellet method [3]. In this study, the fertilizing ability of cryopreserved mouse spermatozoa after being thawed with the slow dilution method [6] was greater on the whole than that reported by Tada *et al.* But the fertilizing ability of frozen-thawed spermatozoa of C57BL/6N mice was very low (25.5%), as in their report (12.9-35.4%). In producing transgenic mice, zygotes from C57BL/6 or its F$_1$ hybrid strains are generally used for microinjection [2], and a large number of transgenic mouse strains have been produced in many laboratories. Therefore, it is necessary to establish the cryopreservation of embryos and gamates of C57BL/6 strains for maintenance of the transgenic mice produced. The embryos and oocytes of the C57BL/6N mouse strain can be cryopreserved successfully by the simple technique of vitrification [4,5], but the fertilizing ability of C57BL/6N mouse spermatozoa after freezing and thawing was the lowest in the inbred strains at this time, even though the fresh C57BL/6N mouse spermatozoa had high motility and fertilizing ability (Data not shown). The sensitivity of the spermatozoa to freezing and thawing may be different among strains, and the low fertilizing ability of cryopreserved C57BL/6N spermatozoa may be due to the low motility at the time of insemination (Table 1). We found that the lower the sperm motility, the fewer were the oocytes which developed to pronuclear oocytes and 2-cell embryos. Further efforts are needed to enhance the fertilizing ability of C57BL/6N spermatozoa after freezing and thawing.

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各種系統マウス精子の凍結保存

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18%ラフィノース＋3%スキムミルクを保存液とし，5系統の近交系（BALB/c，C3H/He，C57BL/6N，CBA/JNおよびDBA/2N）と3組の近交系間交配 F_1（B6C3F_1（C57BL/6N×C3H/He），BDF$_1$（C57BL/6N×DBA/2N）および CDF$_1$（BALB/c×DBA/2N）雄マウスの精巣上体尾部精子を−196℃に凍結保存した。融解後の精子の受精能は，ICR系マウス未受精卵との間で体外受精を行なうことにより検討した。各種凍結マウス精子の活力および受精率（2細胞期へ発生した卵子の割合）は，系統によりかなりのばらつきが見られた（精子活力：23-62%，受精率：26-89%）。また，2細胞期胚の移植により，移植胚の35-62%が新生児へ発生した。