A new method for cryopreservation of mouse spermatozoa

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A new method for the cryopreservation of mouse spermatozoa was developed using a modified egg-yolk TES–Tris diluent containing 0.1% sodium lauryl sulfate and 1.25% (v/v) glycerol (mouse sperm cryoprotectant, MSC). Epididymal spermatozoa collected from 10-week-old CBA males were frozen at a rate of $5^{\circ}\text{C min}^{-1}$ to 4°C and $50^{\circ}\text{C min}^{-1}$ to -70°C using a programmable cell freezer. A percentage of the spermatozoa (25%) regained motility after thawing. *In vitro* fertilization with frozen–thawed spermatozoa resulted in 50% of oocytes developing to the two-cell stage. These two-cell embryos were placed in the oviducts of pseudopregnant recipients (C57BL/CBA) and 16% developed to be viable fetuses, or in the oviducts of pregnant recipients (MF1) and 17% developed to live offspring.

Introduction

In contrast to the cryopreservation of mouse embryos (Whittingham *et al.*, 1972) and oocytes (Whittingham, 1977), the only report of successful cryopreservation of mouse spermatozoa was until recently the unpublished findings by Rapatz and Zimmerman (see Graham *et al.*, 1978). These investigators artificially inseminated two groups of mice; 106 females with spermatozoa cryopreserved in skimmed milk and fructose diluent which resulted in the birth of nine young, and 84 females with spermatozoa cryopreserved in a skimmed milk and fructose diluent containing 1% BSA and 4 mmol calcium chloride l^{-1} , which resulted in the birth of 61 young. With the dramatic increase in the use of transgenic mice there is now a greater urgency to develop methods for cryopreservation of male and female germ cells efficiently and this has led to renewed interest in improving mouse sperm cryopreservation. Tada *et al.* (1990), Yokoyama *et al.* (1990) and Okuyama *et al.* (1990) have indicated that mouse spermatozoa (from several strains) frozen in various combinations of raffinose and glycerol will regain motility after thawing, and when used in combination with *in vitro* fertilization and embryo transfer, live offspring have been produced. In this laboratory, despite repeated trials, we have been unable to obtain fertile mouse spermatozoa using these protocols. However, on screening other potential diluents with a cryomicroscope (L.M. Penfold, unpublished), consistent survival of mouse spermatozoa was obtained following freezing and thawing with a low (1.25% v/v) glycerol and egg yolk cryoprotectant.

Here, we report on the use of modified egg-yolk diluent containing glycerol and sodium lauryl sulfate (SDS) and designated mouse sperm cryoprotectant (MSC) medium. A proportion of

spermatozoa from CBA mice suspended in this diluent retained motility after freezing and thawing and could be recovered from the medium for subsequent *in vitro* fertilization.

Materials and Methods

Animals

Three-week-old, C57Blk/6 $\times$ CBA/CA F_1 females were used as oocyte donors and 10-week-old CBA/CaBlk males as sperm donors (Bantin and Kingman Ltd, Grimston Aldrough, Hull).

Eight- to twelve-week-old C57Blk/6 $\times$ CBA/CA F_1 females and outbred MF1 females (Harlan Olac Ltd, Shaw's Farm, Bicester) were used as foster recipients. Animals were housed at $22^{\circ} \pm 2^{\circ}\text{C}$, 50% relative humidity, 14 h light:10 h dark cycle and fed on Rat and Mouse diet no. 1 (SDS, Witham, Essex) with continuous access to water. When necessary, females were mated with bilaterally vasectomized CBA/CaBlk males so that they became pseudopregnant. Ten-week-old males were anaesthetized with an i.p. injection of 0.015–0.017 ml 2.5% Avertin g^{-1} (Aldrich Chemicals, Gillingham) of body weight. Under sterile conditions, a midline abdominal incision was used to expose the vasa deferentia which were ligated and cut. The wound was closed with suture in the normal manner. These males were carefully checked for sterility before use.

Mouse sperm cryoprotectant (MSC) medium

Screening of various potential cryoprotectant solutions for mouse spermatozoa with the cryomicroscope (Penfold *et al.*, unpublished) led to the formulation of MSC medium consisting of 21.07 mmol *N*-tris [hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES) l^{-1} , 9.49 mmol tris l^{-1} , 2.22 mmol glucose l^{-1} , 1.25% v/v glycerol, 25% v/v egg yolk, 0.1% sodium lauryl sulfate (SDS) and 0.000125% streptomycin. The medium was prepared by the following method. Double distilled, deionised water (12 ml) was added to 4 ml egg yolk and 0.1% sodium lauryl sulfate. The solution was mixed thoroughly and left for

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30 min before centrifuging at 20 000 *g* for 1 h. To 8 ml of supernatant was added 0.483 g TES, 0.115 g Tris, 0.04 g glucose, 0.01 g streptomycin and 100 μ l glycerol. The final osmolarity was approximately 675 mosmol. Cryoprotectant was stored at 4°C for up to 3 days.

Freezing protocol

Four adult male mice (10-weeks-old) were killed by cervical dislocation and their caudae epididymides without vasa deferentia were removed and placed into 1 ml of cryoprotectant at 30°C. Spermatozoa were released by cutting the epididymides and agitating gently. The spermatozoa were dispersed throughout the cryoprotectant by gently pipetting up and down using a Pasteur pipette for 30 s. Aliquots, 200 μ l, were transferred to 1.8 ml polypropylene cryotubes (Nunc, Paisley) which were then placed in a programmable cell freezer (R204, Planer Products Ltd, Sunbury) at 30°C. Preliminary studies using the cryomicroscope indicated that the optimal freezing regimen consisted of an initial rate of 5°C min⁻¹ to 4°C, then 50°C min⁻¹ to -70°C. The cryotubes were subsequently immersed directly in liquid nitrogen and transferred to a storage vessel for a minimum of 24 h.

Thawing protocol

Cryotubes were immersed in water at 37°C until the medium thawed and the sample was transferred to an incubator at 37°C, 5% CO₂ and equilibrated for 10 min. Most of the MSC medium was removed by centrifuging for 4 min at 800 *g*, removing the supernatant and resuspending the pellet in 500 μ l T6 medium (Quinn *et al.*, 1982) containing 15 mg BSA ml⁻¹ (fraction V, Sigma Chemical Co., St Louis). The sperm suspension was placed in an incubator at 37°C in an atmosphere of 5% CO₂ in air. The MSC supernatant was subjected to a further centrifugation at 12 000 *g* for 5 min to remove any residual spermatozoa and used as a negative control (see below).

In vitro fertilization experiments

C57BL/CBA F₁ females were superovulated for use as oocyte donors and CBA males were used as donors of fresh and frozen spermatozoa. *In vitro* fertilization followed a standard procedure with animals and media prepared according to the methods of Glenister *et al.* (1987). Oocytes were collected 14 h after hCG injection in T6 medium under oil. Frozen-thawed spermatozoa (prepared as described above) were diluted to a concentration of 1×10^6 motile cells ml⁻¹ in 300 μ l T6 medium plus BSA under oil and left to capacitate for 2 h from the time of thawing before they were incubated with fresh oocytes (within cumulus) for 4 h under oil. Oocytes were then washed three times in M16 medium containing 4 mg BSA ml⁻¹ before they were incubated overnight at 37°C in 5% CO₂ in air in 100 μ l of M16 medium. Oocytes were incubated with fresh (unfrozen) spermatozoa diluted in T6 medium containing 15 mg BSA ml⁻¹ and transferred to M16 medium containing 4 mg BSA ml⁻¹ before incubating overnight as a positive control. In addition, oocytes were incubated with medium alone or with medium containing 50 μ l MSC supernatant recovered following centrifugation of the frozen-thawed spermatozoa as described above.

Table 1. Development to two-cell of mouse oocytes incubated with fresh or frozen-thawed spermatozoa

Experiment	Number of oocytes	Number of two-cell embryos	% $\pm$ SEM
Frozen-thawed spermatozoa	708	355	50 $\pm$ 4.9*
Fresh spermatozoa	490	375	77 $\pm$ 3.7
Cryoprotectant control	324	56	17 $\pm$ 3.6

*Significantly different from fresh spermatozoa ($P < 0.05$).

Embryo transfer experiments

After fertilization with frozen-thawed or fresh spermatozoa and incubation *in vitro* for 24 h, two-cell embryos (six to ten) were transferred to the oviducts of day 1 pseudopregnant (C57BL/CBA) and pregnant (MF1) females according to the methods of Glenister *et al.* (1987).

On day 15 of pregnancy (day of transfer, day 1), C57BL/CBA females were killed by cervical dislocation and implantation sites and fetuses counted. MF1 females were allowed to go to term and brown offspring were distinguished from the natural albino litter.

Results

Preparation of spermatozoa

A high percentage (80%) of epididymal spermatozoa in the MSC medium displayed lateral tail beating but no progressive motility before freezing. This was considered to be due to the relatively high osmolarity of the medium (650–700 mosmol) affecting the pattern of flagellum beat. After freezing and thawing and then removal from the cryoprotectant, about 25% of spermatozoa exhibited progressive motility.

In vitro fertilization with fresh and frozen spermatozoa

Of the oocytes incubated with frozen-thawed spermatozoa 50 $\pm$ 4.9% developed to the two-cell stage compared with a rate of 77 $\pm$ 3.7% for oocytes with fresh spermatozoa (Table 1). Oocytes not inseminated with spermatozoa, but incubated in the presence of cryoprotectant diluted with T6 medium, showed an activation rate to two-cell of 17 $\pm$ 3.6% compared with a background activation rate of $\leq$ 2% for oocytes incubated with medium alone. Of the oocytes incubated with frozen-thawed spermatozoa (Table 2), 39 $\pm$ 4.9% developed to the morula/blastocyst stage compared with 56 $\pm$ 4.5% for oocytes incubated with fresh spermatozoa (Table 2). None of the activated control oocytes developed beyond the two-cell stage.

In pseudopregnant females, the implantation rate was 36 $\pm$ 0.71% and the fetal development rate 16 $\pm$ 0.25% for oocytes fertilized with frozen-thawed spermatozoa (Table 3). Seventeen per cent of embryos instilled in pregnant foster mice

Table 2. Development of mouse embryos to morula/blastocyst stage following *in vitro* fertilization with fresh and frozen-thawed spermatozoa

Experiment	Number of oocytes	Number of two-cell embryos	Number of morulae/blastocysts	% $\pm$ SEM
Frozen-thawed spermatozoa	312	159	121	39 $\pm$ 4.9*
Fresh spermatozoa	324	249	181	56 $\pm$ 4.5

*Significantly different from fresh spermatozoa ($P < 0.05$).

Table 3. Oviductal transfer of two-cell mouse embryos following *in vitro* fertilization with fresh and frozen-thawed spermatozoa

Experiment	Number of embryos transferred	Number implanted	Number of fetuses or liveborn (%)
Frozen-thawed spermatozoa			
Pseudopregnant	98	35 (36 $\pm$ 0.71)*	16 (16 $\pm$ 0.25)
Pregnant	58		10 (17 $\pm$ 0.53)
Fresh spermatozoa			
Pseudopregnant	93	63 (68 $\pm$ 0.95)	34 (37 $\pm$ 0.52)

*Significantly different from fresh spermatozoa ($P < 0.05$).

resulted in live offspring. For oocytes fertilized with fresh spermatozoa, an implantation rate of $69 \pm 0.95\%$ and a fetal development rate of $37 \pm 0.52\%$ were obtained.

Offspring derived from oocytes fertilized with frozen-thawed spermatozoa were mated to check for fertility. Both male and female offspring had fertile matings resulting in normal litter sizes.

Discussion

After freezing and thawing in MSC medium, a proportion of spermatozoa from CBA mice retained their motility and fertilizing capacity. *In vitro* fertilization with cryostored spermatozoa resulted in the formation of viable two-cell embryos which when transferred to suitable recipients developed to form normal fetuses and live offspring. This technique was consistently successful, although the final number of offspring produced from each batch of *in vitro* fertilization was low. In contrast, in this laboratory (L. M. Penfold, unpublished observation) it has not been possible to obtain fertile spermatozoa cryopreserved using raffinose-glycerol diluent (Tada *et al.*, 1990; Yokoyama *et al.*, 1990; Okuyama *et al.*, 1990).

Several characteristics of the MSC medium probably contribute to the survival of mouse spermatozoa during the freezing and thawing process. First, compared with other cryo-diluents, the relatively low glycerol concentration may have minimized any possible toxic effect on mouse spermatozoa, while still exerting an antifreeze action by sequestering free intracellular

water (Mazur *et al.*, 1970). Second, the egg yolk probably protects the spermatozoa during freezing by binding to the sperm plasmalemma (see Watson, 1975, 1981). With the electron microscope, we observed egg-yolk lipid closely associated with the sperm membrane (unpublished observation). This cryoprotective action of egg yolk during cooling and freezing has been well documented by Kampschmidt *et al.* (1953); Blackshaw (1954) and Pace and Graham (1974). The hyperosmolarity of the cryoprotectant may also have contributed to the survival of spermatozoa by removing intracellular water available for potential lethal ice formation. As a result, the spermatozoa were not progressively motile in the cryoprotectant, although they still moved from side to side. SDS was included in the cryoprotectant to solubilize the egg yolk and allow the recovery of the spermatozoa from the cryoprotectant after thawing. If during preparation of the cryoprotectant, SDS was added before the egg yolk was centrifuged, more motile spermatozoa survived freezing than when cryoprotectant was prepared by adding SDS after the egg yolk had been centrifuged (L. M. Penfold, unpublished observation). This would indicate that, in this instance, the detergent was not acting directly on the sperm plasmalemma, but was solubilizing the protective lipids in the egg yolk (Helenius and Simons, 1975; Arriola and Foote, 1987). A high level of egg activation occurred when unfertilized oocytes were mixed with cryoprotectant recovered from the freezing preparation. Exposure to cryoprotectant has been demonstrated to activate oocytes (Shaw and Trounson, 1989) in some but not all cases (Whittingham, 1977). Thus, residual cryoprotectant or products from dead spermatozoa present in the frozen-thawed spermatozoa sample at the time of

fertilization might have induced premature activation of some oocytes, thereby preventing fertilization. This would account for the failure of some embryos to develop beyond the two-cell stage. This activation rate could be reduced by further removal of the cryoprotectant with additional centrifugation. However, this also led to increased damage to the frozen-thawed spermatozoa and thus poor recovery of motile cells.

Fewer oocytes were fertilized after incubation with frozen-thawed spermatozoa than for those incubated with fresh spermatozoa. Although the concentration of spermatozoa added to the fertilization drop was adjusted for motility to $1 \times 10^{-6} \text{ ml}^{-1}$, dead spermatozoa present in the sample may have impeded viable spermatozoa from reaching the oocyte or may have reduced the survival rate of spermatozoa. As yet we have not developed a method for the elimination of dead spermatozoa. Swim-up methods proved ineffectual owing to the high numbers of immotile cells. Although the proportion of embryos that implanted was less for eggs fertilized with frozen-thawed spermatozoa than for those fertilized with fresh spermatozoa, post-implantation losses were similar for both groups (54% versus 46%). It is anticipated that the cryopreservation of mouse spermatozoa in conjunction with the established techniques for oocytes and embryos will provide greater flexibility for genetic management of laboratory mouse strains and lead to new methods for cryopreservation of spermatozoa in other rodent species.

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