The effects of cooling rates and type of freezing extenders on cryosurvival of rat sperm

Omer Varisli, Hollie Scott, Cansu Agca, Yuksel Agca

University of Missouri, College of Veterinary Medicine, Dept. of Veterinary Pathobiology, 1600 East Rollins Street, Columbia, MO, 65211 USA

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Cryopreservation of rat sperm is very challenging due to its sensitivity to various stress factors. The objective of this study was to determine the optimal cooling rate and extender for epididymal sperm of out-bred Sprague Dawley (SD) and inbred Fischer 344 (F344) rat strains. The epididymal sperm from 10 to 12 weeks old sexually mature SD and F344 strains were suspended in five different freezing extenders, namely HEPES buffered Tyrode's lactate (TL-HEPES), modified Kreb's Ringer bicarbonate (mKRB), 3% dehydrated skim milk (SM), Salamon's Tris-citrate (TRIS), and tes/tris (TES). All extenders contained 20% egg yolk, 0.75% Equex Paste and 0.1 M raffinose or 0.1 M sucrose. The sperm samples in each extender were cooled to 4 °C and then held for 45 min before equilibration before freezing. The equilibrated sperm samples in each extender were placed onto a shallow quartz dish inserted into Linkam Cryostage (BCS 196). The samples were then cooled to a final temperature of -150 °C by using various cooling rates (10, 40, 70, and 100 °C/min). For thawing, the quartz dish containing the sperm samples were randomly removed from the Linkam cryo-stage and placed on a 37 °C slide warmer and held for 1 min before motility analysis. Sperm membrane and acrosomal integrity and mitochondrial membrane potential (MMP) values were compared among cooling rates and extenders. Both cooling rate and type of extender had significant effect on cryosurvival (P < 0.05). Sperm motility increased as cooling rate was increased for both strains (P < 0.05). Highest cryosurvival was achieved when 100 °C/min cooling rate was used in combination with TES extender containing 20% egg yolk, 0.75% Equex paste and either 0.1 M sucrose or raffinose (P < 0.05). This study showed that TES extender containing 0.1 M raffinose or sucrose with 70 °C/min cooling rate improved post-thaw motility of rat sperm.

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Introduction

Cryobanking of reproductive cells and tissues provide benefits for agriculture, animal husbandry programs, human infertility treatments and biomedical research [16]. Rats are commonly used laboratory animals for biomedical and genomic research [28,49]. Molecular and cellular biology techniques have allowed production of thousands of new strains of laboratory animals and this process is expected to accelerate in the future. However, maintenance of live colony is costly and sometimes difficult. Cryopreservation of germplasm circumvents the need for maintenance of live colony and genetic material would still be available for future use. In addition, up to now, many inbred mutant and genetically modified rat strains have not been readily available to investigators around the world [1,28,31,49]. Cryobanking of embryos, sperm, oocytes are becoming very important both for reducing the maintenance cost and improving distribution of strains [1,36]. Cryopreservation of sperm provides a simpler and more economical alternative to cryopreservation of embryos, and reduces the cost and space needed for keeping a large number of rat strains having a single mutation [1,35].

Sperm preservation protocols vary among species due to their inherent characteristics. There are marked species differences in spermatozoa size and morphology. In addition, there are also more subtle differences in membrane phospholipid composition and metabolism of spermatozoa [6]. Rat sperm are known to have
extreme sensitivity to suboptimal conditions such as centrifugation, pipetting, chilling, osmotic stress [34,46,51] freezing and thawing [25,34,35] possibly due to unusually long tail, head shape and membrane composition [12,20,24]. Thus, acceptable and repeatable rat sperm cryopreservation protocol has not been achieved [57].

Post-thaw sperm quality is still unsatisfactory for intrauterine insemination or in vitro fertilization in rats with genetic modifications [34,57]. Despite species variation, there are common stages to any sperm freezing protocol. All protocols involve sperm collection and extension, addition of cryoprotective agents (CPA) and cooling above 0 °C, freezing below 0 °C, storage and thawing [11]. During all of these stages, spermatozoa are exposed a number of potentially damaging stresses such as the change in temperature, osmotic and toxic stresses presented by exposure to high molar concentrations of CPA and the formation and dissolution of ice crystals in the extracellular space [54]. Success of cryopreservation depends on sperm endurance to these insults [45,54]. Extenders, CPA, optimal cooling and thawing rates play important role for successful cryopreservation of sperm [10,20,30,42]. Extender composition and cooling rate have significant effects on sperm viability and there is a strong interaction between extender and cooling rate [55]. If the cooling rate is slower or faster than optimum cooling rate, this may cause irreversible damage to sperm [13,27,29]. An optimum cooling rate must be slow enough to permit water to leave the cells to avoid intracellular ice formation, and fast enough to avoid severe cell dehydration and cryo-injury due to the solution effect [29]. The optimal cooling rate is different among species and which was between 76 and 140 °C/min in bull [55], 30 °C/min in boar [13], 27–130 °C/min in mouse [27] and 10 °C/min in human [21]. But there is no published study that determined the optimal cooling rate of rat sperm.

Determination of optimal extender composition for various species has enabled development of better cryopreservation protocols [14,38,53]. An ideal sperm extender should have optimum pH, buffering capacity, suitable osmotic pressure and protect sperm against cold shock [45]. The solutions of Tris-citrate-EY, skim milk-EY, lactose-EY and Tris-TE are the most commonly used sperm extenders [56]. Krebs–Ringer bicarbonate (mKRB) solution containing raffinose, 0.75% Equex STM, 0.05% sodium dodecyl sulfate (SDS) and EY greatly enhanced the cryosurvival of rat sperm [57]. The use of EY reduces chilling injury to sperm in many mammalian species [38]. In a recent study [51], we found that the addition of 20% lactose-egg yolk (LEY) into extenders reduced motility loss after chilling. In addition, various SDS-based products improve the effectiveness of EY during sperm freezing for several mammalian species including mouse [40], rat [34], cat [5], dog [39] and pig [8]. Equex Paste (EP) and Orvus ES Paste are the commercial forms of SDS which is a water-soluble anionic detergent. Equex Paste is used for horse and swine sperm cryopreservation. Equex Paste with EY has more protective effect against freezing damage and cold shock [41] due to increase of protective activity of EY by changing the structure of lipoproteins of egg yolk [4].

Many previous reports suggest that sperm from different species respond differently to chilling, CPA, and extenders [2,17,31,44,51]. The addition of permeating CPA (e.g. glycerol) and non-permeating sugars (e.g. sucrose, raffinose and trehalose) to extenders has been effective for cryopreservation of sperm from various mammalian species [3,4]. Glycerol is the most common CPA used for freezing sperm from various species [42,45]. However, addition of glycerol to extenders was found to be detrimental to mouse sperm [26] and not effective for rat sperm freezing [34]. Furthermore, many reports suggested that raffinose is an effective CPA for mouse and rat [25,26,32,33,37,57]. For successful cryopreservation, careful selection of extender as well as an appropriate CPA that works well with the chosen extender to maintain high sperm motility after freezing is necessary [51]. In this study we performed series of experiments to determine appropriate CPA, extender and cooling rate to improve post thaw rat sperm viability.

Materials and methods

Chemicals

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

Animals

10 to 12 weeks old SD and Fisher 344 (F344) rats were used as sperm donors. 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. Rats were housed in conventional rat cages at 23–24 °C in a controlled light environment (10 h dark/14 h light) and provided free access to water and standard rodent chow.

Sperm collection

Rats were humanely euthanized by CO2 inhalation, cauda epididymides were excised and placed in a 35-mm culture dish containing 3 ml HEPES buffered Tyrode’s lactate (TL-HEPES) solution 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 10–15 min at 22 °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 22 °C in test tubes and were used for further experiments. The final concentrations of sperm samples were about 20–30 × 10⁶ sperm/ml. Each experiment was performed by using a sample from a single donor and was repeated 6 times. Thus total of six rats per rat strain were used in the experiments.

Preparation of sperm extenders

Five different base extenders namely HEPES buffered Tyrode’s lactate (TL-HEPES), Modified Krebs’ Ringer bicarbonate (mKRB), Skim milk (SM), Tris-citrate (TRIS) and TES were used.

TL-HEPES

TL-HEPES contained 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO₃, 0.4 mM NaH₂PO₄, H₂O, 10 mM Lactic Acid, 2 mM CaCl₂.2H₂O, 0.5 mM MgCl₂.6H₂O, 10 mM Hepes, 10 mL/L Penicillin/Streptomycin (10 mg streptomycin and 10,000 U penicillin in 1 mL), Bovine serum albumin (BSA; 3 mg/mL) fraction V and 0.1 M sucrose were added to obtain final freezing extender [7].

mKRB

The mKRB solution was basically the same as that was developed and used by Toyoda and Chang [50] except phenol red and BSA were not included. The modified Krebs–Ringer bicarbonate buffer contained 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂.2H₂O, 1.19 mM MgSO₄.7H₂O, 0.25 mM KH₂PO₄, 25.07 mM NaHCO₃, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 10 mL/L Penicillin/Streptomycin. The mKRB media was equilibrated in 5% CO₂ in air at 37 °C at least 5 h before use. To obtain freezing extender, 0.1 M raffinose was added to the mKRB.
SM

The SM extender was prepared by dissolving 3% (w/v) dehydrated skim milk (Difco 0032-17-3, Becton Dickinson, Franklin Lakes, NJ) and 0.1 M sucrose in TL-HEPES without NaCl. The mixture was centrifuged at 15,000g for 15 min, and the supernatant was filtered through 0.45 μm filter to obtain a final working extender.

TRIS

The TRIS extender contained 27.0 g/l Tris(hydroxymethyl)aminomethane (TRISMA Base, catalog no: T6066, Sigma, USA), 14.0 g/l citric acid and 10.0 g/l fructose [45]. To obtain freezing extender either 0.1 M sucrose (TRIS-S) or 0.1 M raffinose (TRIS-R) was added.

TES

The TES base solution consisted of 15.7 g/l N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES, catalog no: T5691, Sigma, USA) and 8.2 g/l Tris. To obtain freezing extender either 0.1 M sucrose (TES-S) or 0.1 M raffinose (TES-R) was added.

The pH and osmolality of each extender was adjusted to approximately 7.4 and 300–400 mOsm, respectively. Egg yolk phospholipids were then solubilized by adding 0.75% (v/v) Equex-Paste (Minitüb, Tiefenbach, Germany) to the extender.

Freezing and thawing

Sperm samples (100 μl) from SD or F344 were transferred into 1.5 ml centrifuge tubes containing 400 μl of each freezing extender and gently mixed by inverting the tube. After dilution, motility analysis was performed using a phase contrast microscope equipped with 20× objective. The sperm samples were then equilibrated at 4 °C for 45 min. After equilibration in the extenders, 150 μl sperm sample from each extender was placed onto a shallow quartz dish (14 mm inner diameter and 2.56 mm deep) and covered with a round coverslip and then inserted into Linkam cryostage (TMS-94) that was mounted on a Nikon microscope. The samples were then cooled by using various cooling rates (10, 40, 70 and 100 °C/min) to final temperature of −150 °C. For thawing, the quartz dish containing the sperm samples was rapidly removed from the Linkam cryostage and placed on a 37 °C slide warmer in order to have direct contact with the warm surface to achieve about 1000 °C/min warming rate. After warming, motility analysis was performed and the samples were transferred into 1.5 ml Eppendorf tubes containing 150 μl TL-HEPES base solution. All samples were underwent mitochondrial, acrosome and membrane integrity assessment.

Evaluation of sperm plasma membrane and acrosomal integrity

SYBR-14/Propidium iodide (Live/Dead sperm viability kit, catalog no: L-7011, Molecular Probes, Eugene, OR, USA) and Alexa Fluor-488-PNA (catalog no: L-21,409, Molecular Probes, Eugene, OR, USA) conjugate were used to determine rat sperm plasma membrane and acrosome integrity, respectively. For plasma membrane integrity, 200 μl TL-HEPES solution was gently added to the tube containing 100 μl thawed sperm (1–2 × 10⁶ spermatozoa/ml). Diluted sperm samples were incubated with 5 μl PI (0.5 μM final concentration) and 10 μl (0.4 μM final concentration) SYBR-14 at 37 °C for 10 min. After staining, 10 μl of sperm sample was placed on a microscope slide, covered with a coverslip and observed under the epifluorescence microscope (Nikon Eclipse 600 using a dual fluorescence filter). The images of stained sperm samples were classified into two groups: sperm head displaying green fluorescence was considered to be membrane intact, whereas sperm displaying red fluorescence in the head was considered to be damaged membrane. 100 sperm per sample were counted as described previously [52].

To evaluate sperm acrosomal integrity, thawed sperm samples were washed to remove freezing extender. After thawing, 100 μl sperm sample centrifuged at 200g for 3 min than the supernatant was removed and 320 μl TL-HEPES was added to the tube containing the sperm pellet to resuspend the sperm pellet by gentle rotation of the tube) 10 μl (1–2 × 10⁶ spermatozoa/ml) washed sperm sample was smeared onto microscope slide 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 (peanut agglutinin) at 37 °C for 30 min, washed with PBS, and analyzed by using epifluorescent microscope with an appropriate filter. The images of stained sperm samples were classified into two groups: Sperm displaying intensive and moderate bright fluorescence in the acrosomal region were considered to be intact, whereas sperm displaying weak, patchy, or no fluorescence in the acrosomal region were considered to be damaged [52]. 100 sperm on each slide were evaluated to determine the proportion of sperm with intact acrosomes.

Evaluation of sperm mitochondrial membrane potential (MMP)

The sperm MMP was evaluated using the JC-1 fluorescent dye (M34152, Molecular Probes Inc.) by the modified method that was previously described by Guthrie and Welch [18]. The JC-1 fluorescent dye was used to distinguish spermatozoa with poorly and highly functional mitochondria. In poorly functional mitochondria, JC-1 remains in the monomeric state and fluoresces green. However, in highly functional mitochondria, JC-1 forms aggregates that fluoresce orange. For evaluation of MMP in spermatozoa, 300 μl the washed (prepared before acrosomal integrity analysis) sperm suspensions (1–2 × 10⁶ spermatozoa/ml) were mixed with 10 μl JC-1 (0.75 μg final concentration). The mixture was incubated at 37 °C for 30 min and then 100 sperm per sample were analyzed by using epifluorescent microscope with a dual fluorescence filter (Nikon Eclipse 600).

Statistical analysis

Statistical analyses were performed using SPSS software (version 11.5 for Windows; SPSS Inc., Chicago, IL). The data were analyzed to determine the effects of extenders and freezing rate on motility, membrane and acrosome plasma integrity and MMP. Parametric data were analyzed by analysis of variance (Two-Way ANOVA) and if there were significant differences, Tukey test for multiple comparisons was used for post hoc analysis. The non-parametric data were analyzed Kruskal–Wallis and if there were significant differences between groups, Mann–Whitney test was used to determine the differences in groups. Statistical significance was set at P < 0.05. Values were presented as the mean ± standard error of the mean (SEM).

Results

Most of the extenders tested were effective in maintaining motility after equilibration. Motility of diluted, equilibrated and frozen-thawed SD rat sperm for different extenders and cooling rates are given in Tables 1–3. Fresh and diluted sperm motility before equilibration was between 60.0% and 76.7% for SD rats. Equilibration caused less than 10% motility loss in sperm samples diluted in extenders with the exception of m-KRB in 40 °C/min group. After freezing, sperm motility ranged between 3.7% and
32.5% (p < 0.05). Sperm samples that were frozen in TES-S extender retained the highest motility (32.5%) at 100 °C/min cooling rate (p < 0.05). Sperm samples frozen in TL-HEPES at 10 °C/min cooling rate resulted in the lowest motility (3.7%; p < 0.05). The cooling rate significantly affected sperm motility recovery in TL-HEPES, m-KRB and TES-R treatment groups (p < 0.05). Sperm motility was significantly decreased in 10 °C/min cooling rate compared to 100 °C/min cooling rate and sperm motility increased as cooling rate increased.

Membrane integrity, acrosomal integrity and MMP of frozen-thawed SD rat sperm are shown in Tables 4–6, respectively. Post-thaw membrane integrity ranged between 7.5% and 22.3% (p < 0.05). The SM, TES-R and TES-S extenders were superior for maintaining membrane integrity in sperm frozen (p < 0.05). Sperm acrosome integrity was not different among the extenders and cooling rates (p > 0.05). However, the cryopreservation caused disruption in MMP compared to fresh sperm (p < 0.05) in SD rat sperm.

**Table 1**

| Groups       | Fresh sperm | TL-HEPES | m-KRB | SM  | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|-----|--------|--------|-------|-------|
| 10 °C/min    | 74.2 ± 2.0^a| 65.0 ± 3.4^a| 55.0 ± 2.2^a| 60.0 ± 2.6^a| 60.0 ± 2.6^c| 63.3 ± 2.1^b| 60.0 ± 2.6^a| 58.3 ± 1.7^c|
| 40 °C/min    | 73.3 ± 3.3^c| 63.3 ± 3.3^b| 48.3 ± 3.1^b| 58.3 ± 1.7^b| 61.7 ± 1.7^b| 61.7 ± 1.7^b| 58.3 ± 1.7^b| 60.0 ± 0.1^b|
| 70 °C/min    | 75.0 ± 2.2^c| 60.0 ± 2.6^a| 58.3 ± 3.1^b| 65.0 ± 2.2^b| 63.3 ± 2.1^b| 65.0 ± 2.2^b| 61.7 ± 1.7^b| 56.7 ± 2.1^b|
| 100 °C/min   | 76.7 ± 2.1^b| 60.0 ± 4.5^b| 56.7 ± 4.2^a| 60.0 ± 5.2^b| 61.7 ± 4.8^b| 61.7 ± 4.8^b| 56.7 ± 4.2^b| 58.3 ± 1.7^b|
| Significance | –            | –         | –     | –   | –      | –      | –     | –     |

Values are mean percentages ± SEM (n = 6).
Differences between the same columns are not significant (p > 0.05).

**Table 2**

| Groups       | Fresh sperm | TL-HEPES | m-KRB | SM  | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|-----|--------|--------|-------|-------|
| 10 °C/min    | 74.2 ± 2.0^a| 65.0 ± 3.4^a| 55.0 ± 2.2^a| 60.0 ± 2.6^a| 60.0 ± 2.6^c| 63.3 ± 2.1^b| 60.0 ± 2.6^a| 58.3 ± 1.7^c|
| 40 °C/min    | 73.3 ± 3.3^c| 63.3 ± 3.3^b| 48.3 ± 3.1^b| 58.3 ± 1.7^b| 61.7 ± 1.7^b| 61.7 ± 1.7^b| 58.3 ± 1.7^b| 60.0 ± 0.1^b|
| 70 °C/min    | 75.0 ± 2.2^c| 60.0 ± 2.6^a| 58.3 ± 3.1^b| 65.0 ± 2.2^b| 63.3 ± 2.1^b| 65.0 ± 2.2^b| 61.7 ± 1.7^b| 56.7 ± 2.1^b|
| 100 °C/min   | 76.7 ± 2.1^b| 60.0 ± 4.5^b| 56.7 ± 4.2^a| 60.0 ± 5.2^b| 61.7 ± 4.8^b| 61.7 ± 4.8^b| 56.7 ± 4.2^b| 58.3 ± 1.7^b|
| Significance | –            | –         | –     | –   | –      | –      | –     | –     |

Values are mean percentages ± SEM (n = 6).
Differences between the same columns are not significant (p > 0.05).

**Table 3**

| Groups       | Fresh sperm | TL-HEPES | m-KRB | SM  | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|-----|--------|--------|-------|-------|
| 10 °C/min    | 74.2 ± 2.0^a| 3.7 ± 0.9^b| 6.7 ± 1.1^b| 10.8 ± 2.4^b| 11.7 ± 3.8^b| 9.2 ± 0.8^b| 17.5 ± 3.1^c| 21.7 ± 3.8^b|
| 40 °C/min    | 73.3 ± 3.3^c| 6.3 ± 1.7^b| 12.5 ± 2.8^b| 20.8 ± 3.3^b| 12.8 ± 3.8^b| 15.8 ± 4.9^b| 25.0 ± 2.2^b| 23.3 ± 1.7^b|
| 70 °C/min    | 75.0 ± 2.2^c| 6.6 ± 1.1^b| 16.7 ± 1.7^b| 16.7 ± 2.1^b| 19.1 ± 3.2^b| 19.2 ± 3.3^b| 30.0 ± 2.9^b| 27.5 ± 3.6^b|
| 100 °C/min   | 76.7 ± 2.1^b| 10.0 ± 1.8^a| 15.8 ± 2.0^a| 19.2 ± 2.7^b| 21.7 ± 2.1^b| 22.5 ± 5.3^b| 31.7 ± 1.7^c| 32.5 ± 3.1^b|
| Significance | –            | –         | –     | –   | –      | –      | –     | –     |

Values are mean percentages ± SEM (n = 6).
Differences between the same columns are not significant (p > 0.05).

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**Table 4**

| Groups       | Fresh sperm | TL-HEPES | m-KRB | SM  | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|-----|--------|--------|-------|-------|
| 10 °C/min    | 59.6 ± 1.9^b| 7.5 ± 1.1^c| 9.7 ± 1.3^b| 14.7 ± 2.4^b| 9.8 ± 1.9^b| 8.7 ± 1.7^b| 14.2 ± 2.2^b| 17.0 ± 1.7^b|
| 40 °C/min    | 55.5 ± 3.3^b| 12.3 ± 2.6^b| 15.0 ± 2.0^b| 22.3 ± 2.1^b| 13.2 ± 3.6^b| 17.3 ± 2.9^b| 16.5 ± 2.2^b| 17.3 ± 1.2^b|
| 70 °C/min    | 59.0 ± 2.7^b| 13.3 ± 1.9^b| 16.8 ± 2.0^b| 15.3 ± 2.6^b| 14.0 ± 1.7^b| 13.2 ± 1.4^b| 18.2 ± 1.5^b| 18.7 ± 1.8^b|
| 100 °C/min   | 61.5 ± 2.1^c| 11.5 ± 1.9^b| 13.2 ± 2.7^b| 16.2 ± 1.7^b| 16.2 ± 1.6^b| 16.0 ± 2.1^b| 21.2 ± 1.8^b| 21.2 ± 1.4^b|
| Significance | –            | –         | –     | –   | –      | –      | –     | –     |

Values are mean percentages ± SEM (n = 6).
Differences between the same columns are not significant (p > 0.05).

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significantly affected motility recovery (p < 0.05) and the highest motility was achieved in sperm exposed to TES-R and TES-S extenders at 70 and 100 °C/min cooling rates. Lower cooling rates were highly detrimental to motility (p < 0.05).

### Table 5
Percent post-thaw acrosome integrity of Sprague–Dawley rat sperm using various cooling rates and freezing extenders.

| Cooling rate | Fresh sperm | TL-HEPES | m-KRB | SM | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|----|--------|--------|-------|-------|
| 10 °C/min    | 76.2 ± 2.9b | 23.3 ± 3.7a | 26.3 ± 3.6a | 22.2 ± 3.6a | 26.5 ± 4.4a | 29.0 ± 4.6a | 25.0 ± 5.0b | 27.3 ± 3.4a |
| 40 °C/min    | 76.0 ± 6.1b | 24.0 ± 4.0a | 26.2 ± 3.5a | 25.3 ± 3.3a | 27.7 ± 2.8a | 32.2 ± 3.4a | 27.3 ± 3.6a | 29.7 ± 4.5a |
| 70 °C/min    | 76.3 ± 5.7b | 23.3 ± 3.7a | 26.8 ± 4.2a | 21.2 ± 2.2a | 24.5 ± 3.1a | 25.5 ± 4.8a | 26.8 ± 3.2a | 26.2 ± 4.5a |
| 100 °C/min   | 74.3 ± 4.6b | 25.0 ± 3.3a | 26.3 ± 3.9a | 23.3 ± 3.7a | 24.7 ± 3.7a | 26.8 ± 3.5a | 27.0 ± 4.1a | 27.8 ± 4.4a |

Values are mean percentages ± SEM (n = 6). Differences between the within the same columns are not significant (P > 0.05).

\(^{a–b}\) Different superscripts within the same row denote significant differences (P < 0.05).

### Table 6
Percent post-thaw mitochondrial membrane integrity of Sprague–Dawley rat sperm using various cooling rates and freezing extenders.

| Cooling rate | Fresh sperm | TL-HEPES | m-KRB | SM | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|----|--------|--------|-------|-------|
| 10 °C/min    | 52.6 ± 3.0c | 3.0 ± 0.8a | 2.5 ± 0.5a | 4.5 ± 0.8a,b | 4.5 ± 1.2a,b | 4.0 ± 0.9a,b | 5.7 ± 0.8b | 5.8 ± 0.7b |
| 40 °C/min    | 45.5 ± 5.6c | 2.5 ± 0.5a | 2.5 ± 0.3a | 4.7 ± 0.8a,b | 5.0 ± 1.5a,b | 5.3 ± 0.7a | 3.3 ± 0.4a | 4.3 ± 0.4b |
| 70 °C/min    | 50.8 ± 3.0c | 2.3 ± 0.6a | 4.2 ± 0.9b | 4.3 ± 0.4a | 4.8 ± 0.7b,c | 4.7 ± 1.4b | 4.3 ± 0.8b | 5.3 ± 0.8b |
| 100 °C/min   | 52.8 ± 3.0c | 2.3 ± 0.2a | 2.0 ± 0.7a | 5.5 ± 0.4b | 4.2 ± 0.7a | 4.2 ± 0.7b | 3.3 ± 0.7a | 3.3 ± 0.7a |

Values are mean percentages ± SEM (n = 6). Differences between the same columns are not significant (P > 0.05).

\(^{a–d}\) Different superscripts within the same row denote significant differences (P < 0.05).

### Table 7
Percent motility of F344 rat sperm diluted different extenders.

| Groups       | Fresh sperm | TL-HEPES | m-KRB | SM | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|----|--------|--------|-------|-------|
| 10 °C/min    | 71.7 ± 3.1b | 66.6 ± 3.3a,b | 61.7 ± 1.7a | 63.3 ± 2.1a,b | 61.7 ± 1.7a | 60.0 ± 2.6a | 60.0 ± 2.6a | 58.3 ± 3.1a |
| 40 °C/min    | 71.7 ± 3.1c | 70.0 ± 2.6a | 65.0 ± 2.2a | 66.7 ± 2.1a | 65.0 ± 2.2a | 66.7 ± 2.1a | 63.3 ± 2.1a | 63.3 ± 3.3a |
| 70 °C/min    | 74.2 ± 2.0d | 68.3 ± 1.7a | 65.0 ± 2.2a | 65.0 ± 2.2a | 63.3 ± 2.1a | 61.7 ± 3.1a | 61.7 ± 1.7a | 61.7 ± 3.1a |
| 100 °C/min   | 75.8 ± 2.0b | 66.7 ± 3.3a | 61.7 ± 1.7a | 65.0 ± 2.2a | 61.7 ± 3.1a | 61.7 ± 3.1a | 60.0 ± 2.6a | 58.3 ± 3.1a |

Values are mean percentages ± SEM (n = 6). Differences between the same columns are not significant (P > 0.05).

\(^{a–b}\) Different superscripts within the same row denote significant differences (P < 0.05).

### Table 8
Percent motility of F344 rat sperm equilibrated in different extenders.

| Groups       | Fresh sperm | TL-HEPES | m-KRB | SM | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|----|--------|--------|-------|-------|
| 10 °C/min    | 71.7 ± 3.1b | 61.7 ± 3.1a,b | 53.3 ± 4.2a | 55.0 ± 2.2a | 58.3 ± 1.7a | 60.0 ± 2.6a | 55.0 ± 3.4a | 56.7 ± 2.1a |
| 40 °C/min    | 71.7 ± 3.1c | 68.3 ± 1.7a,c | 55.8 ± 3.3a | 60.0 ± 2.6a,b,c | 61.7 ± 3.1a,b,c | 61.7 ± 3.1a | 61.7 ± 3.1a | 61.7 ± 3.1a |
| 70 °C/min    | 74.2 ± 2.0d | 66.7 ± 2.1a,b | 58.3 ± 3.1a | 61.7 ± 1.7a | 61.7 ± 1.7a | 60.0 ± 2.6a | 60.0 ± 0.0a | 60.0 ± 2.6a |
| 100 °C/min   | 75.8 ± 2.0b | 61.7 ± 3.1a | 51.7 ± 4.0a | 55.0 ± 2.2a | 63.3 ± 2.1a,b | 61.7 ± 3.1a | 56.7 ± 4.2a | 55.0 ± 2.2a |

Values are mean percentages ± SEM (n = 6). Differences between the same columns are not significant (P > 0.05).

\(^{a–c}\) Different superscripts within the same row denote significant differences (P < 0.05).

### Table 9
Percent post-thaw motility of F344 rat sperm in different extenders and cooling rates.

| Cooling rate | Fresh sperm | TL-HEPES | m-KRB | SM | TRIS-R | TRIS-S | TES-R | TES-S |
|--------------|-------------|----------|-------|----|--------|--------|-------|-------|
| 10 °C/min    | 74.2 ± 2.0d | 3.2 ± 0.8a | 9.2 ± 1.5b | 11.7 ± 2.1b,c | 7.5 ± 1.1b | 13.3 ± 1.7b,c | 19.2 ± 2.7c | 15.0 ± 2.6a,b,c |
| 40 °C/min    | 71.7 ± 3.1c | 4.7 ± 1.8a | 14.2 ± 2.7b,c | 14.2 ± 2.0b,c | 15.0 ± 2.6b | 14.2 ± 3.5b | 25.0 ± 2.2b | 21.7 ± 3.1c |
| 70 °C/min    | 74.2 ± 2.0c | 9.3 ± 0.9a | 15.0 ± 1.8a | 18.3 ± 1.1a | 15.0 ± 3.7a | 9.2 ± 2.0a | 33.3 ± 2.5b | 30.0 ± 2.6a |
| 100 °C/min   | 75.8 ± 2.0b | 8.3 ± 1.1a | 15.0 ± 3.2b | 15.0 ± 1.3a | 14.2 ± 3.7b,c | 11.7 ± 3.6b,c | 29.2 ± 0.8a | 32.5 ± 3.1a |

Values are mean percentages ± SEM (n = 6). Differences between the same columns are not significant (P > 0.05).

\(^{a–d}\) Different superscripts within the same row denote significant differences (P < 0.05).

\(^*\) Differences between the same columns are significant (P < 0.05).

\(^*\) Differences between the same columns are significant (P < 0.05).
Membrane and acrosome integrity and MMP of frozen-thawed F344 rat sperm for different extenders and cooling rates are given in Tables 10–12, respectively. Membrane integrity after freezing and thawing processes were between 8.8% (for TRIS-S, at 100 °C/min cooling rate) and 21.3% (for TES-S, at 70 °C/min cooling rate; p < 0.05). Post-thaw membrane integrity was lower than motility except for TL-HEPES. Sperm acrosome integrity was not affected significantly from the extenders or cooling rates (p > 0.05). But cryopreservation procedure caused the greatest disruption in MMP (p < 0.05) in F344 rat sperm. The sperm that was frozen in m-KRB containing 0.1 M raffinose, 0.75% Equex STM and 20% EY. It has been widely reported that non-permeating CPAs preserved Wistar rat sperm in m-KRB provided better recovery of post-thaw motility compared to TL-HEPES and m-KRB extenders served as good extenders but SM was not effective against chilling injury [52]. In our previous study, TL-HEPES, SM, Lactose, Tris and TES extenders served as good extenders but SM was not effective against chilling injury [51]. On the other hand, extender containing SM and 0.1 M raffinose in this study was effective against chilling injury, but it was ineffective during freezing. Cryopreservation in extender containing 0.1 M raffinose or 0.1 M sucrose prepared in TES medium with 0.75% Equex-Paste and 20% egg yolk significantly improved the sperm motility compared to TL-HEPES and m-KRB for both SD and F344 strains. Yamashiro et al. [52] found that cryopreserving Wistar rat sperm in m-KRB provided better recovery of its effectiveness for mouse sperm, skim milk was not effective in protecting rat sperm from freezing injury, even sperm from very closely related species (i.e. rats and mice) have their specific cryobiologic characteristics and emphasized the importance of developing species-specific freezing protocols.

Compared to mouse sperm [33,48], there have been little success in freezing rat sperm [22,23,34,57,58]. Yamashiro et al. [57] reported higher (39.3%) post-thaw motility for epididymal rat sperm in mKRB containing 0.1 M raffinose, 0.75% Equex STM and 20% EY. It has been widely reported that non-permeating CPAs are more effective than permeating CPAs for both rat and mouse sperm freezing [25,26,32,33,37,57]. The current study also showed that freezing extender containing 0.1 M raffinose provided good cryoprotection for rat sperm. In addition, while the protective effect of non-permeating CPAs against cooling [51] was reported, few studies showed ineffectiveness of permeating CPA, glycerol, during rat sperm cryopreservation [34,57].

In general, damage to sperm during cryopreservation have been attributed to several factors including cold shock, freezing injury, oxidative stress, alterations in membrane compositions, chemical toxicity of CPA, and osmotic stress [9]. In order to develop an optimized rat sperm freezing procedure, we studied optimal cooling rate in various sperm extenders. A simple and effective freezing medium consisting of 18% raffinose and 3% skim milk without any permeating CPA has been successfully used to cryopreserve sperm from many inbred and outbred mouse strains [26,43]. One of the interesting findings of the current study is that, in contrast to its effectiveness for mouse sperm, skim milk was not effective in protecting rat sperm from freezing injury, even sperm from very closely related species (i.e. rats and mice) have their specific cryobiologic characteristics and emphasized the importance of developing species-specific freezing protocols.

**Table 10** Percent post-thaw plasma membrane integrity of F344 rat sperm using various cooling rates and freezing extenders.

| Cooling rates | Fresh sperm | TL-HEPES | m-KRB | SM  | TRIS-R | TRIS-S | TES-R | TES-S |
|---------------|-------------|----------|-------|-----|--------|--------|-------|-------|
| 10 °C/min     | 59.5 ± 1.3 b| 9.3 ± 1.9 | 13.0 ± 2.0 | 15.2 ± 1.7 | 10.8 ± 1.3 | 12.8 ± 2.1 | 17.0 ± 1.2 | 13.0 ± 1.0 |
| 40 °C/min     | 58.0 ± 3.2 b| 9.3 ± 2.7 | 16.8 ± 1.9 | 16.0 ± 2.0 | 14.0 ± 1.7 | 17.3 ± 2.4 | 17.8 ± 1.2 | 18.8 ± 2.8 |
| 70 °C/min     | 56.0 ± 3.0 | 12.5 ± 1.9 | 15.3 ± 1.6 | 20.2 ± 0.9 | 12.0 ± 1.2 | 15.8 ± 2.1 | 19.3 ± 1.3 | 21.3 ± 2.5 |
| 100 °C/min    | 60.2 ± 2.4 a| 11.3 ± 1.1 | 12.7 ± 2.0 | 15.0 ± 2.0 | 11.3 ± 2.3 | 8.8 ± 2.2 | 15.2 ± 1.6 | 18.0 ± 1.2 |

Values are mean percentages ± SEM (n = 6). Differences between the same columns are not significant (P > 0.05).

**Table 11** Percent post-thaw acrosome integrity of F344 rat sperm using various cooling rates and freezing extenders.

| Cooling rates | Fresh sperm | TL-HEPES | m-KRB | SM  | TRIS-R | TRIS-S | TES-R | TES-S |
|---------------|-------------|----------|-------|-----|--------|--------|-------|-------|
| 10 °C/min     | 66.5 ± 6.8 | 18.5 ± 3.1 | 18.8 ± 2.2 | 19.7 ± 2.1 | 21.3 ± 2.3 | 26.8 ± 3.5 | 20.3 ± 2.7 | 24.3 ± 2.6 |
| 40 °C/min     | 68.7 ± 7.8 | 25.3 ± 5.6 | 23.2 ± 2.9 | 23.7 ± 2.9 | 25.0 ± 3.7 | 32.2 ± 4.9 | 23.2 ± 3.0 | 24.5 ± 2.7 |
| 70 °C/min     | 78.5 ± 4.4 | 32.0 ± 5.0 | 30.7 ± 3.2 | 29.8 ± 2.7 | 19.5 ± 1.3 | 23.3 ± 5.0 | 24.0 ± 3.5 | 25.0 ± 2.1 |
| 100 °C/min    | 68.7 ± 3.8 | 25.7 ± 3.8 | 29.3 ± 1.0 | 29.8 ± 2.4 | 25.0 ± 2.4 | 28.2 ± 3.0 | 30.0 ± 3.2 | 27.3 ± 2.6 |

Values are mean percentages ± SEM (n = 6). Differences between the same columns are significant (P < 0.05).

**Table 12** Percent post-thaw mitochondrial membrane integrity of F344 rat sperm using various cooling rates and freezing extenders.

| Cooling rates | Fresh sperm | TL-HEPES | m-KRB | SM  | TRIS-R | TRIS-S | TES-R | TES-S |
|---------------|-------------|----------|-------|-----|--------|--------|-------|-------|
| 10 °C/min     | 42.2 ± 3.4 | 1.7 ± 0.2 | 3.7 ± 0.6 | 4.2 ± 1.3 | 3.8 ± 0.9 | 3.5 ± 0.7a | 5.7 ± 0.6b | 5.0 ± 1.2b |
| 40 °C/min     | 44.2 ± 4.0 | 3.2 ± 0.7 | 4.7 ± 1.0 | 4.8 ± 1.2 | 3.2 ± 0.5 | 3.3 ± 0.7a | 4.5 ± 0.9a | 4.0 ± 0.8a |
| 70 °C/min     | 44.7 ± 2.8 | 4.0 ± 0.6 | 4.5 ± 1.1 | 4.3 ± 1.1 | 4.7 ± 1.1 | 3.3 ± 0.7a | 4.8 ± 1.3a | 4.7 ± 1.1a |
| 100 °C/min    | 45.3 ± 4.1 | 3.7 ± 0.8 | 4.7 ± 1.4 | 5.8 ± 1.2 | 4.0 ± 0.9 | 3.7 ± 1.0a | 5.7 ± 1.2a | 5.2 ± 1.2a |

Values are mean percentages ± SEM (n = 6). Differences between the same columns are not significant (P > 0.05).

**Discussions**

In general, damage to sperm during cryopreservation have been attributed to several factors including cold shock, freezing injury, oxidative stress, alterations in membrane compositions, chemical toxicity of CPA, and osmotic stress [9]. In order to develop an optimized rat sperm freezing procedure, we studied optimal cooling rate in various sperm extenders. A simple and effective freezing medium consisting of 18% raffinose and 3% skim milk without any permeating CPA has been successfully used to cryopreserve sperm from many inbred and outbred mouse strains [26,43]. One of the interesting findings of the current study is that, in contrast to its effectiveness for mouse sperm, skim milk was not effective in protecting rat sperm from freezing injury, even sperm from very closely related species (i.e. rats and mice) have their specific cryobiologic characteristics and emphasized the importance of developing species-specific freezing protocols.

Compared to mouse sperm [33,48], there have been little success in freezing rat sperm [22,23,34,57,58]. Yamashiro et al. [57] reported higher (39.3%) post-thaw motility for epididymal rat sperm in mKRB containing 0.1 M raffinose, 0.75% Equex STM and 20% EY. It has been widely reported that non-permeating CPAs are more effective than permeating CPAs for both rat and mouse sperm freezing [25,26,32,33,37,57]. The current study also showed that freezing extender containing 0.1 M raffinose provided good cryoprotection for rat sperm. In addition, while the protective effect of non-permeating CPAs against cooling [51] was reported, few studies showed ineffectiveness of permeating CPA, glycerol, during rat sperm cryopreservation [34,57].
motility (39.3%) and acrosome (89.3%) integrity. Sperm cryopreserved in mKRB in this current study had lower motility (16.7% and 15.0% for F344 and SD rats). However, in a recent study, Yamashiro et al. [58] reported lower post-thaw sperm motility (21.5%) when m-KRB was used as an extender. These inconsistencies in post-thaw sperm characteristics in epididymal rat sperm may be attributed to (1) uncontrolled cooling rate (2) lack of optimal sperm extender components and (3) suboptimal handling throughout the cryopreservation procedure. For example although mouse sperm freezing protocol developed by Nakagata et al. [3], has been universally used to cryobank sperm from thousands of mouse strains, there are still undetermined aspects of the freezing protocol that can lead to significant differences in outcome.

Medium composition and cooling rate have significant effects on sperm survival and the interaction between medium and cooling rate was previously observed [55]. To date, freezing extender containing 23% (v/v) egg yolk, 8% (w/v) lactose monohydrate has been most commonly used extender used to cryopreserve rat sperm [34]. All of these previous studies cooled straws containing rat sperm by holding 2 cm above the liquid nitrogen level (at ~150 to −170 °C) for 10–15 min before plunging into LN2 [34,56–58]. However, they did not report exact cooling rates. To date there has been only one fundamental cryobiologic study investigating optimal cooling rate for rat sperm. Hagiwara et al. [19] evaluated the biophysics (membrane permeability) of rat sperm to better understand the cooling rate response that contributes to cryopreservation success. A differential scanning calorimeter studies pre-cooling and experimentally tested optimal cooling rates that ranged from 53 to 70 °C/min for rat sperm. Maximum motility was obtained with cooling rates between 50 and 80 °C/min.

This is one of the first studies which aimed at determining optimal cooling rate using a Linkam Cryostage. Optimal cooling rate varies from species to species. It has been shown for mouse sperm that cryo-survival is dependent significantly on the cooling rate, and less strongly associated with the warming rates as long as rapid warming (~1000 °C/min) is used. In this study we also used the rapid warming (~1000 °C/min). Cooling rate significantly affected post-thaw motility of SD sperm in TL-HEPES, m-KRB and TES-R extenders and motility of F344 sperm in TL-HEPES, SM, TES-R and TES-S extenders. In these extenders, post-thaw motility decreased significantly in 10 °C/min cooling rate compared to 100 °C/min cooling rate. The highest motility was obtained when rat sperm was cooled between 40 and 100 °C/min. This is consistent with the previous report from Hagiwara et al. [19] in that maximum motility was obtained with cooling rates between 50 and 80 °C/min. In this study we did not investigate cooling rate higher than 100 °C/min because of the limitation of Linkam cooling stage. Most commonly used cooling machines in laboratories cannot reach controlled cooling rate of 100 °C/min and above. It is accepted that constant cooling of rat sperm cannot be achieved in LN2 which cools sperm between 100 and 130 °C/min. Stacy et al. [47] has elegantly demonstrated low reproducibility of freezing protocols due mainly to variation in cooling rate in LN2 vapor. For mouse sperm, cooling rate of 114 °C/min resulted in higher motility than cooling rate of 39 °C/min but, cooling rate of 192 °C/min led to the lowest motility [47]. Similarly, Koshimoto and Mazur [27] showed that cooling rate between 27 and 130 °C/min resulted in more motile sperm compared to the lower or higher rates.

In this study, freezing and thawing of rat sperm resulted in decreased motility, plasma membrane integrity, acrosome integrity and MMP. Our results indicate that MMP was highly affected from freezing for both SD and F344 rat strains. The motility and acrosome integrity of SD rat sperm were approximately 32% and 27% for TES-R and TES-S extenders at 100 °C/min cooling rate. On the other hand, plasma and mitochondrial membrane integrity were approximately 21% and 4% for TES-R and TES-S, respectively. These results suggest that freezing injury and lower progressive motility in rat sperm may be mostly caused by damage to MMP. Yamashiro et al. [58] previously showed that supplementation adenosine 5-triphosphate (ATP) to extender, before freezing, enhanced sperm cryosurvival by improving the metabolic capacity of rat sperm. Similarly, Kim et al. [25] in our laboratory obtained slightly higher total (36.5%) and progressive (6.0%) motility after adding 2 g/L ATP to TES-sucreo-EY extender. However, plasma membrane integrity and MMP showed only a slight increase compared to this study.

Sperm motility is the most commonly used assay to evaluate fresh or frozen-thawed sperm quality. But this assay is not enough to determine the fertility of sperm samples. Cell viability, acrosom integrity and mitochondrial function evaluation enable more accurate description of spermatozoa’s fertilization capacity [15]. Post-thaw spermatozoa could be motile but incapable of fertilization due to acrosomal damage [43]. For this reason, all sperm parameters should be taken into consideration to evaluate sperm fertility capability. In this study, motility was the least affected parameter from freezing compared to membrane, acrosome and mitochondrial membrane integrity. Acrosome integrity decreased after freezing but was not affected from freezing rate and extenders and ranged 18.5–32.2% for both SD and F344 sperm. This result was lower than the study of Yamashiro et al. [57] who reported 89.3% acrosome integrity in mKRB extender. This conflict may be due to classification of intact and damaged spermatozoa. Another interesting result revealed in our study was that the extenders and cooling rates were not particularly effective in protecting acrosome integrity from freezing injury. In addition, we found that sperm membrane integrity and MMP were highly affected from freezing compared to motility. Besides lower MMP rate, weak membrane integrity may be involved in low progressive motility of rat sperm.

In summary, freezing procedure significantly decreased the motility of rat sperm, but there was no difference between Sprague–Dawley and F344 rat strains. Although SM has been successfully used to cryopreserve mouse sperm, it did not provide cryoprotection for rat sperma. In addition, the results revealed weak interaction between extenders and the cooling rate on the rat sperm viability parameters. Our results indicate that TES extender containing non-penetrating CPA (raffinose or sucrose) with moderate (40 °C/min) and fast (100 °C/min) cooling rate was superior to other extenders and cooling rates tested.

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