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

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ABSTRACT

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 outbred 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 held for 45 min for 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 rapidly 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) were assessed by SYBR-14/Propidium iodide, Alexa Fluor-488-PNA conjugate and JC-1, respectively. The total motility, acrosomal integrity, membrane integrity and 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 and 100 °C/min cooling rate improved post-thaw motility of rat sperm.

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

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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-TES are the most commonly used sperm extenders [56]. Krebs–Ringer bicarbonate (mKRB) solution containing raffinose, 0.75% Equex STM, 0.05% sodium dodecly 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 CO_2 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 experimentations. The final concentrations of sperm samples were about 20–30 $\times$ 10^6 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 Kreb's 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 $NaHCO_3$, 0.4 mM $NaH_2PO_4.H_2O$, 10 mM Lactic Acid, 2 mM $CaCl_2.2H_2O$, 0.5 mM $MgCL_2.6H_2O$, 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 $CaCl_2.2H_2O$, 1.19 mM $MgSO_4.7H_2O$, 1.19 mM KH_2PO_4, 25.07 mM $NaHCO_3$, 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% CO_2 in air at 37 °C at least 5 h before use. To obtain freezing extender, 0.1 M raffinose was added to the mKRB.