Cryobiology 66 (2013) 275–282

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender ☆

Abozar Najafi [a], Mahdi Zhandi [a,*], Armin Towhidi [a], Mohsen Sharafi [a], Abbas Akbari Sharif [b], Mahdi Khodaei Motlagh [c], Felipe Martinez-Pastor [d]

[a] Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran
[b] Jahad-e-Keshavarzi Organization of Tehran Province, Breeding Station of Zandi Sheep, Pishva-Varamin, Iran
[c] Department of Animal Science, Faculty of Agriculture, Arak University, Arak, Iran
[d] INDEGSAL and Molecular Biology, University of León, León, Spain

ARTICLE INFO

Article history:
Received 21 February 2013
Accepted 3 March 2013
Available online 13 March 2013

Keywords:
Ram semen
Trehalose
Soybean lecithin
Cryopreservation
Glycerol

ABSTRACT

The objective of this study was to examine the interaction of different concentrations of trehalose [0 (T0), 50 (T50) or 100 (T100) mM] and glycerol [5% (G5) or 7% (G7)] on post-thawed quality of ram semen, cryopreserved in a soybean lecithin (SL)-based extender. Twenty-eight ejaculates were collected from four rams and diluted with six trehalose/glycerol combinations: T0G5, T50G5, T100G5, T0G7, T50G7, and T100G7. Sperm motility (CASA), membrane integrity (eosin/nigrosin) and functionality (HOST), abnormal forms, capacitation status (CTC), mitochondrial activity (rhodamine 123), apoptotic features (Annexin V/propidium iodide) and lipoperoxidation (malondialdehyde production) were evaluated after thawing. Extender T100G5 yielded the highest results for total and progressive motility, sperm velocity, normal morphology, functional membranes, active mitochondria and membrane integrity, with $P < 0.05$ in general, except for T50G7 ($P > 0.05$). The combinations T0G5, T0G7 and T100G7 yielded the lowest post-thaw quality. We could not detect significant changes in other kinematic parameters, capacitation status or lipoperoxidation. We conclude that, in our SL-based extender, a combination of 100 mM trehalose and 5% glycerol was the most adequate combination to achieving post-thawing quality in our soybean lecithin-based extender, and our results support that a synergistic effect among trehalose and glycerol exists. We suggest that other combinations could improve these results.

Introduction

Sugars play an important role during the cryopreservation of biological material, not only due to their osmotic effects, but also to the interaction with the phospholipid bilayers at the low hydration conditions occurring during the freezing process, contributing to stabilize them [16]. Sugars can also depress the membrane phase transition temperature of dehydrated lipids, preventing or delaying this phase transition, and thus reducing shedding of components and membrane fusion. Therefore, sugars have been considered for sperm cryopreservation, not only as a source of energy for the spermatozoa (glucose or fructose), but also to prevent structural and sub-structural damage of sperm during this dehydrated reduced-water state [1,21].

Many authors have been used isoosmotic extenders for cryopreservation of ram semen. However, hyperosmotic extenders using different sugars and sugar concentrations have been shown to improve ram semen quality after the freeze–thawing process [4,5,27,52,53]. Among them, trehalose is a non-penetrating disaccharide that seems to protect cells both by increasing the tonicity of the extender and by stabilizing the plasma membrane, possibly due to direct interaction with phospholipid polar head groups of membrane phospholipids [15]. Trehalose seems to be more efficient than other sugars for protection of spermatozoa in cryopreservation media, and many authors have reported its beneficial effect for semen cryopreservation in different species, such as ram [4,27,38], goat [1,2], bull [13,55], boar [25] and mouse [51]. In contrast, several studies have reported no significant positive effect of trehalose for cryopreserving spermatozoa from stallion [49], Iberian red deer [21], European brown hare [30], rooster [33] and emu [48].

☆ Experiment was designed by M. Zhandi and A. Towhidi. Semen freezing and post thawed sperm evaluations were done by A. Najafi, M. Sharafi, A. Akbari Sharif and M. Khodaei Motlagh. Manuscript was written by A. Najafi, M. Sharafi, M. Zhandi and F. Martinez-Pastor.

* Corresponding author. Fax: +98 26 32246752.
E-mail address: mzhandi@ut.ac.ir (M. Zhandi).

0011-2240/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cryobiol.2013.03.002

Whereas sugars are non-permeating cryoprotectants, other substances such as glycerol penetrate within the cell, stabilizing the intracellular components [18,42]. The effects of these substances are multiple, preventing intracellular ice formation, contributing to stabilize lipids and proteins in the sperm membrane and increasing dehydration as well as membrane fluidity [22,24]. It seems that combining of penetrating and non-penetrating cryoprotectants in semen extender would be better than that of single cryoprotectant [10]. Previous reports have been shown the combination effect of trehalose and glycerol in egg yolk-based or LDL-based extenders [25,32]. Although we could expect a synergic effect between glycerol and trehalose, due to their different properties, few studies have tested the interaction of different concentrations of these two cryoprotectors [20,31,50].

Moreover, to our knowledge, there are no reports about the synergistic effects of trehalose and glycerol in a soy lecithin (SL)-based extender for cryopreservation of ram semen. Therefore, the objective of this study was to examine the combined effect of different concentrations of trehalose and glycerol in a SL-based extender on some ram sperm parameters after the freeze–thawing process.

Materials and methods

Chemicals

Unless otherwise indicated, all chemicals used in this study were obtained from Sigma (St. Louis, MO, USA), and Merck (Darmstadt, Germany).

Semen collection, processing and extender preparation

Semen were collected from four mature Zandi ram (3 and 4 years of age), of superior genetic merit and proven fertility. A total of 28 ejaculations (seven ejaculates for each ram) were collected twice a week from each ram using an artificial vagina, during the breeding season (autumn). The primary criteria for evaluation of sperm were: volume of 0.75–2 mL; semen concentration above than 3×10^9 sperm/mL; progressive motility higher than 70%; less than 10% abnormal sperm. To eliminate individual differences, semen were pooled and processed for extension.

The basic extender used in this study was composed of 27.1 g/L Tris, 10 g/L fructose, and 14 g/L citric acid. Soybean lecithin was added to the basic extender at 1% (wt/vol). The osmolarity and pH of this base extender were set at 320 mOsm and 7.2, respectively. The base extender was supplemented either with 5% (G5) or 7% (G7) glycerol and either 0 mM (T0), 50 mM (T50) or 100 mM (T100) trehalose. Each pooled ejaculate was split into six equal aliquots and diluted (37 °C) with each of the six extenders: T0G5, T50G5, T100G5, T0G7, T50G7 and T100G7, for a total of six experimental groups. Diluted samples were loaded into 0.25 mL French straws (IMV, L'Aigle, France) at a final concentration of 4×10^8 sperm/mL and equilibrated at 4 °C for a period of 2 h. After equilibration, the straws were horizontally frozen in liquid nitrogen vapors (5 cm above liquid nitrogen) for 12 min, and then plunged into liquid nitrogen for storage. For sperm evaluation, straws were thawed individually at 37 °C for 30 s in a water bath. Sperm evaluation was performed on all semen samples immediately after thawing.

Semen evaluation

Analysis of standard semen parameters

Motility and motion parameters of sperm were estimated by computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA). The following variables were analyzed: total motility (TM, %); progressive motility (PM, %); average path velocity (VAP, μm/s); straight-line velocity (VSL, μm/s); curvilinear velocity (VCL, μm/s); amplitude of lateral head displacement (ALH, μm); beat/cross frequency (BCF, Hz); linearity (LIN, %); straightness (STR, %).

Viability was assessed by means of the eosin–nigrosin stain method [17]. Sperm suspension smears were prepared by mixing a drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide. Viability was assessed by counting 200 cells at ×400 (CKX41; Olympus, Tokyo, Japan). Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable.

The evaluation of functional membrane integrity of sperm was performed by using the hypo-osmotic swelling test (HOST), considering positive those spermatozoa with curled or swollen tails [44]. In brief, 25 mL semen was added to 200 mL of hypo-osmotic solution (100 mOsm/L, 57.6 mM fructose and 19.2 mM sodium citrate). After 30 min incubation, the mixtures was homogenized and evaluated under a phase-contrast microscope (CKX41; Olympus, Tokyo, Japan). A total of 200 spermatozoa were counted in at least five different microscopic fields at ×400. The percentage of spermatozoa with swollen and curved tails was recorded.

For the evaluation of total abnormalities in the semen samples, at least three drops of the semen were pipetted into 1.5 mL tubes, containing 1 mL Hancock's solution [45]. One drop of this mixture was placed on a microscope slide and covered with a cover slip. The percentage of sperm abnormalities was recorded by counting a total of 200 sperm under a phase-contrast microscope. Hancock's solution was prepared by mixing 62.5 mL formalin (37% formaldehyde), 150 mL of sodium saline solution, 150 mL of buffer solution and 500 mL of double-distilled water. Sodium saline solution: 9.01 g NaCl in 500 mL of double-distilled water; buffer solution: (1) 21.7 g $Na_2HPO_4 \times H_2O$ in 500 mL of double-distilled water; (2) 22.254 g KH_2PO_4 in 500 mL of double-distilled water; 100 mL of (1) and 80 mL of (2) were mixed to obtain 180 mL of buffer solution.

Chlortetracycline (CTC) staining

Chlortetracycline staining was used for the evaluation of capacitation status as described by Perez et al. [40] with a little modification [19]. A CTC working solution (750 mM) was freshly prepared in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM D,L-cysteine at a pH of 7.4. Five microliters of semen were mixed with 20 mL CTC working solution. After 20 s, the reaction was stopped by the addition of 5 μL glutaraldehyde (1% (v/v) in 1 M Tris–HCl, pH 7.8). Smears were prepared on a clean microscope slide, covered with a cover glass, sealed with nail varnish, and kept in the dark at 4 °C. The evaluations were carried out using an epifluorescent microscope (Nikon PCM 2000™ Personal Laser Scanning Confocal Microscope, USA) at ×1000. Two hundred spermatozoa were classified into three categories: uniform fluorescent head (uncapacitated: CTC-F), fluorescent-free band in the post-acrosomal region (capacitated: CTC-B), and non-fluorescent head or a thin fluorescent band in the equatorial segment (acrosome-reacted: CTC-AR).

Malondialdehyde (MDA) concentrations

The thiobarbituric acid reaction was used for measurement of MDA, which is indicative of lipid peroxidation in phospholipids of the sperm membrane. This method was adapted from Placer et al. [41]. Briefly, 1 mL of diluted sperm (250×10^6 mL^{-1}) were mixed with 1 mL of cold 20% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifuging (900*g* for