

Effects of cryopreservation on sperm quality, nuclear DNA integrity, *in vitro* fertilization, and *in vitro* embryo development in the mouse

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Abstract

Efficient freezing, archiving, and thawing of sperm are essential techniques to support large scale research programs using mouse models of human disease. The purpose of this study was to investigate the effects of variable combinations and concentrations of cryoprotectants on sperm-assessment parameters of frozen–thawed mouse sperm in order to optimize cryopreservation protocols. Sperm was frozen using combinations of 3% skim milk + 0.2 or 0.3 M nonpermeating raffinose with either permeating glucose, fructose, propylene glycol, ethylene glycol, glycerol, or sodium pyruvate in CD-1, C3FeB6F1/J, B6129SF1, C57BL/6NCrIBR, 129S/SvPaslco, and DBA/2NCrIBR mice. Sperm-assessment parameters included progressive motility, plasma membrane integrity (SYBR-14 + PI), *in vitro* fertilization rate, and *in vitro* embryo development rate to blastocyst. DNA content analysis of sperm was measured by the sperm chromatin structure assay (SCSA). 0.3 M raffinose with 0.1 M fructose significantly improved post-thaw sperm-assessment parameters for CD-1, C3B6F1, B6129SF1 mice ($P < 0.05$ – 0.01), whereas 0.2 M raffinose with 0.1 M glycerol or 0.1 M fructose enhanced sperm assessment values for C57BL/6 and 129S mice ($P < 0.01$), compared to 0.3 M raffinose alone. DNA fragmentation during cryopreservation was significantly increased in all strains evaluated when compared with fresh control sperm in a strain-dependent manner ($P < 0.01$). Supplementation with permeating glycerol or fructose to the cryoprotectant (CPA) solution showed a significant protective effect to DNA integrity when cryopreserving sperm from C57BL/6 and 129S mice. Damage to sperm DNA significantly decreased the rate of *in vitro* embryo development to blastocyst in C57BL/6 mice. The type of monosaccharide sugar or polyols, CPA molarity, and combination of permeating and nonpermeating cryoprotectant are significant factors for improving progressive motility, plasma membrane integrity, DNA integrity, *in vitro* fertilization rate, and *in vitro* embryo development rate to blastocyst in cryopreserved mouse sperm.

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Introduction

The mouse has become a significant research tool in genetic and molecular biology allowing the study of many models of human diseases based on transgenic or targeted mutations (Thornton *et al.* 1999, Crister & Moberaten 2000). The mouse is a convenient model because of its well-characterized genome and ease of genetic and experimental manipulation. Due to increasing demand for archiving through cryopreservation of newly emerging lines of outbred, hybrid, and inbred mutant mice, many laboratories have been working to better understand the freezing and thawing properties of mouse sperm in order to find more efficient methods for

sperm storage and recovery. The primary problem in mouse sperm cryopreservation is that it becomes very sensitive to cold and osmotic effect (Critser & Moberaten 2000). The most commonly described adverse effect of freeze–thawing on mouse sperm is the dramatic and sharp decrease in sperm motility and plasma membrane integrity (Songsasen & Leibo 1997a, Szein *et al.* 2000, 2001, Nishizono *et al.* 2004). Therefore, factors such as the type of cryoprotectant agents (CPA) used and genetic background of the mice all contribute to the successful cryopreservation of mouse sperm.

The CPA used in mouse sperm cryopreservation, whether permeating, nonpermeating, or combined is a key element. Polge *et al.* (1949) first reported that

glycerol had a protective action on the survival of frozen-thawed spermatozoa of chicks and humans. The CPA provides a protective action as an energy source for spermatozoa (Watson 1979) and maintains the osmotic pressure by forming hydrogen bonds with membrane phospholipids and sugars reducing membrane damage and minimizing membrane destabilization during freezing and thawing (Strauss *et al.* 1986). The first successful CPA used with mouse sperm consisted of a combination of 3% skim milk and 18% raffinose (Tada *et al.* 1990, Takeshima *et al.* 1991). This CPA combination was further improved by Nakagata (1995) and Nakagata *et al.* (1992). The CPA produced normal offspring that were derived from cryopreserved oocytes fertilized *in vitro* by cryopreserved sperm (Nakagata 1993). Another successful CPA for mouse sperm consists of a mixture of raffinose, glycerol, and egg yolk (Songsasen & Leibo 1997a, 1997b). Other studies have used mono-, di- and tri- saccharides, polyols (e.g. glycerol, or DMSO), and macromolecules (e.g. polyvinylpyrrolidone, or metrizamide) alone or in combination with conventional CPAs (Storey *et al.* 1998, An *et al.* 2000, Sztein *et al.* 2001, Thampson *et al.* 2001, Koshimoto & Mazur 2002) to improve the efficiency of mouse sperm cryopreservation.

It is well established that the genetic background of outbred, hybrid, and inbred strains of mice also influence sperm-assessment parameters (Songsasen & Leibo 1997a, Sztein *et al.* 2000, Nishizono *et al.* 2004), *in vitro* fertilization rate (Kasai *et al.* 1978, Hoppe 1980, Niwa *et al.* 1980, Sztein *et al.* 2000), and *in vitro* embryo development rate (Dandekar & Glass 1978, Roudebush & Duralia 1996, Scott & Whittingham 1996, Sztein *et al.* 2000). Unfortunately, inbred strains, such as C57BL/6 and 129S that are among the most commonly used strains of mice for the production of mutant lines still pose a challenge to obtaining consistently high freeze-thaw sperm quality, *in vitro* fertilization rate, and live-birth rate compared to most outbred and hybrid strains (Songsasen & Leibo 1997a, Sztein *et al.* 2000, Nishizono *et al.* 2004).

The integrity of mammalian sperm DNA is of prime importance for the paternal genetic contribution to normal offspring. Damaged DNA can have a significant negative impact on oocyte fertilization, embryo development rate, and live-birth rate. A significant correlation between the presence of nuclear DNA alterations in mature spermatozoa and poor sperm parameters or impaired reproductive efficiency is reported in both humans and animals (Ron-el *et al.* 1991, Hughes *et al.* 1996, Edwards & Beard 1999). To date, studies show that the cryopreservation process causes DNA damage to mammalian sperm in human (Royere *et al.* 1988, 1991, Donnelly *et al.* 2001, Hammadeh *et al.* 2001), boar (Fraser & Strzerek 2004), and ram (Peris *et al.* 2004).

The purpose of this study was to examine seven different CPAs in a prototypic hybrid strain of mice to determine the optimal CPA for sperm cryopreservation.

Using this optimal CPA, different molar concentrations and combinations were then used to assess its effects on progressive motility, plasma membrane integrity, DNA integrity, *in vitro* fertilization rate, and *in vitro* embryo development rate to blastocyst in a number of outbred, hybrid and inbred strains of mice.

Materials and Methods

Animals

Outbred male (CD-1), hybrid female (B6C3F1/CrIBR), and inbred male mice (DBA/2NCrIBR, 129S/SvPaslco, C57BL/6NCrIBR) were obtained from Charles River Canada (St-Constant QC, Canada). Hybrid B6129SF1 mice were obtained from Taconic (Germantown, NY, USA). Hybrid C3FeB6F1/J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). All sperm-donor mice ranged from 12 to 15 weeks of age and were sacrificed by cervical dislocation. Females ranged from 4 to 5 weeks of age and were sacrificed in the same manner. During all experiments, mice were housed under controlled light conditions (12 h light:12 h darkness) at the Laboratory Animal Services facilities of The Hospital for Sick Children, maintained in pathogen free holding, and received standard mouse diet and water *ad libitum*. Their use and care at the Hospital were reviewed in advance and performed according to standards of the Canadian Council on Animal Care (CACC).

Reagents

Unless otherwise stated, all reagents were purchased from Sigma Chemical Company.

Summary of experimental design

Experiment 1 evaluated the effects of seven different cryoprotectants, sugars, and polyols, on sperm-assessment parameters. Fresh sperm samples were obtained from B6129SF1 mice and frozen using a programmable controlled-rate freezer. The results obtained from experiment 1 were analyzed and the two cryoprotectants that provided the best results were used for further evaluation in experiment 2.

Experiment 2 assessed the optimal CPA concentrations of 0.2 and 0.3 M raffinose alone and combined with permeating CPA in CD-1, C3B6F1, B6129SF1, C57BL/6, 129S, and DBA mice. Sperm obtained from donors was frozen using a manual freezing technique and tested for post-thaw progressive motility, plasma membrane integrity, and DNA integrity.

Experiment 3 studied the effects of the optimal CPA combinations identified in experiment 2 in C3B6F1 and C57BL/6 mice on unassisted *in vitro* fertility rate and *in vitro* embryo development rate to blastocyst.

Cryoprotectant agents

The seven CPA used in these studies were prepared according to the method described by Nakagata (2000). A measure of 0.2 M raffinose (D+raffinose pentahydrate) sugar was combined with either glucose, fructose, propylene glycol, ethylene glycol, glycerol, or sodium pyruvate (0.1 M and different amounts). Osmotic pressures were adjusted to 430–450 and 470–490 mOsm/l as shown in Table 1; 330–350 and 600–620 mOsm/l as in Tables 2 and 3 (Advanced Instruments, Inc., Model 3300, Norwood, Massachusetts, USA).

The buffer used was Bacto Difco powder (Becton Dickinson, Franklin Lakes, NJ, USA) (w/v) dissolved in distilled water at 45 °C. The solution was centrifuged at 14 000 r.p.m. for 15 min at room temperature and the resulting supernatant was filtered through 0.45 µm Millipore filters (Carrigtwohill, Co. Cork, Ireland). The filtered solution was stored at –20 °C in 1.0 ml aliquots in sterile 1.5 ml polystyrene Eppendorf tubes (Eppendorf, Mississauga, Canada). Each aliquot was thawed and warmed to 37 °C prior to use.

Sperm freezing and thawing

Both epididymides and vasa deferentia from male mice were removed and transferred to a 35 mm petri dish (Falcon 351008, Becton-Dickinson, NJ, USA) containing 1 ml CPA pre-stored in an incubator at 37 °C, 5% CO₂. They were then pierced eight to ten times using sterile forceps allowing spermatozoa to swim out. Each vas deferens was then gently compressed along its length to expel residual spermatozoa with fine-tipped sterile forceps. The CPA with spermatozoa was then incubated for 10 min at 37 °C. After incubation, debris and tissue was manually removed and the sperm-CPA sample was distributed as 100 µl aliquots into four cryovials (1.8-cc Nunc cryotubes; Nunc Cryotubes, Roskilde, Denmark) using wide pipette tips. Cooling rate was automatically

controlled according to Songsasen & Leibo (1997b). The cryovials were placed into a controlled-rate freezer (Thermo Forma 7452, Marietta, Ohio, USA) at 20 °C. After 10 min in the freezer, the cryovials were cooled at 5 °C/min to +4 °C and held for 3 min. The samples were cryopreserved without seeding. Sperm samples were cooled directly from +4 °C at 20 °C/min to –100 °C and finally were plunged into liquid nitrogen (LN₂). Sperm freezing for each CPA group was replicated six times by using B6129SF1 mice (Table 1).

The sperm-CPA samples were separately distributed as 100 µl aliquots into eight labeled 1.8-cc cryovials by using wide pipette tips.

For manual rate freezing, a styrofoam box was used measuring 20.5 cm (length) × 20.5 cm (width) × 20 cm (depth). It was filled to 2.5 cm from the bottom with LN₂. Cryovials containing sperm-CPA were placed into a rack at 2 cm above the surface of LN₂ exposing them to LN₂ vapor (approximately –120 °C) for 10 min at a cooling rate of 20–40 °C/min. Sperm-CPA samples were then plunged directly into LN₂ for storage. One frozen-thawed sperm sample was analyzed for sperm-assessment parameters and recorded prior to thawing and *in vitro* fertilization. Sperm freezing for each CPA group was replicated between 6 and 15 times using different mice (Tables 2 and 3). Samples were rapidly thawed by transferring them from LN₂ into a 37 °C water bath for 2 min until the ice melted.

Sperm analysis

Progressive motility

Progressive motility of sperm samples was determined using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) according to World Health Organization (WHO 1999) guidelines. Frozen sperm samples were diluted 1:4 or 1:6 with Human Tubal Fluid (HTF; Quinn *et al.* 1985) depending on sperm cell concentration. After 15-min incubation of frozen

Table 1 Post-thaw progressive motility and membrane integrity results for B6129SF1 mouse sperm using raffinose alone and or various combinations of permeating cryoprotectants under the same molar or same osmotic pressure ($n=6$; mean \pm s.e.).

Type of CPA	Osmotic pressure (mOsm/l)	Progressive motility (%)	Intact membrane (%)
0.3 M=raffinose 0.3 M+skim milk (SM)	470–490	11.1 \pm 1.6 ^{b,c}	27.9 \pm 1.6 ^a
0.3 M=raffinose 0.2 M+glucose 0.1 M+SM	430–450	13.6 \pm 2.1 ^{a,b}	25.8 \pm 4.3 ^a
0.3 M=raffinose 0.2 M+fructose 0.1 M+SM	430–450	19.2 \pm 2.3 ^a	29.1 \pm 1.5 ^a
0.3 M=raffinose 0.2 M+propylene glycol 0.1 M+SM	430–450	9.55 \pm 2.1 ^{b,c,e}	12.9 \pm 0.2 ^b
0.3 M=raffinose 0.2 M+ethylene glycol 0.1 M+SM	430–450	11.9 \pm 1.9 ^{b,c}	16.9 \pm 1.9 ^{b,d}
0.3 M=raffinose 0.2 M+glycerol 0.1 M+SM	430–450	14.2 \pm 3.1 ^{a,b}	26.1 \pm 2.8 ^a
0.3 M=raffinose 0.2 M+sodium pyruvate 0.1 M+SM	430–450	6.9 \pm 2.1 ^{c,d,e}	13.8 \pm 0.5 ^b
Raffinose 0.2 M+glucose 280 mg+SM	470–490	12.8 \pm 1.7 ^{a,b}	31.6 \pm 2.1 ^a
Raffinose 0.2 M+fructose 280 mg+SM	470–490	16.1 \pm 1.5 ^{a,b}	25.6 \pm 0.4 ^{a,d}
Raffinose 0.2 M+propylene glycol 133 µl+SM	470–490	3.7 \pm 0.5 ^d	14.1 \pm 1.1 ^b
Raffinose 0.2 M+ethylene glycol 98.7 µl+SM	470–490	5.6 \pm 0.5 ^{c,d,e}	15.3 \pm 1.3 ^b
Raffinose 0.2 M+glycerol 128.8 µl+SM	470–490	12.9 \pm 1.9 ^{a,b}	28.2 \pm 2.1 ^a
Raffinose 0.2 M+sodium pyruvate 120 mg+SM	470–490	7.1 \pm 1.5 ^e	22.5 \pm 3.0 ^{a,b}
Skim milk (no. of CPA=negative control)	85	0.1 \pm 0.08 ^f	1.5 \pm 0.2 ^e

n , number of replicated experiment. a–f: $P < 0.05$; different superscripts within column indicate significant differences.

Table 2 Post-thaw progressive motility results for tested outbred, hybrid, and inbred strains of mice using alone or combined permeating and nonpermeating cryoprotectants (mean \pm s.e.).

Type of CPA	Progressive motility (%)					
	CD-1 (n=10)	C3B6F1 (n=8)	B6129SF1 (n=10)	C57BL/6 (n=15)	129S (n=8)	DBA (n=8)
Raffinose 0.3 M	24.3 \pm 2.3 ^b	20.5 \pm 3.4 ^{d,e}	21.8 \pm 3.2 ^d	3.4 \pm 1.2 ^d	1.5 \pm 1.3 ^d	27.6 \pm 8.2 ^c
Raffinose 0.2 M	17.9.0 \pm 2.4 ^b	13.3 \pm 0.6 ^e	16.3 \pm 1.7 ^d	11.1 \pm 4.7 ^c	7.5 \pm 4.3 ^c	19.9 \pm 6.5 ^c
Raffinose 0.3 M + Fru. 0.1 M	33.2 \pm 2.7 ^a	32.7 \pm 1.7 ^c	34.9 \pm 2.3 ^c	12.1 \pm 2.1 ^c	9.9 \pm 2.2 ^c	26.6 \pm 6.4 ^c
Raffinose 0.3 M + Gly. 0.1 M	25.1 \pm 5.3 ^{a,b}	27.14 \pm 2.4 ^{c,d}	22.1 \pm 3.1 ^d	13.7 \pm 3.7 ^c	9.7 \pm 1.3 ^c	24.5 \pm 6.6 ^c

Osmotic pressure: 0.3 MRaf. = 470–490 mOsm/l, 0.2 MRaf. = 330–350 mOsm/l, 0.3 MRaf. + 0.1 MFru. or 0.3 MRaf. + 0.1 MGly. = 600–620 mOsm/l. n = number of replicated experiment. a, b: $P < 0.05$; c–e, c, d: $P < 0.01$; different superscripts within column indicate significant differences.

sperm-CPA with HTF in a 37 °C water bath, 10 μ l of sperm-CPA sample was placed into a heated (37 °C) Makler counting chamber and evaluated for progressive motility of sperm. The number of motile and nonmotile spermatozoa was quantified by counting at least six predetermined fields (sequences) with a minimum of 200 spermatozoa. The routine sperm analysis was performed using an inverted phase contrast microscope (Zeiss, Axiovert 100; Zeiss, Oberkochen, Germany) with 10 \times magnification.

Plasma membrane integrity

Cell plasma membrane integrity was evaluated by the fluorescent staining method for sperm viability (Live/Dead Sperm Viability Kit, Molecular Probes Inc., Eugene, OR, USA) according to manufacturer's guidelines. This vital stain contains two nucleic acid dyes: (1) SYBR-14 that permeates intact plasma membrane causing viable spermatozoa to fluoresce green and (2) propidium iodide that permeates only damaged plasma membrane, causing nonviable spermatozoa to fluoresce red. Stain was prepared according to *Songsasen & Leibo (1997b)*. After staining, 10 μ l of sperm sample was placed on a microscope slide covered with a cover slip and observed under a fluorescence microscope (Zeiss Model IM35, filter set 487709; excitation filter 450–490 nm). For each mouse, 200 spermatozoa were counted on each slide.

DNA analysis

DNA analysis was performed as described by *Larson et al. (2000)* and *Traina et al. (2003)*. Briefly, fresh sperm

cells were expelled from the isolated vas deferens into 1 ml of TNA (0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4) using watchmaker's forceps. After 15-min incubation, sperm samples were filtered through a 70 μ m nylon mesh and were counted to determine sperm concentration. From each fresh sperm sample, 1–2 $\times 10^6$ cells were taken to a 15 ml conical tube and a pellet was prepared after centrifugation at 300 *g* for 4 min. Frozen sperm samples were archived in LN₂ for 12 weeks prior to DNA analysis. Frozen-thawed sperm aliquots (0.1 ml) were mixed with 1 ml TNA and incubated for 10 min in an incubator at 37 °C, 5% CO₂, then the solution was centrifuged at 300 *g* for 4 min, the pellet re-suspended in 0.2 ml TNA, and then mixed with 0.4 ml acid-detergent solution (0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCL, pH 1.2). After 30 s, the cells were stained by adding 1.2 ml of a solution containing 6 μ g/ml of Acridine Orange (AO; Molecular Probes Inc.) in staining buffer (0.1 M citric acid, 0.2 M Na₂HPO₄, 1 mM EDTA, 0.15 M NaCl, pH 6). When excited with blue light AO intercalated into double-stranded DNA fluoresces green. AO associated with single-stranded DNA emits red fluorescence. Abnormal sperm chromatin structure was measured by flow cytometry (FCM), on a cell-by-cell basis, and expressed as green (native DNA) to red (denatured, single-stranded DNA) AO fluorescence intensity shift using an index called α T. Alpha-T represents the ratio of red to total (red+green) fluorescence. Normal, native chromatin remains structurally sound and produces a narrow α T distribution. DNA in sperm with abnormal chromatin structure has increased red fluorescence, thereby

Table 3 Post-thaw plasma membrane integrity results for tested outbred, hybrid, and inbred strains of mice using alone or combined permeating and nonpermeating cryoprotectants (mean \pm s.e.).

Type of CPA	Intact membrane (%)					
	CD-1 (n=10)	C3B6F1 (n=8)	B6129SF1 (n=10)	C57BL/6 (n=15)	129S (n=8)	DBA (n=8)
Raffinose 0.3 M	38.6 \pm 3.1 ^{a,b}	33.2 \pm 5.3 ^{a,b}	36.0 \pm 2.4 ^{a,b}	20.8 \pm 5.8 ^a	18.7 \pm 3.5 ^a	38.0 \pm 4.5 ^a
Raffinose 0.2 M	28.3 \pm 1.9 ^b	24.0 \pm 2.8 ^b	27.9 \pm 1.2 ^b	23.0 \pm 7.3 ^a	25.0 \pm 6.1 ^a	27.3 \pm 3.9 ^a
Raffinose 0.3 M + Fru. 0.1 M	40.3 \pm 2.3 ^a	39.7 \pm 2.4 ^a	39.3 \pm 3.9 ^a	25.4 \pm 4.7 ^a	22.2 \pm 4.5 ^a	33.6 \pm 5.3 ^a
Raffinose 0.3 M + Gly. 0.1 M	35.3 \pm 2.6 ^{a,b}	36.9 \pm 3.9 ^a	38.0 \pm 4.8 ^{a,b}	28.6 \pm 2.1 ^a	25.8 \pm 3.6 ^a	35.4 \pm 4.4 ^a

Osmotic pressure: 0.3 MRaf. = 470–490 mOsm/l, 0.2 MRaf. = 330–350 mOsm/l, 0.3 MRaf. + 0.1 MFru. or 0.3 MRaf. + 0.1 MGly. = 600–620 mOsm/l. (n = number of replicated experiment). a and b: $P < 0.05$; different superscripts within column indicate significant differences.

producing a broader α T distribution. Cells were analyzed using the FACSCalibur flow cytometer (Becton Dickinson, Serial no. E0939, Biosciences, NJ, USA). Green fluorescence was measured between 515 and 545 nm band pass filter and red fluorescence using an LP670 nm long pass filter. The two colors were separated by a 560 nm dichroic filter. Data was stored in list-mode at 10 bit resolution. A total of 5×10^4 gated events were accumulated for each measurement. A gate was set on FL1 vs FL3 to exclude debris. Flowjo software (Version 4.0, Tree Star, Inc. Ashland, OR, USA) was used to analyze values of α T. Fresh sperm from B6129SF1 mice sonicated for 5 min using a sonicator (power rate 5, pulsar duty cycle 75%/s; Ultrasonic Processor XL Sonicator, Hert Systems, Farmingdale, NY, USA) adjusted to increase DNA fragmentation was used as positive control for the sperm chromatin structure assay (SCSA) system (Fig. 1).

In vitro fertilization

In vitro fertilization was performed as modified by Sztein *et al.* (2000). Four to five-week-old B6C3F1 hybrid mice were superovulated by i.p. injection of 0.5 IU of pregnant mare serum gonadotrophin (PMSG) followed by 0.5 IU of human chorionic gonadotrophin (hCG) 48 h later. Female mice were sacrificed by cervical

dislocation 14 h after the hCG injection and their oviducts removed into a plastic culture dish containing 2 ml of HTF medium. The cumulus–oocyte complexes were released from the ampulla into the HTF by rupturing with fine sterile forceps, and then placed into a 250 μ l HTF fertilization drop. Dishes with HTF drops for fertilization and culture were overlaid with mineral oil for sealing, and then placed overnight in a modular incubator chamber at 37 °C in a humidified atmosphere of 5% CO₂. IVF drop for C3B6F1 sperm+B6C3F1 oocyte combinations was used per three oocyte donors with a low concentration, 5×10^5 of progressively motile sperm per milliliter of fresh or frozen sperm.

Frozen sperm samples were thawed in a 37 °C water bath for 3 min and centrifuged at 3000 *g* (Mikro 20, D-78532, Tuttlingen, Germany) for 4 min. The supernatant was discarded and replaced with 50 μ l of HTF. The sperm samples were re-suspended by gently tapping of the tube and then incubated for 20 min at 37 °C to allow a minimal 'swim up'. A 40 μ l aliquot after incubation was taken for the IVF assay from each sperm sample. The fertilization drop was maintained for 4 h at 5% CO₂ in an incubator and then the oocytes were washed with HTF to eliminate excess sperm. The inseminated oocytes were cultured overnight in a 100 μ l drop of HTF. The next morning, the number of two-cell embryos was scored, and transferred to a 100 μ l drop of KSOM/AA medium

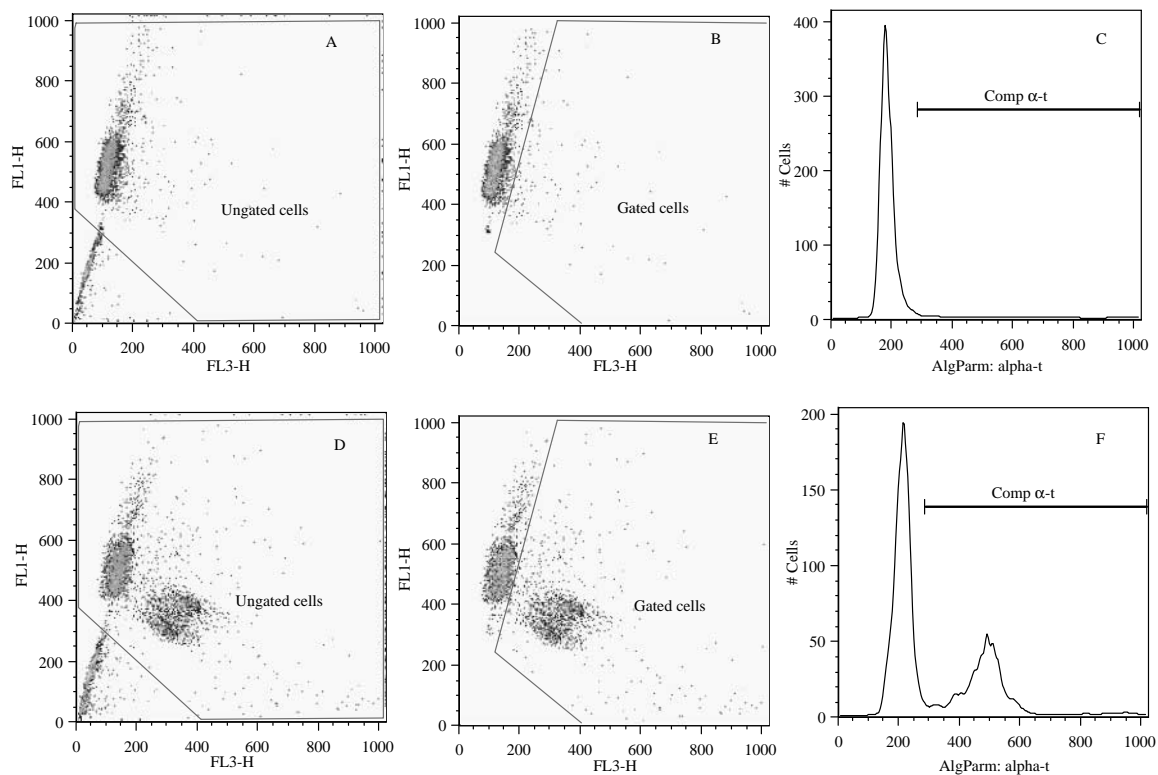


Figure 1 SCSA data with cytogram and histogram (α -t) showing the effects of sonication in unsonicated B6129SF1 fresh mouse sperm (control sample; A, B, and C) and with sonication (D, E, and F). Cells (gated cells : comp- α T) outside the main population have a larger ratio of red:green fluorescence, indicative of abnormal chromatin structure.

(Ho *et al.* 1995) for *in vitro* embryo development to the blastocyst stage. This IVF procedure was replicated three or four times for fresh and frozen sperm using different males for each strain of mice evaluated.

Zona-thinned oocytes for *in vitro* fertilization

To test the *in vitro* fertilization rate and *in vitro* embryo development rate to blastocyst of C57BL/6 sperm IVF zona-thinned B6C3F1 oocytes were used. A minimum count of 1×10^6 progressively motile sperm per milliliter was used in combination with C57BL/6 fresh or frozen sperm + B6C3F1 oocytes. To account for any parthenogenetic effects on oocytes in the zona-thinned group, cleaved parthenogenetic oocytes were identified and removed from culture according to Nagy *et al.* (2002).

The oocyte–cumulus complexes were collected from superovulated C3B6F1 mice. Cumulus cells were removed by using 0.05 mg/ml hyaluronidase (Sigma; H-3506) for 5 min in HTF medium. After washing the oocytes with HTF, zona thinning was performed using zona tyrode solution containing 0.8 g NaCl, 0.02 g KCl, 0.024 g CaCl₂·2H₂O, 0.301 g MgCl₂·6H₂O, 0.1 g Glucose, 0.4 g polyvinylpyrrolidone (PVP) in 100 ml water (pH 3.0). Oocytes were exposed for 45 s to the acid tyrode solution and washed three times with fresh HTF media prior to use.

Statistical analyses

Statistical analyses were performed using Graphpad Prism version 3.0 software (Graphpad software, San Diego, CA, USA). Post-thaw sperm assessment parameter results for DNA integrity, *in vitro* fertilization rate, and *in vitro* embryo development rate to blastocyst were evaluated for significance using repeated-measures one-way ANOVA (Tables 1–6). The Dunnett multiple-comparison test was used to compare differences among groups studied.

Results

Experiment 1

The greatest progressive motility of B6129SF1 sperm was obtained with the combination of 0.2 M raffinose +

0.1 M fructose (430–450 mOsm) compared to 0.3 M raffinose alone ($P < 0.05$). However, there were no significant differences in sperm membrane integrity between the groups with CPA and the negative control. The combination of 0.2 M raffinose + 0.1 M glycerol significantly improved progressive motility and plasma membrane integrity compared to other polyols, such as prophylenglycol or ethylenglycol, propylene glycol, ethylene glycol, glycerol, or sodium pyruvate with osmotic pressure between 430 and 490 mOsm ($P < 0.05$, Table 1). All combinations of CPA in all groups after thawing improved progressive motility and membrane integrity when compared with the negative control group (no CPA; $P < 0.05$; Table 1).

Experiment 2

Raffinose alone, and raffinose combined with fructose and glycerol were evaluated by using sperm from several strains of mice selected for this study (Tables 2 and 3). The optimal molar concentrations were determined to be 0.2 and 0.3 M raffinose in outbred, hybrid, and inbred strains of mice.

The results of comparing different combinations and concentrations of CPA are presented in Tables 2 and 3. Combination of 0.3 M raffinose + 0.1 M fructose had the highest progressive motility rates when compared with 0.3 M raffinose alone and 0.3 M raffinose + 0.1 M glycerol groups in CD-1, C3B6F1, and B6129SF1 mice ($P < 0.05–0.01$). There were no significant differences in rates of DNA fragmentation in frozen–thawed sperm from CD-1, C3B6F1, B6129SF1, and DBA mice when compared with the CPA control groups (Table 4). A 0.2 M concentration of raffinose in CPA solution had a significant beneficial impact on the progressive motility and membrane integrity of sperm from C57BL/6 and 129S mice when compared with 0.3 M raffinose alone after freezing and thawing. Additionally, 0.2 M raffinose + 0.1 M glycerol and 0.2 M raffinose + 0.1 M fructose in C57BL/6 and 129S mice provided for the highest progressive motility when compared with 0.3 M raffinose alone ($P < 0.001$), but did not significantly affect membrane integrity. Post-thaw C57BL/6 and 129S inbred sperm had a higher DNA fragmentation rate when

Table 4 Post-thaw DNA integrity results for mouse sperm using combined permeating and nonpermeating cryoprotectants with skim milk on selected strains ($n = 8$; mean \pm s.e.).

Type of CPA	Fragmented DNA (%)				Type of CPA	Fragmented DNA (%)	
	CD-1	C3B6F1	B6129SF1	DBA		C57BL/6	129S
Fresh sperm	1.6 \pm 0.2 ^b	1.4 \pm 0.6 ^d	1.1 \pm 0.1 ^f	3.7 \pm 1.1 ^h	Fresh sperm	3.2 \pm 0.7 ^l	4.8 \pm 1.3 ^o
Raffinose 0.3 M	6.1 \pm 1.1 ^a	6.8 \pm 1.3 ^c	8.9 \pm 1.6 ^e	9.4 \pm 3.9 ^g	Raffinose 0.3 M	34.1 \pm 1.4 ⁱ	25.4 \pm 2.2 ^m
Raffinose 0.2 M	7.2 \pm 1.6 ^a	8.1 \pm 0.6 ^c	10.4 \pm 0.9 ^e	11.9 \pm 1.2 ^g	Raffinose 0.2 M	30.7 \pm 4.9 ^{ij}	19.4 \pm 2.4 ^{m,n}
Raf. 0.3 M + Fru. 0.1 M	5.1 \pm 1.3 ^a	5.7 \pm 0.4 ^c	9.3 \pm 2.1 ^e	10.8 \pm 2.1 ^g	Raf. 0.2 M + Fru. 0.1 M	22.5 \pm 1.9 ^{jk}	15.1 \pm 0.9 ⁿ
Raf. 0.3 M + Gly. 0.1 M	6.5 \pm 0.6 ^a	6.4 \pm 0.9 ^c	7.9 \pm 3.5 ^e	9.7 \pm 2.1 ^g	Raf. 0.2 M + Gly. 0.1 M	17.4 \pm 2.8 ^k	21.5 \pm 3.4 ^{m,n}

n , number of replicated experiment. a–o: $P < 0.01$: letters within columns indicate significant differences. Fragmented DNA (COMP α T) is the % of sperm with abnormal chromatin.

Table 5 *In vitro* fertilization rate and embryo development rate to blastocyst for C3B6F1 mouse sperm cryopreserved with different cryoprotectants.

Sperm donor	Type of CPA	Type of sperm treatment	Number of oocytes	Number of 2-cell (%)	Number of blastocysts (%)
C3B6F1	No CPA	Fresh	135	97 (71.8) ^a	81 (83.5) ^d
	0.3 M R+SM	Frozen	152	33 (21.7) ^c	20 (71.4) ^d
	0.3 M R+0.1 M F+SM	Frozen	161	57 (35.4) ^b	33 (75.0) ^d

a–d: $P < 0.05$; Different superscripts within column indicate significant differences.

compared with CD-1, C3B6F1, B6129SF1, and inbred DBA mice (Table 4). Interestingly, both 0.2 M raffinose + 0.1 M fructose and 0.2 M raffinose + 0.1 M glycerol combination in C57BL/6 or 129S mice resulted in more robust sperm cell DNA integrity when compared with raffinose alone ($P < 0.01$). In other words, supplementation of CPA solution with glycerol or fructose showed a protective effect for DNA integrity against freezing damage in C57BL/6 and 129S mice ($P < 0.01$). The combination of 0.3 M raffinose + skim milk resulted in increased values for progressive motility, membrane integrity, and a lower rate of DNA fragmentation after freezing in DBA mice ($P > 0.05$). Although DBA was one of the inbred strains, it showed the highest resistance to DNA damage during freezing when compared with C57BL/6 and 129S inbred mice. In addition, resistance to DNA damage during freezing in the strains of outbred and hybrid mice tested were significantly greater than C57BL/6 and 129S inbred mice ($P < 0.01$). The cryopreservation process increased DNA fragmentation in all strains of outbred, hybrid, and inbred mice when compared with the fresh control sperm ($P < 0.01$; Table 4).

Experiment 3

The combination of 0.3 M raffinose + 0.1 M fructose had a higher *in vitro* fertilization rate than 0.3 M raffinose alone when compared with C3B6F1 oocytes fertilized with C3B6F1 fresh sperm ($P < 0.05$; Table 5). However, there were no significant differences in embryo development rate to blastocyst between fresh C3B6F1 hybrid sperm using 0.3 M raffinose alone or 0.3 M raffinose + 0.1 M fructose. For the inbred C57BL/6 strain, the results from experiment 3 further showed the efficiency of 0.2 M raffinose + 0.1 M glycerol combination in improving the *in vitro* fertilization rate and *in vitro* embryo development rate to blastocyst using C3B6F1 oocytes and

C57BL/6 sperm (Table 6). A measure of 0.3 M raffinose alone resulted in lower *in vitro* fertilization rate when compared with fresh C56BL/6 sperm or 0.2 M raffinose + 0.1 M glycerol combination ($P < 0.05$). On the other hand, the combination of 0.1 M glycerol + 0.2 M raffinose significantly improved *in vitro* fertilization rate for frozen C57BL/6 sperm ($P < 0.05$). In addition, this CPA combination had a lower DNA fragmentation rate that correlated with an increased *in vitro* embryo development rate to blastocyst when compared with 0.3 M raffinose alone for frozen C57BL/6 sperm.

Discussion

Investigation into the effects of variable combinations and concentrations of cryoprotectants on sperm quality is critical to generate more efficient techniques for mouse sperm cryopreservation.

The results of the first set of experiments from this study show that the type (chemical structure) of cryoprotectant and combination of permeating and nonpermeating cryoprotectants are important factors that effect the progressive motility and plasma membrane integrity of B6129S1 hybrid mouse sperm during freezing and thawing. Supplementation of glycerol or fructose to raffinose increased post-freezing progressive motility when compared with raffinose alone or combined permeating cryoprotectants (i.e. glucose or ethylene glycol) in B6129SF1 mice. The beneficial effects of using combined permeating and nonpermeating cryoprotectants in this study concur with previous reports in the literature that showed combinations of 18% raffinose and 1.75% glycerol (Tada *et al.* 1990), 10% raffinose and 5% glycerol (Yokoyama *et al.* 1990), and 6% glycerol plus trehalose (Storey *et al.* 1998) provided for the highest motility and plasma membrane integrity rates after sperm freezing and thawing.

Table 6 *In vitro* fertilization rate and embryo development rate to blastocyst for C57BL/6 mouse sperm cryopreserved with different cryoprotectants.

Sperm donor	Type of CPA	Type of sperm treatment	Number of oocytes	Number of 2-cell (%)	Number of blastocysts (%)
C57BL/6	No CPA	Fresh	142	97 (68.3) ^a	76 (78.3) ^d
	0.3 M R+SM	Frozen	362	78 (21.5) ^c	15 (19.2) ^f
	0.2 M R+0.1 M Gly+SM	Frozen	359	139 (38.7) ^b	56 (40.2) ^e

a–f: $P < 0.05$; different superscripts within column indicate significant differences.

The values for sperm progressive motility and membrane integrity reported in the second set of experiments from this study vary according to the molar concentration of CPA and mouse strain used. The use of raffinose alone or combined with permeating fructose or glycerol provided improved progressive motility and plasma membrane integrity when compared with molar concentration of raffinose (0.2 or 0.3 M). Koshimoto & Mazur (2002) pointed out that combinations of raffinose, sucrose, and glucose in PBS did not improve normalized motility when compared with raffinose alone under the same osmotic pressure (410–427 mOsm) or fixed at 18% (w/v) in outbred ICR mouse sperm. A similar finding is presented in Tables 1 and 2 of the present study for the response of raffinose + glucose or fructose combination in B6129SF1 and CD-1 strains. However, Koshimoto & Mazur (2002) also suggested that there is no special protection conferred by a particular sugar, and that protection against damage during freezing and thawing depends on the mass concentration of the sugar rather than its molar concentration. On the contrary, according to our results presented in Table 2, even though fructose (MW=180.2) has the same molecular weight as glucose, it significantly increased post-thaw progressive motility. The beneficial effects and improved sperm-assessment parameters produced as a result of varying sugar types and CPA combination (i.e. glycerol) have also been reported for ram (Molinia *et al.* 1994), bovine (Garcia & Graham 1989), dog (Yildiz *et al.* 2000), and human sperm (Critser *et al.* 1988, McGonagle *et al.* 2002). The effectiveness of fructose on sperm motility in CD-1, C3B6F1, B6129SF1, C57BL/6, and 129S mice can be explained by its relatively higher permeation rate across the cell plasma membrane and rapid metabolic processing in the cell when compared with other monosaccharides, such as glucose or galactose (Schwartz 1997).

The choice of sugar in the CPA formulation in the second set of experiments presented here had a significant impact on post-thaw mouse sperm motility in a strain-dependent manner. CD-1, C3B6F1, B6129SF1, and DBA sperm were frozen successfully using 0.3 M raffinose alone or 0.3 M raffinose + 0.1 M fructose combination (Table 2). It has been previously reported that the same molar concentration of CPA (i.e. 0.3 M raffinose) is not effective and does not equally preserve the sperm from some strains of mice, such as C57BL/6 and 129S when compared with hybrid and other inbred mice (Songsasen & Leibo 1997a, Szein *et al.* 2000, Szein *et al.* 2001, Nishizono *et al.* 2004). Also C57BL/6 and 129S sperm were frozen successfully using 0.2 M raffinose + 0.1 M fructose or glycerol combination (Table 2). It is also reasonable to assume that there can also be differences in sperm head or biochemical composition of sperm head membrane between C57BL/6 and 129S and other outbred, hybrid, and inbred strains of mice. If a suitable CPA's molar

concentration is not used, the biochemical membrane properties or differences in sperm head dimension may possibly induce cell death caused by intracellular or extracellular ice crystallization during freezing; resulting in inadequate dehydration and intracellular high-solute concentration. Also in the second set of experiments presented here, even though 0.3 M raffinose alone had a lower osmolarity (470–490 mOsm) than 0.3 M raffinose–0.1 M fructose combination (600–620 mOsm), this combination of sugar showed statistically higher progressive motility than 470–490 mOsm group in CD-1, C3B6F1 and B6129SF1 mice. Willoughby *et al.* (1996) reported a critical osmolarity of 500 mOsm in anisotonic PBS for mouse sperm. However, Agca *et al.* (2002) recently reported that egg yolk and skim milk significantly protected sperm motility and extended its osmotic tolerance limit (150–600 mOsm) when compared with NaCl, choline, or sucrose alone in ICR and B6C3F1 mice. Similar results using higher osmotic pressures were also found in this study.

DNA integrity of sperm is essential for accurate transmission of paternal genetic information. Normal condensation and stabilization of sperm chromatin in the nucleus followed by decondensation after sperm penetration and injection into the cytoplasm of the oocyte are pre-requisites for fertilization (Flaherty *et al.* 1995). The effects of cryopreservation on the integrity of the sperm nucleus are not well characterized. It is reported that the cryopreservation process of freezing and thawing can increase inappropriate chromatin condensation in human (Royere *et al.* 1988, Royere *et al.* 1991, Donnelly *et al.* 2001, Hammadeh *et al.* 2001), boar (Fraser & Strzezek 2004), horse (Linfoor & Meyers 2002), and ram (Peris *et al.* 2004) sperm. Recently, Chohan *et al.* (2004) noted that normal chromatin packaging significantly decreases after the freeze–thawing procedure in human sperm. Likewise, the cryopreservation process in our studies significantly increased the rate of abnormal sperm DNA in all tested strains compared to the rate in fresh control sperm (Table 4). However, different from the results presented here, Jiang *et al.* (2005) reported that nuclear DNA of fresh or cryopreserved sperm using isotonic CZB-HEPES solution did not affect the level of DNA fragmentation in outbred Kunming mice. The discrepancy between our findings and this report may result from using isotonic CZB-HEPES solution as a cryoprotectant and a different strain of mice. The second set of experiments reported here showed that the sperm DNA integrity was significantly increased by the cryopreservation process in C57BL/6 and 129S inbred mice, but was not affected by the freezing process in CD-1, C3B6F1, B6129SF1, and DBA strains of mice. This clearly implies that there are significant differences in resistance and susceptibility to DNA fragmentation between strains of outbred, hybrid, and inbred mice. In addition, supplementation of fructose or glycerol to raffinose not only protected

progressive motility and membrane integrity, but also DNA integrity of frozen–thawed C57BL6 and 129S sperm when compared with raffinose alone. It is well known that cryoprotectants are essential for the survival of cells during cryopreservation and the most commonly used cryoprotectants are sugars and glycerol. Strauss *et al.* (1986) and Anchordoguy *et al.* (1987) showed that sugars have cryoprotective properties because they lower the Van der Waals interactions at the membrane hydrocarbon chains that enhance trans-membrane transfer (especially phospholipids). The exact mechanism of action for glycerol in protecting cells from freeze–thaw damage is not completely understood. However, it is known that glycerol is osmotically active and is slow to permeate membranes resulting in cell volume changes from both the addition of glycerol and water loss during the freeze–thaw cycle (Schneider & Mazur 1984, Hammerstedt *et al.* 1990). Glycerol induces interdigitation of the two bilayer leaflets, altering membrane fluidity by increasing the order of the interior fatty acyl chains (Boggs & Rangaraj 1985). The beneficial effects of fructose and glycerol on DNA integrity in our study may be related to the different interactions on the DNA defense mechanisms of fructose or glycerol during the cryopreservation process.

The impact of the chemical formulation and combinations of CPA on unassisted *in vitro* fertility rate and *in vitro* embryo development rate to blastocyst was evaluated by using C3B6F1 and C57BL/6 sperm donors in the third set of experiments reported here (Tables 5 and 6). Our findings show that both inbred and hybrid fresh sperm had higher *in vitro* fertility rates and embryo development rates to blastocyst when compared with frozen–thawed sperm. These findings are consistent with previous reports on *in vitro* fertilization and *in vitro* embryo development rate to blastocyst of inbred strains previously reported (Niwa *et al.* 1980, Songsasen & Leibo 1997a, Sztejn *et al.* 2000). The combination of 0.3 M raffinose+0.1 M fructose with IVF produced higher *in vitro* fertility rates than 0.3 M raffinose alone and a lower *in vitro* fertility rate than C3B6F1 fresh sperm. However, there were no significant differences in embryo development rate to blastocyst between fresh sperm, 0.3 M raffinose alone, or 0.3 M raffinose+0.1 M fructose. Progressive motility is one of the most important criteria for fertilization potential of sperm (WHO 1999). Thus, higher fertilization results reported here that were obtained from the combination of 0.3 M raffinose+0.1 M fructose in C3B6F1 mice may be related to their greater post-thaw progressive motility rate when compared with 0.3 M raffinose alone (Tables 2 and 5). A similar correlation can be assumed between the combination of 0.2 M raffinose+0.1 M glycerol due to the higher post-thaw progressive motility rate observed when compared with 0.3 M raffinose alone in C57BL/6 mice (Tables 2 and 6). Recently, Nishizono *et al.* (2004) reported that using 0.3 M raffinose-skim

milk combination significantly increased the occurrence of abnormal or dysmorphic acrosome after the cryopreservation process in C57BL/6 sperm, and reported a strong correlation between dysmorphic spermatozoa and fertilization rates. Unlike this report, our *in vitro* fertilization rate for C57BL/6 mice using a combination of 0.2 M raffinose+0.1 M glycerol can be related to the better protective effect of raffinose–glycerol–skim milk combination on sperm membrane compared to 0.3 M raffinose–skim milk. Glycerol has previously been shown to be effective in protection against freezing membrane injuries in commonly used species of livestock (Parks & Graham 1992, Curry 1995) and a combination of sucrose and glycerol successfully protects embryos from various mammalian species and also bull sperm (Honadel & Killian 1988, De Leeuw *et al.* 1993) during cryopreservation. Classic cryobiology studies suggest the use of glycerol or any permeating additive to more efficiently protect cells from freezing injuries through colligative or solution effects (Mazur 1970). Therefore, using a CPA combination of glycerol and raffinose in C57BL/6 spermatozoa may have a synergistic effect on the protection of sperm cell membranes (i.e. the acrosome), resulting in higher *in vitro* fertilization rates. The combination of 0.1 M glycerol+0.2 M raffinose in the present studies protected sperm DNA integrity and significantly improved *in vitro* embryo development rate to blastocyst when compared with 0.3 M raffinose alone in C57BL/6 mice (Table 6). Decreases in fertilization rate and poor embryo implantation have been shown to be related to sperm DNA fragmentation (Hughes *et al.* 1996, Edwards & Beard 1999). Likewise, Larson *et al.* (2000) and Tesarik *et al.* (2004) noted that chromatin abnormalities decrease late embryonic development after ICSI treatment with human sperm. Similarly, in the results presented here, higher rates of DNA fragmentation that were obtained using 0.3 M raffinose significantly decreased embryo development rate to blastocyst when compared with fresh sperm and the combination of 0.1 M glycerol+0.2 M raffinose in C57BL6 mice (Table 6). These results suggest that the chemical formulation of CPA is important and varies in its ability to protect the DNA material of sperm during cryopreservation, and that increased levels of DNA fragmentation significantly decreases embryo development rate to blastocyst in C57BL6 mice. Recently, Seli *et al.* (2004) also reported a negative correlation between DNA fragmentation and blastocyst development for human sperm.

In summary, the results obtained from the experiments reported here support conclusion that: (1) the type of monosaccharide sugar or polyols used in CPAs are an important factor for mouse sperm cryopreservation, (2) molar concentrations of nonpermeating raffinose sugar in CPA solution have a significant impact on sperm-assessment parameters in C57BL6 and 129S mice, (3) the cryopreservation process increases DNA fragmentation

in all tested outbred, hybrid, and inbred strains of mice when compared with fresh control, (4) the resistance of sperm DNA to damage during freezing and thawing of inbred strains appears to be strain dependent, (5) C57BL/6 and 129S mice are the most sensitive strains to sperm DNA fragmentation, (6) supplementation of CPA solution with either of glycerol or fructose shows a significant protective effect for DNA integrity during cryopreservation in C57BL/6 and 129S mice, (7) supplementation of fructose or glycerol to raffinose significantly improves *in vitro* fertilization rate in C3B6F1 and C57BL/6 mice, and (8) the rate of sperm DNA fragmentation significantly decreases *in vitro* embryo development rate to blastocyst in C57BL/6 mice.

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References

- Agca Y, Gilmore J, Byers M, Woods EJ, Liu J & Critser JK 2002 Osmotic characteristics of mouse spermatozoa in the presence of extenders and sugars. *Biology of Reproduction* **67** 1493–1501.
- An TZ, Iwakiri M, Edashige K, Sakurai T & Kasai M 2000 Factors affecting the survival of frozen-thawed mouse spermatozoa. *Cryobiology* **40** 237–249.
- Anchordoguy TJ, Rudolph AS, Carpenter JF & Crowe JH 1987 Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology* **24** 324–331.
- Boggs JM & Rangaraj G 1985 Phase transitions and fatty acid spin label behavior in interdigitated lipid phases induced by glycerol and polyoxin. *Biochimica et Biophysica Acta* **27** 816 221–233.
- Chohan KR, Griffin JT & Carrell DT 2004 Evaluation of chromatin integrity in human sperm using acridine orange staining with different fixatives and after cryopreservation. *Andrologia* **36** 321–326.
- Critser JK & Moberaten LE 2000 Cryopreservation of Murine Spermatozoa. *ILAR Journal* **41** 4.
- Critser JK, Huse-Benda AR, Aaker DV, Arneson BW & Ball GD 1988 Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. *Fertility and Sterility* **50** 314–320.
- Curry MR 1995 Cryopreservation of semen from domestic livestock. *Methods in Molecular Biology* **38** 189–197.
- Dandekar PV & Glass RH 1978 Development of mouse embryos *in vitro* is affected by strain and culture medium. *Gamete Research* **17** 279–285.
- De Leeuw FE, De Leeuw AM, Den Daas JHG, Colenbrander B & Verkleij AJ 1993 Effects of various cryoprotectant agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology* **30** 32–44.
- Donnelly ET, McClure N & Lewis SE 2001 Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. *Fertility and Sterility* **76** 892–900.
- Edwards RG & Beard HK 1999 Is the success of human IVF more a matter of genetics and evolution than growing blastocysts? *Human Reproduction* **14** 1–4.
- Flaherty SP, Payne D, Swann NJ & Matthews CD 1995 Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Human Reproduction* **10** 2623–2629.
- Fraser I & Strzezek J 2004 The use of comet assay to assess DNA integrity of boar spermatozoa following liquid preservation at 5 and 16 °C. *Folia Histochemica et Cytobiologica* **42** 49–55.
- Garcia MA & Graham EF 1989 Development of a buffer system for dialysis of bovine spermatozoa before freezing. II. Effects of sugars alcohols on post thaw motility. *Theriogenology* **31** 1029–1037.
- Hammadeh ME, Greiner S, Rosenbaum P & Schmidt W 2001 Comparison between human sperm preservation medium and TEST-yolk buffer on protecting chromatin and morphology integrity of human spermatozoa in fertile and subfertile men after freeze-thawing procedure. *Journal of Andrology* **22** 1012–1018.
- Hammerstedt RH, Graham JK & Nolan JP 1990 Cryopreservation of mammalian sperm: what we ask them to survive. *Journal of Andrology* **11** 73–88.
- Ho Y, Wigglesworth K, Eppig JJ & Schultz RM 1995 Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Molecular Reproduction and Development* **41** 232–238.
- Honadel TE & Killian GJ 1988 Cryopreservation of murine embryos with trehalose and glycerol. *Cryobiology* **25** 331–337.
- Hoppe PC 1980 Genetic influences on mouse sperm capacitation *in vivo* and *in vitro*. *Gamete Research* **3** 343–349.
- Hughes CM, Lewis SE, McKelvey-Martin VJ & Thompson WA 1996 Comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Molecular Human Reproduction* **2** 613–619.
- Jiang MX, Zhu Y, Zhu ZY, Sun QY & Chen DY 2005 Effects of cooling, cryopreservation and heating on sperm proteins, nuclear DNA, and fertilization capability in mouse. *Molecular Reproduction and Development* **72** 129–134.
- Kasai K, Minato Y & Toyoda Y 1978 Fertilization and development *in vitro* of mouse eggs from inbred strains and F1 hybrids. *Japanese Journal of Animal Reproduction* **28** 19–22.
- Koshimoto C & Mazur P 2002 The effect of the osmolality of sugar-containing media, the type of sugar, and the mass and molar concentration of sugar on the survival of frozen-thawed mouse sperm. *Cryobiology* **45** 80–90.
- Larson KL, DeJonge CJ, Barnes AM, Jost LK & Evenson DP 2000 Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Human Reproduction* **15** 1717–1722.
- Linfor JJ & Mayers SA 2002 Detection of DNA damage in response to cooling injury in equine spermatozoa using single-cell gel electrophoresis. *Journal of Andrology* **23** 107–113.
- Mazur P 1970 Cryobiology: the freezing of biological systems. *Science* **22** 168 939–949.
- McGonagle LS, Goldstein M, Feldschuh J & Foote RH 2002 The influence of cryoprotective media and processing procedures on motility and migration of frozen-thawed human sperm. *Asian Journal of Andrology* **4** 137–141.
- Molinia FC, Evans G & Maxwell WMC 1994 *In vitro* evaluation of zwitterion buffers in diluents for freezing ram spermatozoa. *Reproduction, Nutrition, Development* **34** 491–500.
- Nagy A, Gersenstein M, Vintersten K, Gertsenstein M & Behringer R 2002 Manipulating the mouse embryo: a laboratory manual. In *Recovery, culture, and transfer of embryos and germ cells*. 3rd edn, pp 129–188. Cold Spring Harbor Laboratory Press: USA.
- Nakagata N 1993 Production of normal young following transfer of mouse embryos obtained by *in vitro* fertilization between cryopreserved gametes. *Journal of Reproduction and Fertility* **99** 77–80.
- Nakagata N 1995 Studies on cryopreservation of embryos and gametes in mice. *Experimental Animals* **44** 1–8.

- Nakagata N** 2000 Cryopreservation of mouse spermatozoa. *Mammalian Genome* **11** 572–576.
- Nakagata N, Matsumoto K, Anzai M, Takahashi A, Takahashi Y, Matsuzaki Y & Miyata K** 1992 Cryopreservation of spermatozoa of a transgenic mouse. *Jikken Dobutsu* **41** 537–540.
- Nishizono H, Shioda M, Takeo T, Irie T & Nakagata N** 2004 Decrease of fertilizing ability of mouse spermatozoa after freezing and thawing is related to cellular injury. *Biology of Reproduction* **71** 973–978.
- Niwa K, Araki M & Iritani A** 1980 Fertilization *in vitro* of eggs and first cleavage of embryos in different strains of mice. *Biology of Reproduction* **22** 1155–1159.
- Parks JE & Graham JK** 1992 Effects of cryopreservation procedures on sperm membranes. *Theriogenology* **38** 209–222.
- Peris SI, Morrier A, Dufour M & Bailey JL** 2004 Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. *Journal of Andrology* **25** 224–233.
- Polge C, Smith AU & Parkes AS** 1949 Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* **164** 666–668.
- Quinn P, Kerin JF & Warnes GM** 1985 Improved pregnancy rate in human *in vitro* fertilization with the use of a medium based on the composition of human tubal fluid. *Fertility and Sterility* **44** 493–498.
- Ron-el R, Nachum H, Herman A, Golan A, Caspi E & Soffer Y** 1991 Delayed fertilization and poor embryonic development associated with impaired semen quality. *Fertility and Sterility* **55** 338–344.
- Roudebush WE & Duralia DR** 1996 Superovulation, fertilization, and *in vitro* embryo development in BALB/cByJ, BALB/cJ, B6D2F1/J, and CFW mouse strains. *Laboratory Animal Science* **46** 239–240.
- Royere D, Hamamah S, Nicolle JC, Barthelemy C & Lansac J** 1988 Freezing and thawing alter chromatin stability of ejaculated human spermatozoa: fluorescence acridine orange staining and Feulgen-DNA cytophotometric studies. *Gamete Research* **21** 51–57.
- Royere D, Hamamah S, Nicolle JC & Lansac J** 1991 Chromatin alterations induced by freeze-thawing influence the fertilizing ability of human sperm. *International Journal of Andrology* **14** 328–332.
- Schneider U & Mazur P** 1984 Osmotic consequences of cryoprotectant permeability and its relation to the survival of frozen-thawed embryos. *Theriogenology* **21** 68–79.
- Schwartz NB** 1997 Carbohydrate metabolism II: special pathways. In *Textbook of Biochemistry*, 4 edn, p 307. Ed. TM Devlin. John Wiley and Son: New York, USA.
- Scott L & Whittingham DG** 1996 Influence of genetic background and media components on the development of mouse embryos *in vitro*. *Molecular Reproduction and Development* **43** 336–346.
- Seli E, Gardner DK, Schoolcraft WB, Moffatt O & Sakkas D** 2004 Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after *in vitro* fertilization. *Fertility and Sterility* **82** 378–383.
- Songsasen N & Leibo SP** 1997a Cryopreservation of mouse spermatozoa II. Relationship between survival after cryopreservation and osmotic tolerance of spermatozoa from three strains of mice. *Cryobiology* **35** 255–269.
- Songsasen N & Leibo SP** 1997b Cryopreservation of mouse spermatozoa I. Effect of seeding on fertilizing ability of cryopreserved spermatozoa. *Cryobiology* **35** 240–254.
- Storey BT, Noiles EE & Thampson KA** 1998 Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. *Cryobiology* **37** 46–58.
- Strauss G, Schurtenberger P & Hauser H** 1986 The interaction of saccharides with lipid bilayer vesicles: stabilization during freeze-thawing and freeze-drying. *Biochimica et Biophysica Acta* **858** 69–180.
- Sztejn JM, Farley JS & Mobraaten LE** 2000 *In vitro* fertilization with cryopreserved inbred mouse sperm. *Biology of Reproduction* **63** 1774–1780.
- Sztejn JM, Noble K, Farley JS & Mobraaten LE** 2001 Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. *Cryobiology* **42** 28–39.
- Tada N, Sato M, Yamanoi J, Mizorogi T, Kasai K & Ogawa S** 1990 Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol. *Journal of Reproduction and Fertility* **89** 511–516.
- Takeshima T, Nakagata N & Ogawa S** 1991 Cryopreservation of mouse spermatozoa. *Jikken Dobutsu* **40** 493–497.
- Tesarik J, Greco E & Mendoza C** 2004 Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Human Reproduction* **19** 611–615.
- Thompson KA, Richa J, Liebhaber SA & Storey BT** 2001 Dialysis addition of trehalose/glycerol cryoprotectant allows recovery of cryopreserved mouse spermatozoa with satisfactory fertilizing ability as assessed by yield of live young. *Journal of Andrology* **22** 339–344.
- Thornton CE, Brown SD & Glenister PH** 1999 Large numbers of mice established by *in vitro* fertilization with cryopreserved spermatozoa: implications and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses. *Mammalian Genome* **10** 987–992.
- Traina ME, Rescia M, Urbani E, Mantovani A, Macri C, Ricciardi C, Stazi AV, Fazzi P, Cordelli E, Eleuteri P et al.** 2003 Long-lasting effects of lindane on mouse spermatogenesis induced by *in utero* exposure. *Reproductive Toxicology* **17** 25–35.
- Watson PF** 1979 *The preservation of semen in mammals*, Oxford Reviews of Reproduction Biology, Oxford: Oxford University Press, pp 283–351.
- WHO Health Organization** 1999 *Organization Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction*, 4 Cambridge: Cambridge University Press.
- Willoughby CE, Mazur P, Peter AT & Critser JK** 1996 Osmotic tolerance limits and properties of murine spermatozoa. *Biology of Reproduction* **55** 715–727.
- Yildiz C, Kaya A, Aksoy M & Tekeli T** 2000 Influence of sugar supplementation of the extender on motility, viability, and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology* **54** 579–585.
- Yokoyama M, Akiba H, Katsuki M & Nomura T** 1990 Production of normal young following transfer of mouse embryos obtained by *in vitro* fertilization using cryopreserved spermatozoa. *Jikken Dobutsu* **39** 125–128.

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