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Effect of Chilling on the Motility and Acrosomal Integrity of Rat Sperm in the Presence of Various Extenders

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Studies were conducted to determine the effect of chilling on rat sperm and optimal components (extenders) to avoid chilling-induced injury. In the first experiment, the effects of chilling (at 4, 10, or 22 °C) on the motility and acrosomal integrity of epididymal sperm from 2 strains of rats (Sprague–Dawley and Fischer 344, F344) were compared. In the second experiment, the motility of epididymal Sprague–Dawley rat sperm after exposure to extenders (HEPES-buffered Tyrode lactate, skim milk, lactose monohydrate, Tris–citrate, and TEST) and cooling and warming was determined. We tested the effects of supplementing base extender solutions with 20% lactose–egg yolk (LEY) alone or in combination with a commercial SDS-based paste (0.5%, v/v) in preventing chilling injury. The motility after each treatment was determined after both cooling and warming. In the third experiment, the motility of Sprague–Dawley rat sperm were compared after supplementing the base extenders with either 0.4 M permeating cryoprotective agent (CPA; glycerol, ethylene glycol, propylene glycol, or DMSO) or 0.1 M nonpermeating CPA (raffinose and sucrose) after cooling and warming. The results showed that chilling significantly reduced the motility—but not acrosomal integrity—of Sprague–Dawley and F344 sperm. Neither motility nor acrosomal integrity differed between Sprague–Dawley and F344 strains. The addition of LEY into each extender significantly prevented motility loss after chilling. These results will be useful during the preparation of optimal extenders and development of successful cryopreservation protocol for rat sperm.

Abbreviations: CPA, cryoprotective agent; LEY, 20% lactose–egg yolk; TEST, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonicd acid plus Tris; TL-HEPES, HEPES-buffered Tyrode lactate.

Rats are commonly used laboratory animals for biomedical and genomic research.[13,18,38] Assisted reproductive technologies and gene modification methods for rats have improved markedly over the last decade.[1] Successful cryopreservation of gametes and embryos has great importance for genome banking of transgenic and mutant rat lines as well as for studies of rat sperm biology.[23,28] In the context of genome banking, the postmortem collection of epididymal sperm is much simpler than is the collection of embryos because superovulation requires hormone injection to obtain abundant numbers of embryos from a single donor. Efficient cryopreservation of rat sperm has been difficult, and few published studies have aimed at understanding the underlying physiologic and cryobiologic mechanisms for the low survival.[23,33] The determination of chilling sensitivity and effective extender media in which rat sperm maintain its integrity are necessary for the development of optimal handling and cryopreservation protocols.

In general, damage to sperm before freezing and after thawing have been attributed to several factors including cold shock, freezing injury, oxidative stress, alterations in membrane compositions, chemical toxicity of cryoprotective agents (CPA), and osmotic stress.[9] Furthermore, there is considerable variation among species in their sensitivity to these effects.[17] The sensitivity of sperm to these damaging factors depends on the temperature at which they are handled as well as interactions between the chemical composition and temperature of the extending environment and osmolality. Therefore, determination of the optimal sperm handling temperature, osmolalities, and extender composition would enable development of better cryopreservation protocols.[12,25,43]

Semen freezing extenders are used to prepare multiple insemination doses for artificial insemination and in vitro fertilization. Tris–citrate, skim milk, egg yolk (EY), lactose, and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonicd acid (TES) are the most commonly used semen extenders.[46] Extenders are understood to provide temperature protection for sperm while reducing their metabolic rate in cool storage.[25,40] Extenders also function as membrane stabilizers at subphysiologic temperatures, energy substrates for sperm metabolism, and pH buffering and ionic balancers.[10,11] The addition of permeating CPA (that is, glycerol) to extenders has been effective for cryopreservation of sperm from various mammalian species, and nonpermeating sugars (that is, sucrose, raffinose, and trehalose) are added to withdraw intracellular water by increasing osmotic pressure and providing cryoprotection.[3-5] However, the efficacy of these supplemented extenders depends on the storage temperature as well as the buffer used.

Many previous reports suggest that sperm from different species respond differently to chilling, CPA, and extenders.[2,14,20,29] However, these studies are mostly limited to sperm from mice and livestock species. Therefore direct application of these protocols to other species often results in low postthaw survival rates. For example, mouse sperm from various strains could be

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successfully cryopreserved in a solution containing 2 readily available compounds (skim milk and raffinose) without the need for permeating CPA, such as glycerol and DMSO.[21] However, the same approach leads to low postthaw motility when used to cryopreserve rat sperm, supporting the notion that sperm cryopreservation protocols developed for 1 species may not work for sperm from another.[17]

The use of EY reduces chilling injury of sperm from many mammalian species.[25] In addition, various SDS-based products improve the effectiveness of EY during sperm freezing for several mammalian species including mouse,[27] rat,[22] cat,[6] dog,[26] and pig.[8] In the present study, we performed experiments to determine the (1) sensitivity of rat sperm from 2 different strains to chilling, (2) effect of adding 20% lactose–EY (LEY) alone or in combination with an SDS-based product to prevent chilling injury of rat sperm; and (3) effect of adding or removing 0.4 M permeating or 0.1 M nonpermeating CPA to rat sperm before and after chilling and warming in the presence of various semen extenders. We considered that gaining information about some of the manipulations and reagents used before freezing would facilitate the development of effective cryopreservation protocols for rat sperm.

Materials and Methods

All chemicals were purchased from Sigma Chemical (St Louis, MO) unless otherwise stated.

Animals and sperm collection. Outbred Sprague–Dawley and inbred Fischer 344 (F344) male rats (age, 16 to 20 wk) were used as sperm donors in this study. The rats were housed in accordance with the policies of the University of Missouri Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals*.[16] For sperm collection, male rats were humanely euthanized by CO_2 inhalation, their cauda epididymides were excised and placed in a 35-mm culture dish containing HEPES-buffered Tyrode lactate (TL-HEPES) solution[7] supplemented with 3 mg/mL bovine serum albumin (fraction V). The cauda epididymides were dissected with fine scissors to allow sperm to swim out for 10 to 15 min at 37 °C. The sperm suspension was gently drawn into a plastic transfer pipette (inner diameter, 2 mm; Samco, San Fernando, CA) and placed in a 5-mL tube for further experimentation. The sperm samples were held at 30 °C in test tubes and were equally distributed and used for further experimentation. Each experiment was performed by using a sample from a single donor without pooling and were repeated 6 times. The final concentration of sperm samples was about 30×10^6 sperm/mL for Sprague–Dawley and 20×10^6 sperm/mL for F344 rats.

Preparation of sperm extenders. During the studies, 5 different base extenders were used and tested: TL-HEPES, skim milk, lactose monohydrate, Salamon Tris–citrate,[30] and TEST. The effect of chilling on rat sperm motility was determined in TL-HEPES solution first (experiment 1). These 5 base extenders then were supplemented with either LEY only or with 0.5% of an SDS-based product (Equex Paste, Minitüb, WI; experiment 2) or with 0.4 M glycerol, 0.4 M DMSO, 0.4 M ethylene glycol, 0.4 M propylene glycol, 0.1 M raffinose, or 0.1 M sucrose (experiment 3) to determine the chilling effect on rat sperm motility in the presence of these base extenders. All solutions contained 0.475 g/L sodium penicillin and 0.8 g/L streptomycin sulfate. To prepare LEY, 23 mL homogenized EY was mixed with 77 mL 8% (v/v) lactose solution and centrifuged in sterile tubes at 15,000 × *g* for 15 min. The supernatant was filtered through 0.45-µm filters. The pH and osmolality of the LEY solution were adjusted by using 10% stock Tris solution to 7.00 ± 0.2 and 330 ± 5 mOsm, respectively. The osmolalities of the extenders were determined by using a vapor-pressure osmometer (VAPRO 5520, Wescor, Logan, UT).

The TL-HEPES solution containing 3 mg/mL bovine serum albumin fraction V was prepared as described.[7] The pH and osmolality were adjusted to 7.2 and 290 ± 5 mOsm, respectively. Skim milk solution was prepared by dissolving 3% (w/v) dehydrated skim milk (Difco 0032-17-3, Becton Dickinson, Franklin Lakes, NJ) in TL-HEPES without NaCl. The mixture was centrifuged at 15,000 × *g* for 15 min, and the supernatant was removed and filtered through 0.45-µm filters to obtain a final working solution at pH 7.2 and 390 ± 5 mOsm. Lactose monohydrate solution was prepared by dissolving 8% lactose monohydrate (w/v) in water purified by reverse osmosis and adjusting the pH and osmolality to 7.0 and 330 ± 5 mOsm, respectively. Tris–citrate extender solution was adapted from Salamon modified Tris-citrate and comprised 27.0 g/L Tris, 14.0 g/L citric acid, and 10.0 g/L fructose. The osmolality of this extender was adjusted to 416 ± 5 mOsm by adding water purified by reverse osmosis, and the pH was adjusted to 7.0. TEST solution consisted of 15.7 g/L TES and 8.2 g/L Tris. The osmolality of TES was adjusted to 330 ± 5 mOsm by adding water purified by reverse osmosis, and the pH was adjusted to 7.0.

Experiment 1: Effect of chilling on rat sperm motility and acrosomal integrity. Sperm samples (100 µL each) from Sprague–Dawley or F344 rats were transferred into 1.5-mL centrifuge tubes and kept at 30 °C. The sperm samples then were placed in a water bath at 4, 10, 22, or 37 °C (control) for 30 min. The samples were mixed gently with 900 µL TL-HEPES solution at 37 °C and held for 10 min before motility analysis. A 20-µL sperm sample from each treatment and control was smeared onto a microscope slide, air dried, and fixed to evaluate acrosomal integrity.

Experiment 2: Effect of LEY alone or in combination with an SDS-based product in preventing chilling injury of rat sperm exposed to TL-HEPES, skim milk, lactose, Tris–citrate, or TEST. This experiment was designed to determine whether the inclusion of LEY alone or combination with the base extenders has beneficial effect on reducing chilling injury of rat sperm and to test whether combination of LEY with SDS-based product (Equex Paste, Minitüb) would further reduce chilling injury. The motility of untreated sperm was determined first. Then 100-µL sperm samples from Sprague–Dawley rats ($n = 6$) were transferred into 1.5-mL centrifuge tubes and gently mixed with 900 µL of each base extender alone, extender containing 20% LEY, or extender containing 20% LEY + 0.5% Equex paste (30 °C). A second motility analysis was performed about 10 min later. The sperm samples from the different treatments then were plunged into a 4 °C water bath and held for another 30 min. A final motility analysis was performed 10 min after returning them to physiologic conditions by dilution in TL-HEPES at 37 °C.

Experiment 3: Effect of chilling on rat sperm motility in the presence of glycerol, DMSO, ethylene glycol, propylene glycol, raffinose, or sucrose. This experiment was designed to determine the optimal CPA–extender combination that could be used for freezing Sprague–Dawley rat sperm. In this experiment, 100 µL rat sperm suspension ($n = 6$) was added to the 900 µL of each base extender (at 30 °C) containing the following CPA: 0.4 M glycerol, 0.4 M DMSO, 0.4 M ethylene glycol, 0.4 M propylene glycol, 0.1 M raffinose, and 0.1 M sucrose. Sperm samples (10 µL each) were exposed for 5 min to the CPA–extender solution before motility was evaluated. The samples then were placed in a 4 °C water bath, held for 30 min, diluted in TL-HEPES solution at 37 °C, and analyzed for motility.

Assessment of acrosomal integrity. Epifluorescent microscopy was used to assess rat sperm acrosomal integrity after staining with Alexa Fluor-488-PNA (peanut agglutinin) conjugate (Molecular Probes, Eugene, OR). The treated and control samples were smeared onto microscope slides and air-dried. The specimens were fixed with 99% methanol and kept at room temperature until fluorescence staining. For staining, slides were incubated with 20 µg/mL Alexa Fluor-488-PNA at 37 °C for 30 min, washed with PBS, and analyzed by epifluorescent microscopy (Zeiss Axiophot, Jena, Germany) by using an appropriate filter. The images of stained sperm samples were classified into 2 groups. Spermatozoa displaying intensively and moderately bright fluorescence in the acrosomal region were considered to be intact, whereas spermatozoa displaying weak, patchy, or no fluorescence in the acrosomal region were considered to be damaged. Approximately 80 to 100 sperm on each slide were evaluated to determine the proportion of sperm with intact acrosomes. At least 5 biologic replicates were conducted for each treatment.

Computer-assisted sperm motility analysis. Computer-assisted sperm motility analysis (Hamilton Thorne Biosciences, Beverly, MA) was used to determine total motility, progressive motility, and average path velocity by using fixed-depth (80 µm), dual-sided sperm-counting chambers (2×CELL, Hamilton Thorne Biosciences). A total of 6 fields were counted for each sample. The settings and definition of sperm motion parameters for computer-assisted sperm motility analysis have been described.[34,40]

Statistical analysis. Statistical analysis was performed by using general linear models (SAS version 9.1, SAS Institute, Cary, NC)[31] to determine differences associated with chilling, various extenders, the SDS-based product, and CPA on motility and acrosomal integrity of the rat sperm. Data were normalized by using arc-sine transformation when needed. Separation of means was assessed by using the Duncan Multiple Range test. Data given as mean ± SEM. For all tests, statistical significance was defined as a P value less than 0.05.

Results

Experiment 1: effect of chilling on rat sperm motility and acrosomal integrity. Table 1 shows the total motility, progressive motility, velocity, and acrosomal integrity (mean ± SEM) of epididymal Sprague–Dawley and F344 rat sperm that were subjected to 4, 10, or 22 °C for 30 min and returned to 37 °C in TL-HEPES solution. Chilling below 22 °C caused a 30% to 40% ($P < 0.05$) loss in motility for both Sprague–Dawley and F344 epididymal sperm but did not have any effect on velocity of F344 sperm. Motility did not differ between Sprague–Dawley and F344 rat sperm, except that Sprague–Dawley sperm showed a significant ($P < 0.05$) loss in total motility at 22 °C. Chilling did not have a detrimental effect on acrosomal integrity of sperm for either strain studied.

Experiment 2: Effect of LEY alone or in combination with an SDS-based product in preventing chilling injury of rat sperm after exposure to various extenders. Table 2 and 3 show the total and progressive motility, respectively, of Sprague–Dawley rat sperm before and after exposure to 4 °C in TL-HEPES, skim milk, lactose, Tris–citrate, and TEST base extenders in the presence of LEY or LEY plus 0.5% SDS-based product. Experiment 2 showed that none of the extenders studied protected rat sperm motility against chilling ($P < 0.05$). However, with the exception of TL-HEPES after dilution, supplementation of each base extender with LEY significantly ($P < 0.05$) improved motility during dilution as well as after chilling to 4 °C and returning to isosmotic condition at 37 °C. The TL-HEPES+LEY combination was the most effective in maintaining motility during the dilution phase, but TEST+LEY was better during the chilling–rewarming phase.

When supplemented with LEY, most extenders were effective in maintaining motility after chilling to 4 °C and returning to isosmotic condition at 37 °C ($P < 0.05$). However, skim milk and LEY both caused a significant ($P < 0.05$) loss in motility after chilling. In contrast, supplementation of the base extenders with the SDS-based product did not have any additional beneficial effects on rat sperm motility after dilution, chilling to 4 °C, and returning to isosmotic conditions at 37 °C. Although the SDS-based product caused significant ($P < 0.05$) reduction in motility (approximately 60%) when it was used to supplement TES+LEY, its addition into Tris–citrate+LEY prevented any reduction in motility as compared with the control ($P > 0.05$).

Experiment 3: Effect of chilling on rat sperm motility in the presence of various base extenders plus 0.4 M glycerol, 0.4 M DMSO, 0.4 M ethylene glycol, 0.4 M propylene glycol, 0.1 M raffinose, or 0.1 M sucrose. Table 4 shows the total motility and progressive motility values of Sprague–Dawley rat sperm before and after exposure to 4 °C in TL-HEPES, skim milk, lactose, Tris–citrate, and TEST base extenders that were supplemented with either 0.4 M glycerol, 0.4 M DMSO, 0.4 M ethylene glycol, 0.4 M propylene glycol, 0.1 M raffinose, or 0.1 M sucrose. This experiment showed that TL-HEPES, skim milk, and lactose extenders containing 0.4 M glycerol, 0.4 M ethylene glycol, or 0.4 M propylene glycol led to significant losses in sperm motility after chilling to 4 °C and returning to isosmotic condition at 37 °C ($P < 0.05$). Compared with the other CPA tested, ethylene glycol caused particularly ($P < 0.05$) low motility (0% to 20%) during dilution and after chilling and warming, except when added to TEST extender ($P > 0.05$). The TES extender gave consistently good motility recovery rates for all CPA after chilling to 4 °C and returning to isosmotic condition at 37 °C ($P > 0.05$). Compared with the other combinations, sucrose and raffinose dissolved in TL-HEPES and LEY extenders were superior in protecting rat sperm motility after chilling and warming. Tris–citrate extender containing 0.4 M propylene glycol had the highest motility recovery rate after chilling and warming (approximately 70%) of any CPA tested ($P < 0.05$).

Discussion

This study showed that chilling damages rat sperm, although to a lesser degree than for other species, including swine, sheep, horses, and bovines.[25,44] The genetic background of the donor had significant influence on cryosurvival and other cryobiologic characteristics of mouse sperm.[21,41] Therefore, we initially compared the motility and acrosomal integrity of F344 and Sprague–Dawley sperm with regard to their sensitivity to chilling and found no difference between these 2 strains. The current study also revealed a potential relation between sperm and EY, skim milk components, and CPA, which affected sperm motility differently. The deleterious effects of chilling and osmotic stress on sperm were significantly reduced when extenders were supplemented with EY (in particularly) and skim milk (to some extent).[2,11,15,25] In 1 study,[2] mouse sperm increased in osmotic tolerance in the presence of either EY or skim milk. The study also confirmed that motility was maintained at a high level when rat sperm underwent chilling and osmotic stress in the presence of EY.[2]

A simple freezing medium consisting of 18% (w/v) raffinose and 3% (w/v) skim milk without any permeating CPA has successfully been used to cryopreserve sperm from many

Table 1. Motility characteristics and acrosomal integrity (mean ± SEM) of Sprague–Dawley and F344 rat sperm subjected to chilling at various temperatures and returned to 37 °C (n = 5)

	Sprague–Dawley				F344			
°C	Total motility (%)	Progressive motility (%)	Velocity (μm/s)[a]	Acrosomal integrity (%)	Total motility (%)	Progressive motility (%)	Velocity (μm/s)[a]	Acrosomal integrity (%)
37 (control)	81.2 ± 4.0	27.7 ± 1.5	121.5 ± 3.94	96.9 ± 1.4	76.6 ± 4.9	26.2 ± 2.1	129.7 ± 3.8	95.5 ± 1.6
22	67.2 ± 4.3[b]	23.4 ± 2.4	125.9 ± 5.55	96.6 ± 2.1	67.6 ± 5.0	20.1 ± 2.1	132.8 ± 3.8	84.1 ± 8.5
10	53.4 ± 4.6[b]	15.0 ± 1.8[b]	107.0 ± 6.47[b]	85.7 ± 3.3	57.9 ± 5.3[b]	15.9 ± 1.2[b]	123.0 ± 4.8	86.0 ± 3.7
4	59.7 ± 2.6[b]	16.4 ± 2.1[b]	107.7 ± 5.74[b]	88.2 ± 6.4	51.4 ± 2.6[b]	14.5 ± 2.1[b]	121.5 ± 8.7	87.5 ± 3.4

[a]Average path velocity
[b]$P < 0.05$ versus control

Table 2. Total motility (% total sperm; mean ± SEM) of Sprague–Dawley rat sperm after dilution and after chilling (4 °C) and warming (37 °C) in TL-HEPES, skim milk, lactose monohydrate, Tris–citrate, and TEST extenders containing either 20% lactose egg yolk (LEY) or 20% LEY plus 0.5% SDS-based product (EP; n = 6)

	TL-HEPES	Skim milk	Lactose	Tris–citrate	TEST
After dilution					
Extender only	73.9 ± 2.4[a] (untreated control)	59.5 ± 4.1[c]	46.9 ± 2.6[d]	57.8 ± 2.5[c]	60.9 ± 4.2[c]
Extender + LEY	77.4 ± 2.3[a]	68.3 ± 1.9[a]	80.1 ± 2.7[a]	68.7 ± 2.6[b]	70.4 ± 2.2[a]
Extender + LEY + EP	70.6 ± 2.9[a]	66.0 ± 4.3[a]	62.5 ± 4.6[b]	64.2 ± 4.0[b]	68.8 ± 2.7[a]
After chilling and warming					
Extender only	57.6 ± 3.6[c]	34.7 ± 4.8[e]	36.4 ± 4.2[e]	50.2 ± 4.7[d]	45.5 ± 4.7[d]
Extender + LEY	75.5 ± 3.9[a]	59.6 ± 4.0[c]	65.3 ± 2.0[b]	64.0 ± 5.0[b]	70.9 ± 1.4[a]
Extender + LEY + EP	53.2 ± 7.0[d]	54.9 ± 3.8[c]	51.5 ± 5.0[d]	63.2 ± 5.2[b]	33.3 ± 3.3[e]

Different letters indicate significant difference ($P < 0.05$) between values.

Table 3. Progressive motility (% total sperm; mean ± SEM) Sprague–Dawley rat sperm after dilution and chilling (4 °C) and warming (37 °C) in TL-HEPES, skim milk, lactose monohydrate, Tris–citrate, and TEST extenders containing either 20% lactose–egg yolk (LEY) or 20% LEY plus 0.5% SDS-based product (EP; n = 6)

	TL-HEPES	Skim milk	Lactose	Tris–citrate	TEST
After dilution					
Extender only	27.1 ± 1.6[a]	13.2 ± 1.1[b]	16.7 ± 1.7[b]	14.9 ± 1.6[b]	10.6 ± 2.1[b]
Extender + LEY	28.3 ± 2.2	14.5 ± 2.4[b]	25.2 ± 7.0	19.7 ± 2.0	20.5 ± 2.3
Extender + LEY + EP	23.5 ± 4.6	12.6 ± 2.5[b]	19.3 ± 1.7[b]	19.1 ± 2.5	10.8 ± 1.2[b]
After chilling and warming					
Extender only	22.0 ± 1.6	15.4 ± 1.5[b]	13.7 ± 1.6[b]	7.7 ± 1.1[b]	17.7 ± 1.8[b]
Extender + LEY	29.2 ± 1.3	17.8 ± 1.4[b]	23.1 ± 2.9	11.7 ± 1.2[b]	27.3 ± 2.9
Extender + LEY + EP	36.1 ± 3.0	15.5 ± 1.9[b]	18.7 ± 2.3[b]	12.4 ± 2.1[b]	40.4 ± 9.8

[a]Untreated control
[b]$P < 0.05$ versus untreated control

inbred and outbred mouse strains.[21,37] However, the efficacy of this protocol could not be validated in every mouse strain. In contrast to its effectiveness for mouse sperm, our data suggest that skim milk is not particularly effective in protecting rat sperm from chilling injury. However, preservation of motility comparable to control levels was obtained after rat sperm was chilled in a solution containing 3% skim milk and 0.4 M DMSO. In this regard,1 study[36] compared the cryoprotection provided by permeating (glycerol, propylene glycol, DMSO) or nonpermeating (lactose, raffinose, sucrose, trehalose) CPA in 3% skim milk for cryosurvival of mouse sperm. The results showed that after thawing, mouse sperm diluted with raffinose, trehalose, and sucrose sustained the best motility (60%), whereas the best permeating CPA was DMSO (42%). Further, the least sperm injury occurred in samples frozen in trehalose, raffinose, and sucrose. Overall, our study was in agreement with the cited study,[36] in that DMSO was the best permeating CPA if combined with skim milk, and disaccharides are effective in protecting rodent sperm from stresses related to cryopreservation.

Semen extenders containing EY or skim milk consist of a variety of substances, and the nature of their protective action during cryopreservation is not fully understood.[25] The optimal extender and choice of CPA for sperm cryopreservation usually is determined empirically. Previous studies[25] suggest that permeating and nonpermeating CPA and biological products such as EY and skim milk interact with each other, leading to variable subsequent effects on sperm survival. In addition, the inclusion of an antioxidant, α-monothioglycerol, in a mouse sperm freezing extender (18% raffinose and 3% skim milk) improved the postthaw fertilization efficiency of C57BL/6J sperm.[24] In the context of rat sperm cryopreservation, a recently reported method for cryopreservation of epididymal rat spermatozoa involved a medium containing 23% EY, 8% lactose monohydrate, 0.7% of an SDS-based product, and 3% glycerol and

Table 4. Total and progressive motility (% total sperm, mean ± SEM) of Sprague–Dawley rat sperm after dilution and after chilling (4 °C) and warming (37 °C) in TL-HEPES, skim milk, lactose monohydrate, Tris–citrate, and TEST extenders containing 0.4 M glycerol, 0.4 M DMSO, 0.4 M ethylene glycol, 0.4 M propylene glycol, 0.1 M raffinose, or 0.1 M sucrose (n = 6)

	After dilution		After chilling and warming	
Extender	Total motility	Progressive motility	Total motility	Progressive motility
TL-HEPES (control)	82.7 ± 3.3	24.0 ± 1.3	not applicable	not applicable
TL-HEPES + glycerol	63.6 ± 6.9[a]	18.8 ± 5.4	2.6 ± 2.6[a]	1.4 ± 1.4[a]
TL-HEPES + DMSO	84.4 ± 3.5	28.0 ± 2.8	62.8 ± 6.3[a]	15.6 ± 3.8[a]
TL-HEPES + ethylene glycol	6.6 ± 5.6[a]	0.6 ± 0.6[a]	1.4 ± 1.4	0.0 ± 0.0[a]
TL-HEPES + propylene glycol	79.4 ± 4.0	23.4 ± 1.8	5.4 ± 4.4[a]	0.4 ± 0.4[a]
TL-HEPES + raffinose	75.4 ± 8.8	18.0 ± 4.3	61.6 ± 9.4[a]	18.2 ± 4.6
TL-HEPES + sucrose	85.4 ± 6.2	26.8 ± 2.7	60.8 ± 4.4[a]	21.0 ± 2.8
Skim milk + glycerol	77.8 ± 5.7	21.0 ± 4.2	20.6 ± 14.9[a]	5.2 ± 4.5[a]
Skim milk + DMSO	85.6 ± 2.9	23.0 ± 4.8	64.6 ± 6.3[a]	14.8 ± 3.1[a]
Skim milk + ethylene glycol	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0	0.0 ± 0.0[a]
Skim milk + propylene glycol	74.4 ± 7.7	19.8 ± 2.7	5. 0 ± 4.5[a]	0.6 ± 0.6[a]
Skim milk + raffinose	78.8 ± 8.3	22.8 ± 4.1	47.4 ± 10.2[a]	12.2 ± 4.3[a]
Skim milk + sucrose	78.6 ± 7.8	20.8 ± 5.0	54.0 ± 6.6[a]	14.2 ± 3.2[a]
Lactose + glycerol	71.4 ± 4.9	20.0 ± 3.3	34.6 ± 9.81[a]	8.2 ± 3.4[a]
Lactose + DMSO	80.8 ± 7.4	25.6 ± 4.1	47.8 ± 14.5[a]	21.8 ± 6.8
Lactose + ethylene glycol	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0	0.0 ± 0.0[a]
Lactose + propylene glycol	49.0 ± 4.7[a]	13.2 ± 5.4[a]	30.4 ± 9.8	13.0 ± 5.2[a]
Lactose + raffinose	86.2 ± 7.1	26.6 ± 4.4	63.6 ± 10.4[a]	20.8 ± 5.3
Lactose + sucrose	84.7 ± 3.4	24.0 ± 3.9	60.7 ± 10.9[a]	22.7 ± 4.7
Tris–citrate + glycerol	80.0 ± 8.2	26.8 ± 5.4	60.2 ± 8.1[a]	23.0 ± 4.8
Tris–citrate + DMSO	85.4 ± 5.0	26.4 ± 4.7	52.6 ± 7.8[a]	11.2 ± 2.2[a]
Tris–citrate + ethylene glycol	74.0 ± 12.0	19.4 ± 4.6	24.4 ± 2.8[a]	5.0 ± 1.7[a]
Tris–citrate + propylene glycol	77.6 ± 4.9	20.6 ± 2.7	69.8 ± 6.5	17.6 ± 3.8
Tris–citrate + raffinose	32.6 ± 8.2[a]	5.2 ± 2.5[a]	41.8 ± 14.7	12.2 ± 4.5[a]
Tris–citrate + sucrose	53.0 ± 6.5[a]	6.2 ± 1.6[a]	45.4 ± 14.0	12.0 ± 4.0[a]
TEST + glycerol	86.0 ± 4.2	26.4 ± 1.9	55.0 ± 5.7[a]	19.0 ± 2.5
TEST + DMSO	83.8 ± 4.4	30.6 ± 2.4	64.4 ± 5.2[a]	22.0 ± 2.5
TEST + ethylene glycol	60.8 ± 12.0[a]	22.4 ± 4.2	53.4 ± 7.2	12.2 ± 3.7[a]
TEST + propylene glycol	84.6 ± 7.6	27.0 ± 2.9	56.2 ± 3.8[a]	17.0 ± 1.9
TEST + raffinose	76.8 ± 4.0	26.6 ± 3.0	61.0 ± 6.7[a]	18.2 ± 6.0
TEST + sucrose	73.2 ± 6.6	26.4 ± 1.7	51.6 ± 9.1[a]	15.8 ± 3.5[a]

[a] $P < 0.05$ versus untreated control.

successfully produced live pups after artificial insemination.[22] However, the results of that study also showed that addition of glycerol in the freezing solution did not enhance postthaw motility and plasma membrane integrity of rat sperm, and the percentage of membrane-intact spermatozoa was significantly higher in sperm that had been frozen in solution without 3% glycerol than in samples frozen in medium with 3% glycerol. A study comparing the effectiveness of modified Krebs–Ringer bicarbonate buffer[39] and Dulbecco PBS solutions revealed that cryopreservation of epididymal rat sperm in modified Krebs–Ringer bicarbonate containing 0.1 M raffinose, 0.75% of an SDS-based product, and 20% EY provided significantly higher postthaw motility (39%) than did DPBS solution (6%) under the same conditions.[45] Although we did not perform freezing experiments, the current study is in agreement with the cited work[45] in that 0.1 M raffinose dissolved in a comparable media (TL-HEPES) provided optimal holding conditions after chilling and warming. Furthermore, the earlier study[45] suggested that rat sperm that were not treated with glycerol had greater motility and more intact acrosomes than those exposed to any concentration of glycerol (1, 2, 4, or 8%). Our study is consistent with both previous studies[39,45] in that the addition of glycerol to TL-HEPES or any other extender tested was detrimental to rat sperm motility.

With bolus addition of CPA solution at a concentration of 0.4 to 1 M in combination with skim milk or EY, Tris (10% to 20%) extenders are a common medium for freezing of sperm from many species including mice[21,35] and rats.[23] We have used somewhat lower concentrations (0.1 M) of nonpermeating CPA because high (that is, molar) concentrations of nonpermeating CPA cause excessive dehydration and subsequent cell death in human, mice, and rat sperm.[12,33,45] Whereas permeating CPA such as glycerol and DMSO protect cells from freezing injury through their colligative effects, nonpermeating CPA such as disaccharides help to minimize the intracellular water content and subsequently increase the likelihood of intracellular vitrification.[19,32] Our data suggest that rat sperm exposed to 0.4 M glycerol underwent significant motility loss unless combined with Tris–citrate or TEST extenders. Moreover, the presence of ethylene glycol had a similarly detrimental effect on rat sperm motility, suggesting toxicity of ethylene glycol to rat sperm when introduced in TL-HEPES, skim milk, or Tris–citrate solutions. Interestingly, this detrimental effect was not present when ethylene glycol was introduced in TEST. With the exception of DMSO, permeating CPA caused significant motility loss after rat sperm were chilled in all extenders and returned to isosmotic conditions at 37 °C. However, rat sperm also had good postchilling total motility when the extenders contained sucrose or raffinose. The results collectively suggest that extenders containing DMSO plus either raffinose or sucrose merit further optimization studies and freezing experiments.

Rat sperm is quite sensitive to the osmotic stress created by nonpermeating compounds.[33] Therefore, we adjusted the osmolality of the Tris–citrate and TEST extenders in our study to minimize loss of sperm motility due to osmotic stress and to observe the actual effects of the type of extender on sperm motility. Although rat sperm exposed to DPBS containing 0.9 M DMSO showed a large (greater than 90%) decrease in motility, only a small decrease in motility and slight acrosomal damage occurred after exposing to 0.9 M glycerol, ethylene glycol, or propylene glycol in PBS.[33] However, in this study high motility was obtained after exposure of rat sperm to a lower molar concentration of DMSO but prepared in TL-HEPES, suggesting the need for careful selection of holding media when preparing ex-

tenders for freezing rat sperm. In this study, Tris–citrate extender containing 0.4 M propylene glycol resulted in favorable motility rates which were comparable to those of the untreated control. Moreover, TEST was the optimal extender for all CPA, providing consistently better motility recovery rate after their bolus addition, chilling, and removal. This result suggests that TEST also may serve as an optimal extender for rat sperm freezing, although actual freezing studies must be performed to confirm this hypothesis. These results overall suggest the importance of the extender solution in which CPA is introduced and therefore underscore the need to determine appropriate CPA and extender mixtures for sperm cryopreservation.

Addition of SDS-based products to freezing solutions has been found to increase the cryosurvival and longevity of spermatozoa from various species including mouse,[27] rat,[22] cat,[6] dog,[26] and boar.[8] As the active compound in these commercial preparations, SDS has been suggested to modify the structure of EY lipoproteins in the extracellular medium.[5] In 1 study,[6] the addition of an SDS-based product to the freezing solution protected the acrosomes of cat epididymal spermatozoa during the freezing process but reduced sperm longevity during in vitro incubation at 38 °C. In the present study, we only investigated the effects of a single SDS-based product during the addition of extenders and after chilling and warming. Under these conditions, the SDS-based product we used did not protect rat sperm motility against chilling although it had a protective effect on motility during the incubation period in all extenders except TEST. Therefore, our current data suggest that the addition of an SDS-based product to EY during cold storage has no additional benefit on the motility of epididymal rat sperm. Overall, SDS-based products appear to exert their cryoprotective action during the freezing procedure. In fact, 1 study[22] found that the percentage of membrane-intact spermatozoa was significantly higher in the presence of 0.7% of an SDS-based product after thawing.

In summary, chilling decreased the motility of rat sperm, but no difference was found between Sprague–Dawley and F344 rat strains. Although EY is beneficial to alleviate chilling injury, addition of an SDS-based product at 0.5% did not bolster viability under similar conditions. Although skim milk has been used widely to cryopreserve sperm from other species including mice, it was the least protective compound against chilling injury for rat sperm. In addition, the results revealed strong interaction between extenders and the type of CPA on rat sperm motility. This finding suggests the need for careful selection of extender as well as an appropriate CPA that works well with the chosen extender to maintain high motility. Based on the experimental conditions and extender components described in the current study, we offer the following suggestions to those attempting to cryopreserve epididymal rat sperm. For nonpermeating CPAs TL-HEPES, lactose monohydrate, and TEST extenders (plus LEY) and 0.1 M raffinose or sucrose should be investigated further because the total and progressive motility rates obtained from sperm stored in these combinations are comparable to those of the untreated control. Regarding permeating CPA, we found that the addition of 0.4 M glycerol or 0.4 M propylene glycol to either Tris–citrate or TEST extender (plus LEY) or of 0.4 M DMSO into lactose monohydrate, Tris–citrate, or TEST extender (plus LEY) resulted in optimal motility rates; therefore these combinations should be investigated further in freezing trials. These data provide a basis for selection of an optimal extender and CPA combination for epididymal rat sperm and may facilitate preparation of an optimal freezing solution that maintains rat sperm viability as well as acceptable postthaw survival. Our current effort is directed to use this information to develop more effective cryopreservation procedures for rat sperm.

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