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Comparison of Permeating and Nonpermeating Cryoprotectants for Mouse Sperm Cryopreservation

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Mouse sperm has proven to be more difficult to cryopreserve than sperm of other mammalian species. Published reports show that only three cryoprotectant agents (CPAs), alone or combined, have been studied: glycerol and dimethyl sulfoxide (DMSO), as permeating agents, and raffinose, as a nonpermeating agent. To date, the most consistent results for mouse sperm cryopreservation have been achieved by use of raffinose/skim milk as cryoprotectant with rapid cooling at 20°C per minute. In this study, we compared the cryoprotection provided by permeating (glycerol, formamide, propanediol, DMSO, adonitol) or nonpermeating (lactose, raffinose, sucrose, trehalose, D-mannitol) compounds for freezing mouse sperm. Different solutions were made using 3% skim milk solution as the buffer or extender in which all different cryoprotectant agents were dissolved at a concentration of 0.3 M, with a final osmolality of approx. 400 mOsm. Sperm samples from CB6F1 (hybrid) and C57BL/6J (inbred) mice collected directly into each CPA were frozen/thawed under identical conditions. After thawing and CPA elimination (centrifugation) raffinose (59%), trehalose (61%), and sucrose (61%) sustained the best motility ($P = < 0.1$) of the nonpermeating agents, whereas the best of the permeating agents was DMSO (42%). Membrane integrity was analyzed and showed that the simple exposure (prefreeze) to sugars was less harmful than the exposure to glycols. Coincidentally, sperm frozen in trehalose (41%), raffinose (40.5%), and sucrose (37.5%) were the samples less injured among all different postthawed CPA tested. The *in vitro* fertilization results demonstrated that hybrid mouse spermatozoa frozen with sugars (lactose 80%, raffinose 80%, trehalose 79% of two-cell embryos production) were more fertile than those frozen with glycols (glycerol 11%).

The reviews on sperm preservation by A. U. Smith (32) and J. K Sherman (31) trace the first attempts at conservation at low temperatures to as early as 1776. In 1938 Luyet and Hodapp (13) published the first report of sperm vitrified in liquid air (−192°C); for that experiment the authors used frog spermatozoa dehydrated by a 2 M sucrose solution. Later, Luyet and Keane (14) reported that the same procedure was not successful for rat sperm. They also found that a single exposure of sperm to hypertonic solutions was enough to kill 40% of cells; however, nearly 100% of those that survived exposure to sucrose also survived the vitrification. In 1949 Polge and collaborators (28) discovered that glycerol protects fowl and human spermatozoa exposed to low temperatures. Since then, sperm from a variety of mammals have been successfully frozen as a result of the addition of this "cryoprotectant."

Successful mouse sperm cryopreservation was reported in 1990 (23, 40, 41, 44). From reports that have been published since, only three cryoprotectant agents (CPAs), alone or combined, have been studied. Two of them have properties that facilitate their permeation through the cell membrane: glycerol and dimethyl sulfoxide (DMSO). The other one is raffinose, a trisaccharide classified as a nonpermeating compound. Up to now, more consistent results with mouse sperm cryopreservation have been obtained by use of the raffinose/skim milk combination (1, 15, 19–23, 38, 39, 44). Glycerol, the commonly used sperm cryoprotectant, did not provide the expected protection for mouse sperm when used at the usual concentration (5 to 10%). However, glycerol at low concentration (± 1.7%) combined with egg yolk

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and raffinose has been shown to protect mouse sperm during freezing (26, 34, 35).

Other mouse sperm cryopreservation studies such as those investigating osmotic tolerance (3, 10, 21, 35, 48), collection temperature (38, 43), or mechanical damage (11, 12, 40) have been done with solutions containing either glycerol or raffinose. Therefore, since the optimal working conditions were not similar among those studies, their results cannot be directly compared.

The goal of this project was to demonstrate that cryoprotection for mouse sperm can be achieved without the use of a permeating cryoprotectant. With the freezing protocol elaborated for the 18% raffinose–3% skim milk (38) cryoprotectant solution as reference for the present study, we analyzed the degree of cryoprotection that different permeating and nonpermeating compounds provided to mouse sperm.

A hypothesis of the difference seen between the nonpermeating and the permeating compounds is that whereas they both bring about dehydration of the spermatozoon (necessary for successful cryopreservation), it may be that the mouse spermatozoa does not tolerate the intracellular presence of permeating cryoprotective compounds as well as spermatozoa from other species. Maybe this has something to do with the fact that mouse spermatozoa have a smaller fraction of water content than other mammalian sperm.

MATERIALS AND METHODS

Sperm Donors

Inbred C57BL/6J male mice were obtained from the Animal Resources colonies at The Jackson Laboratory and hybrid CB6F1/J (BALB/cBy × C57BL/6By) mice from our own colony. Mice were maintained under routine husbandry procedures according to standards set forth in the Guide for the Care and Use of Laboratory Animals (5). They include a light–dark cycle of 14 h light and 10 h dark. Males were housed singly or at maximum of two per cage at least 5 days before the experiment. Food and water were provided *ad libitum*.

Cryoprotectant Solution

The cryoprotectant solution was prepared using 3% skim milk as the buffer or extender in which all different cryoprotectant agents were tested. The CPAs used in this study were classified as permeating (acetamide, glycerol, formamide, propanediol, dimethyl sulfoxide, adonitol, and perseitol) or as nonpermeating (raffinose, lactose, sucrose, trehalose, and mannitol) (tissue culture grade; Sigma).

The 3% skim milk (skim milk dehydrated, Bacto Difco; Becton–Dickinson) was dissolved in culture-grade water (Millie Q Plus PF system) and centrifuged for 30 min at 16,000*g* in a microcentrifuge (Eppendorf; Model 5410). The supernatant was removed and filtered through a 0.22-μm pore. This clear solution was stored at 4°C in a refrigerator for no more than 2 weeks. All sugars and polyols tested in this study were dissolved in this solution at 0.3 M final concentration. For complete dissolution of the sugars, the solution was warmed (~60°C), centrifuged at 10,000*g* for 10 min, and filtered again. All skim milk solutions containing CPAs had a final osmolality between 380 and 450 mOsm.

To analyze whether the 3% skim milk, the lactose that the skim milk contained, or the 18% raffinose alone has cryoprotectant activity, sperm samples were frozen in 3% skim milk solution, in 0.4 M lactose (0.4 M to obtain the correct osmolality), or in 0.3 M raffinose dissolved in phosphate-buffered saline (PBS).

Sperm Collection

Sperm were collected from 3- to 5-month-old CB6F1 and C57BL/6J males. After an animal was euthanized, both epididymides and vas deferentia were removed and placed into a 35-mm sterile plastic dish (Falcon 1008; Becton–Dickinson) containing 1 ml of CPA. Each cauda epididymis with vas deferens from one animal was combined with that from another animal to diminish individual variation. The tissue was cut five to seven times with the edge of a 30-g injection needle directly in the CPA equilibrated at 37°C, and sperm were allowed to "swim out" for 10 min.

Freezing and Thawing Procedures

Sperm samples collected into different cryoprotectants were frozen and thawed using the same protocol (38). Briefly, vials (1.8-cc Nunc Cryotubes) with 100-μl sperm aliquots collected at 37°C were frozen by exposure to liquid (LN_2) vapors for 10 min (approx. −20°C) per minute) before storage under LN_2. Samples were rapidly thawed by transfer from LN_2 into a 37°C water bath until the ice melted. Subsequently, the sample was centrifuged at 735*g* for 4 min. The supernatant (cryoprotectant) was discarded and replaced either with 50 μl of human tubal fluid (HTF) (29) for *in vitro* fertilization (IVF) or with bovine serum albumin (BSA)–Hepes-buffered saline for fluorescence microscopy. The sperm sample was gently resuspended by tapping the tube with fingertips and incubated for 10 min at 37°C to allow a minimal selection by "swim up".

Sperm Analysis

Sperm samples—control (collected in HTF or Hepes–BSA), prefreeze (collected in CPA), and postthawed—were analyzed using a Hamilton Thorn IVOS computerized semen analyzer. Parameters of concentration (total number of cells per milliliter), motility, and progressive motility were determined in each sample. Motility was defined as the percentage of spermatozoa that showed any movement of the sperm head. Progressive motility was the percentage of spermatozoa that moved with a linear velocity greater than 50 μm per second and whose straightness, derived from the ratio of absolute straight-line velocity to average path velocity, was not less than 50%.

Live/Dead Fluorescence

The cell membrane integrity was measured by the fluorescent staining method for determining sperm viability (Live/Dead Sperm Viability Kit; Molecular Probes). Live spermatozoa with intact cell membranes fluoresce bright green with the membrane-permeant nuclei acid stain SYBR 14, whereas dead cells or cells with damaged membranes fluoresce red with propidium iodide. Control samples were collected directly into Hepes–BSA buffer, as the staining protocol required. Prefreeze and frozen/thawed sperm were centrifuged 4 min at 750*g*; the supernatant was removed and replaced with Hepes–BSA buffer before being stained. After being stained, 15 μl of sperm sample was placed onto a microscope slide and covered with a cover slip. A Nikon Diaphot microscope with an epifluorescence system was used for observation.

In Vitro Fertilization

The 20- to 23-day-old CB6F1 hybrid females were superovulated by intraperitoneal injection of 2.5 IU of PMSG (Sigma), followed by 2.5 IU of HCG (Sigma) 48 h later. At 13 h later, the females were euthanized and their oviducts were removed. The oocyte–cumulus complexes were isolated in a sterile culture dish containing 2 ml of HTF medium.

Forty microliters of the thawed sperm sample, with a minimum count of 1 million progressively motile sperm per milliliter, were added to a 250-μl drop of HTF covered with light mineral oil (Sigma; embryo tested). The oocyte–cumulus complexes collected from three to five females were placed into each fertilization drop containing sperm that had been incubated for at least 10 min. Dishes were placed into a sealed modular incubator chamber (Billups–Rothenberg) gassed with 5% CO_2, 5% O_2 balanced in 90% N_2 and maintained in the incubator at 37°C for 5 h. After that time, eggs were washed to eliminate excess sperm and then cultured overnight in a 250-μl drop of HTF under the same conditions. The following morning the two-cell embryos were counted and either were transferred to a 200-μl drop of KSOM/AA medium (8) for culture until the blastocyst stage or were surgically transferred at the two-cell stage into a surrogate mother for *in vivo* evaluation. For each strain, an IVF control was done using only 10 μl of the 1-ml sample of fresh sperm collected in HTF. Fertility was considered the percentage of two-cell embryos produced by IVF scored 24 h after insemination.

Embryo Transfer

Forty-five two-cell embryos from each group selected for *in vivo* evaluation were surgically

transferred into the infundibulum of three pseudopregnant CB6F1 females. In most cases a maximum of 15 embryos per recipient were transferred using both uterine horns. Embryos were allowed to develop to term and the number of pups born was recorded.

Statistics

The arcsine transformation values of motility and progressive motility percentages were evaluated by repeated-measures ANOVA test. The analysis was corroborated by Dunnett's test for comparison of a control group (raffinose) with all the experimental groups.

Fertility, considered the percentage of two-cell-stage embryos scored 24 h after insemination, was compared by paired Student's *t* test and expressed as the mean ± SE. Differences were considered significant when $P < 0.01$. All analyses were carried out using the GraphPad prism version 2.0 computer program (GraphPad Software).

RESULTS

Effect of CPA Composition on Sperm Motility

The sperm motility rate before freezing varied with the composition of the CPA in which it was collected. For both strains, sperm freshly collected in any of the sugars (nonpermeating) showed greater motility ($P < 0.01$) than sperm collected in all of the permeating CPAs used in the study. Although the postthaw motility was reduced for all groups, spermatozoa had greater motility when frozen in sugars than when frozen in glycols. Sucrose, slightly (but not statistically significantly) better than trehalose and raffinose, produced the highest motility (Figs. 1a and 1b). Sperm did not survive freezing when protected by perseitol or mannitol; 4 M lactose and 3% skim milk alone had very low protective activity.

The results for progressive motility (Figs. 2a and 2b) were similar to those for motility. The prefreeze values were higher in the sugar ($P < 0.01$) than in the glycol groups. Although the progressive motility rates obtained for all groups diminished abruptly upon postthaw evaluation, sugars retained the highest rates. Progressive motility was slightly higher in sucrose than in raffinose or trehalose ($P < 0.05$) for CB6F1 hybrid sperm, but not for C57BL/6J sperm in which DMSO produced progressive motility levels statistically similar ($P > 0.05$) to those of raffinose and trehalose.

Cryoprotectants Evaluated by Live/Dead Fluorescence

Sperm collected directly into different CPA solutions showed no significant decrease in motility after 10-min exposure. The motility and integrity of freshly collected spermatozoa were similarly maintained in the sugars and in glycerol, but not in the rest of the glycols ($P < 0.05$).

The postthaw evaluation showed that raffinose and trehalose provided the best protection for hybrid sperm. However, trehalose was less effective than raffinose in protecting C57BL/6J sperm. In general, glycols were less effective than sugars in protecting the spermatozoa membrane integrity, and from that group DMSO was better than glycerol ($P < 0.05$) (Figs. 3a and 3b).

In Vitro Fertilization

The fertilization rates achieved with hybrid sperm frozen in lactose, raffinose, trehalose, and glycerol are presented in Fig. 4.

The frozen/thawed hybrid sperm cryoprotected with lactose (80.4% (626/776)), raffinose (80% (591/736)), or trehalose (79% (476/600)) retained significantly better fertility ($P < 0.001$) than the sperm frozen in glycerol (11% (27/244)). For C57BL/6J sperm, trehalose (9% (54/541)) supported fertilization best among the sugars ($P < 0.01$), and lactose (4% (26/623)) and raffinose (2% (14/493)) were better than glycerol (0% (1/521)).

Embryo Transfer

When embryos derived from hybrid sperm frozen in raffinose or lactose in skim milk were transferred into pseudopregnant recipients, the percentage of offspring born was the same as that for the unfrozen control (56%); those derived from sperm frozen in sucrose and

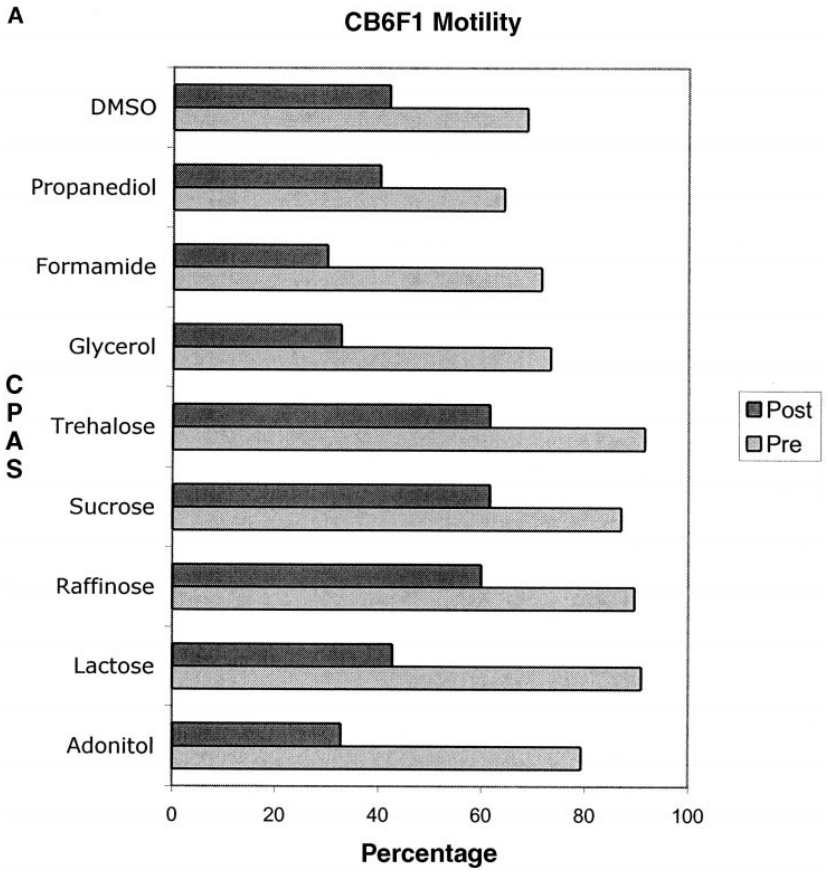

FIG. 1. CB6F1(a) and C57BL/6J (b) prefreeze and postthaw motility of sperm exposed to permeating and nonpermeating cryoprotectants at the same molal solution. The count was performed by computer-assisted sperm analysis.

trehalose resulted in 42 and 33% live born, respectively.

DISCUSSION

Rodent sperm has proven to be more difficult to cryopreserve than other mammalian sperm. Rat sperm cryopreservation has not yet been accomplished and mouse sperm cryopreservation was reported only 10 years ago. The studies published then, by Takeshima *et al.* (42), Tada *et al.* (41), Okuyama *et al.* (24), and Yokoyama *et al.* (45), followed different approaches concerning the CPA selected for freezing mouse sperm: glycerol or DMSO combined with raffinose or raffinose alone.

Difficulties in reproducing the original results (4, 26) inspired modifications (21, 26, 33) to protocols that make freezing generally more reliable but still not equally successful for all mouse strains.

Classic cryobiology studies suggest the use of glycerol or any permeating additive to effi-

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