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Optimizing the freezing rate for ovine semen cryopreservation: Phospholipid profiles and functions of the plasma membrane and quality and fertilization of spermatozoa

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ABSTRACT

The objective of this study was to investigate the effects of freezing rate on plasma membrane (PM) phospholipids and the function and fertilization of ram spermatozoa during freezing. Semen was obtained from 10 Merino rams using an artificial vagina, diluted with glucose-egg yolk buffer, and frozen in a programmable freezer at rates of −1, −20, −40, −60, and −80 °C/min. After thawing, we examined PM phospholipid content and distribution, fluidity, permeability, potential, spermatozoa quality, and *in vivo* and *in vitro* fertilization rates. The results showed that phospholipid, phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) contents of the PM decreased significantly ($P < 0.05$), whereas diphosphatidylglycerol (DPG) content and the cholesterol: phospholipid (CH: PL) ratio increased significantly compared to those of fresh semen after freezing ($P < 0.05$). PE, phosphatidylinositol (PI), and DPG were transferred to the outer leaflet, whereas PS and PG were transferred to the inner leaflet after freezing. Spermatozoa cryopreserved at −40 °C/min resulted in distributions of PS, PE, and DPG, as well as increased PM fluidity, permeability, and potential that was greater than those of the other freezing-rate groups ($P < 0.05$). No differences in vitality, PM and acrosome integrity, *in vitro* fertilization hatching rate, or artificial insemination conception rate were observed in any of the freezing-rate groups ($P > 0.05$), whereas cleavage rate (69.67 ± 5.11%) at −40 °C/min increased significantly ($P < 0.05$). Therefore, the optimal freezing rate to cryopreserve ram spermatozoa based on cryoinjury of phospholipids, membrane functions, and fertilization rate was −40 °C/min.

1. Introduction

The success of semen cryopreservation is influenced by freezing rate, which is of special interest, as it can lead to different degrees of chemical and physical damage to spermatozoa (William et al., 2005). An optimal freezing protocol minimizes cryoinjury caused by formation of intracellular ice, cell shrinkage, and exposure to multiple osmotic gradients and is strongly dependent on the composition and function of the plasma membrane (PM) (Bank and Brockbank, 1987).

Ram spermatozoa are particularly vulnerable to extreme temperature changes compared to those of humans, cows, and mice due to the high unsaturated fatty acid content on their PM (Samadian et al., 2010). The freezing rate range (10–100 °C/min) currently used to cryopreserve ram semen is too broad to prevent spermatozoa from suffering due to subtle cryoinjury (Fiser and Fairfull, 1984). Ice crystals form at −10 to −25 °C, which strongly affects survival of spermatozoa, and is freezing rate dependent (Salamon and Maxwell, 2000). However, strict control and pairing of these rates minimizes cryoinjury caused by intracellular ice (Woods et al., 2004). Although freezing rates have been optimized in previous studies based on sheep spermatozoa viability, motility, and morphological characteristics (Ollero et al., 1998; Ashrafi et al., 2011; Byrne et al., 2000), reduced motility and viability are less important factors for successful fertilization than structural and functional damage to the spermatozoa PM (Salamon and Maxwell, 2000). Few studies have fully evaluated the composition and distribution of ram spermatozoa PM phospholipids during cryopreservation.

Abbreviations: PM, Plasma membrane; PL, Phospholipids; PC, Phosphatidylcholine; PS, Phosphatidylserine; PI, Phosphatidylinositol; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; DPG, Diphosphatidylglycerol; CH, Cholesterol.

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The composition and distribution of phospholipids in the outer and inner leaflets of the spermatozoa PM are particularly important for PM permeability and fluidity. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are mainly localized in the inner leaflet of the PM, whereas sphingomyeline (SM) and phosphatidylcholine (PC) are mainly localized in the outer leaflet (Gadella et al., 1999; Hammerstedt et al., 1990). The strong association between phospholipids in the spermatozoa PM and freezing has been emphasized, as they are most susceptible to temperature change, osmotic imbalance (Khan and Ijaz, 2008), and cryoprotectant toxicity (Gadella and Harrison, 2000; Gadella et al., 1995).

Therefore, the aims of this study were: (1) to study phospholipid composition, distribution, and membrane function in the spermatozoa PM after freezing at different freezing rates and (2) to investigate spermatozoa quality and fertilization to optimize the ram spermatozoa freezing procedure.

2. Materials and methods

The use of animals and the experimental procedure were approved by the Animal Care Committee of the Institute of Geography and Agroecology, Chinese Academy of Sciences, Jilin, China. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Animals and diet

Ten Merino breeding rams (age, 2.5–3.0 years; body weight, 65–75 kg) were used. All animals were fed the same diet according to the National Research Council recommended 60:40 forage: concentrate ratio *ad libitum*, and water was freely available.

2.2. Semen collection and spermatozoa examination

Ejaculates were collected using an artificial vagina twice weekly for 10 weeks. A total of 170 s ejaculates (first ejaculates were discarded) were obtained from the 10 rams. Ejaculates with volumes of 0.7–2.0 mL, spermatozoa concentrations $>2.5 \times 10^9$/mL, and >80% motility were pooled.

2.3. Semen dilution, freezing, and thawing

The basic cryopreservative (Tris-egg-yolk) was an 80% (v:v) solution of 3.0% glucose, 3.0% sodium citrate, penicillin (10 IU), and streptomycin (10 IU) in distilled water plus 20% (v:v) egg yolk (fresh). The freezing solution contained 94% (v:v) of the basic solution and 6% (v:v) glycerol.

All ejaculates were mixed thoroughly and incubated at 37 °C for 30 min. After diluting the spermatozoa to 1.0×10^9 cells/mL, the pool was divided into six aliquots, placed in 0.25 mL straws, and chilled at 4 °C for 2 h. Then, each set of straws was placed in a programmable freezer (Minidigitcool; IMV-Technologies, L'Aigle, France) and frozen at −1, −20, −40, −60, or −80 °C/min. The straws were placed immediately into liquid nitrogen for storage when their core temperature reached −120 °C. Samples were stored for at least 2 weeks. The straws were thawed in a 40 °C water bath for 15 s. Fresh semen was used as the control.

2.4. PM lipid extraction and analysis

1 mL of thawed or fresh semen was diluted with 1 mL phosphate-buffered saline (PBS) and centrifuged at 1000*g* for 10 min at room temperature to separate the seminal plasma. The spermatozoa pellets were washed in 10 mL PBS and re-centrifuged. The intact spermatozoa were mixed with 1.25 mM EDTA to dissociate the PM, and the spermatozoa were dispersed in a two-phase polymer system consisting of 5.5% 252-Kd dextran and 4.2% 20-Kd polyethylene glycol prior to centrifugation at 9700*g* for 30 min; the membrane fraction sediments are at inter-phase when the two polymer phases separate (Folch et al., 1957). The resulting membrane fraction was further purified by repeating the two-phase fractionation step. Membrane yield was 35–40%, based on recovery of the membrane-bound marker enzymes alkaline phosphatase and 5′-nucleotidase. The isolated PMs were highly pure, as judged by phase-contrast (Nikon E-200, Tokyo, Japan) and electron microscopic studies (an aliquot of the PM was fixed with phosphotungstic acid) (Rana and Majumder, 1987).

After adding an organic solvent mixture (1:1 [v/v] chloroform: methanol), the mixed sample was centrifuged at 1000*g* and room temperature for 5 min to accelerate the phase separation. The lower phase (chloroform) was removed carefully with a syringe, and the homogenate was filtered. The filtrate contained the lipids accompanied by non-lipid substances, which were removed with a five-fold volume of distilled water (Bligh and Dyer, 1959). Cholesterol was measured with a CHOD PAP Kit (Biolabo, Maizy, France) according to the manufacturer's instructions, and a cholesterol calibration curve was prepared. Thin-layer chromatography (TLC) was performed to separate the different lipid classes (Douard et al., 2000). Lipid methyl esters were obtained by acidic transesterification using the Metcalf method (Folch et al., 1957).

The quantities of individual phospholipids in the outer leaflet were calculated by subtracting the quantity of phospholipid in the inner leaflet from total phospholipids obtained. 2,4,6-trinitrobenzenesulfonic acid (TNBS) has been used extensively to study the distribution of amino phospholipids in the PM. In brief, the amino groups were labeled by incubating the spermatozoa samples at 4 °C in the dark in 5 mL of medium containing 280 mM mannitol buffer (pH 7.4), 70 mM sucrose, 40 mM sodium bicarbonate, 1 mM $MgCl_2$, 2 mM succinate, and 2 mM TNBS for 20 min. After the incubation, the supernatants were discarded, and the spermatozoa were washed twice with 10 mL of the suspending medium mentioned above without TNBS. The derivatized phospholipids appeared as a yellow mark on the TLC plate.

Phospholipids were detected and identified by gas chromatography according to the method described by Beirao et al. (2012). Phospholipid standards were purchased from Sigma. Results are presented as μg/mg protein or as a relative proportion.

2. :5 PMpermeabilization, potential, and fluidity

PM permeabilization was measured using the commercial LIVE/DEAD BacLight kit (Invitrogen, Carlsbad, CA, USA), which is based on the nucleic acid-specific viability dyes propidium iodide and SYTO9, as described previously (Higgins et al., 2005; Leuko et al., 2004). The reaction is based on the observation that spermatozoa with an intact PM stain with the SYTO9 PM-permeant green fluorescent dye. If the membrane is compromised and membrane permeabilization has occurred, SYTO9 fluorescence is quenched by entry of propidium iodide into the cytoplasm. SYTO9 fluorescence was measured by a laser confocal scanning microscope (KLM 510; Carl Zeiss Inc., Zena, Germany) following excitation at 488 nm and emission at 510 nm.

The PM potential-sensitive 3,3-dipentyoxacarbocyanine assay was used to measure changes in spermatozoa PM potential, as described previously (Arias et al., 2011). Fluorescence was measured at an excitation wavelength of 622 nm and emission wavelength of 670 nm. Loss of red fluorescence represented the PM potential.

PM fluidity was determined using the 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescent probe (Bayer et al., 2000). Briefly, fresh and frozen-thawed semen samples were centrifuged for 15 min at 700*g* at room temperature and washed twice with PBS.