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Addition of superoxide dismutase mimics during cooling process prevents oxidative stress and improves semen quality parameters in frozen/thawed ram spermatozoa

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ABSTRACT

High levels of reactive oxygen species (ROS), which may be related to reduced semen quality, are detected during semen cryopreservation in some species. The objectives of this study were to measure the oxidative stress during ram semen cryopreservation and to evaluate the effect of adding 2 antioxidant mimics of superoxide dismutase (Tempo and Tempol) during the cooling process on sperm motility, viability, acrosomal integrity, capacitation status, ROS levels, and lipid peroxidation in frozen and/or thawed ram spermatozoa. Measuring of ROS levels during the cooling process at 35, 25, 15, and 5 °C and after freezing and/or thawing showed a directly proportional increase ($P < 0.05$) when temperatures were lowering. Adding antioxidants at 10 °C confered a higher motility and sperm viability after cryopreservation in comparison with adding at 35 °C or at 35 °C/5 °C. After freezing and/or thawing, sperm motility was significantly higher ($P < 0.05$) in Tempo and Tempol 1 mM than that in control group. Percentage of capacitated spermatozoa was lower ($P < 0.05$) in Tempo and Tempol 1 mM in comparison with that in control group. In addition, ROS levels and lipid peroxidation in group Tempo 1 mM were lower ($P < 0.05$) than those in control group. These results demonstrate that ram spermatozoa are exposed to oxidative stress during the cooling process, specifically when maintained at 5 °C and that lipid peroxidation induced by high levels of ROS decreases sperm motility and induces premature sperm capacitation. In contrast, the addition of Tempo or Tempol at 0.5 to 1 mM during the cooling process (10 °C) protects ram spermatozoa from oxidative stress.

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1. Introduction

Oxidative stress, related to altered sperm function [1], is caused by high levels of reactive oxygen species (ROS) produced by ram [2] and human spermatozoa [3] or by activated leukocytes present in the seminal plasma [4]. Ram spermatozoa produce high levels of hydrogen peroxide [5]. High ROS levels result in reduced sperm motility [6,7], peroxidation of unsaturated fatty acids in sperm membranes [8], and sperm DNA damage [7].

Increased ROS levels have been observed during cryopreservation of human spermatozoa, specifically during the cooling process, with maximum levels observed at 4 °C [9,10]. Similarly, gradual reduction of temperature stimulated the generation of superoxide anion in bovine spermatozoa [11]. At temperatures below 0, sperm ROS levels

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are extremely low [9]; however, there is an increase in nitric oxide content during sperm thawing in bovine [11] and canine [12] spermatozoa. Consequently, high ROS levels during the cooling process could be responsible for reduced sperm motility, lipid peroxidation, and/or DNA fragmentation observed after cryopreservation. Conversely, elevated ROS levels after freezing and/or thawing may not necessarily be related to sperm function.

In ram semen, various antioxidants have been used during cryopreservation [2,13] indicating that it is possible to reduce oxidative stress during cryopreservation and prevent the loss of motility, viability, and membrane integrity; lipid peroxidation; and DNA damage after freezing and/or thawing. However, superoxide dismutase (SOD) does not improve sperm function after cryopreservation [14]. In other species, Tempo and Tempol have been studied during sperm cooling and freezing. Tempo and Tempol are nitroxide radicals, which have similar activity to superoxide dismutase, and, compared with SOD, have low molecular weight, high solubility, and penetrate cell membranes easily [15]. In bulls, the addition of 0.2 to 2 mM Tempo and 0.2 to 6 mM Tempol in a milk-based extender at the beginning of the cooling process has detrimental effect on sperm motility after cryopreservation [16]. Similarly, incubating ram spermatozoa with Tempol at 37 °C decreases sperm motility [17]. However, the addition of 1-mM Tempo or Tempol at 10 °C (during the cooling process) prevents loss of motility and DNA fragmentation during cryopreservation of alpaca semen [18]. The temperature at which Tempo or Tempol are added to the extender could affect their protective effect on spermatozoa. In addition, other studies indicate a positive effect of antioxidants on sperm viability and motility during cooling of ram [19], bull [20], and tom turkey [21] spermatozoa. Fertility was also improved when Tempol was used for storing ram spermatozoa at 15 °C [19].

The objectives of this study were to (1) evaluate ROS production during the cooling process for cryopreservation, (2) the timing and temperature of addition of SOD mimics to extender, and (3) the effect of different concentrations of SOD mimics on quality of frozen and/or thawed ram spermatozoa.

2. Materials and methods

2.1. Semen collection, dilution, and freezing and/or thawing

Semen was collected by artificial vagina from 6 adult Romney rams during the breeding season. Animals were housed at La Frontera University, Temuco, Chile. Only ejaculates with at least 80% progressive motility and 2.5×10^9 spermatozoa/mL were processed. Semen was diluted using two fractions of extender. The first fraction of the extender was made of skim milk supplemented with 5% egg yolk (v:v). The second fraction included the addition of 48.5 g/L fructose (Sigma–Aldrich; St. Louis) and 14% of glycerol (v:v; Ultra Pure Glycerol; Agtech, Manhattan) to the base extender. After mixing semen and the first fraction at 1:1 ratio, semen was cooled from 35 °C to 5 °C within 1.5 hours (−1 °C/3 minutes) using a controlled rate freezer (Bio-Cool IV; FTS Systems, NY). At 5 °C, the second fraction was added in equal volume to extended semen, and samples were kept at this temperature for 30 minutes. The extended semen was packaged in 0.5-mL French straws, exposed to liquid nitrogen vapor for 15 minutes, and then stored in liquid nitrogen for at least 24 hours. Straws were thawed in water bath at 38 °C for 45 seconds.

2.2. Assessment of ROS levels and semen quality

Reactive oxygen species levels were determined in duplicate by measuring luminescence over a period of 200 seconds with a luminometer (Lumat LB0501; Berthold, Wildbad, Germany) [22]. Reactive oxygen species levels were expressed in relative light units with subtraction of the background and shown as relative light units/10^7 viable cells.

Percentages of spermatozoa with progressive motility were assessed under light microscopy (magnification: ×400) on a warm glass slide covered with a coverslip (18 mm × 18 mm). For viability and acrosomal integrity, the double stain technique [23] was used. Briefly, 200-μL sperm suspension aliquots were mixed 1:1 with 2% trypan blue (T-6146; Sigma–Aldrich, St. Louis) for 10 minutes at 37 °C, washed two or three times by centrifugation with SP-Talp medium at 600 *g* for 6 minutes, placed on a slide, and stained for 40 minutes with Giemsa 10% (Merck, Darmstadt, Germany). Spermatozoa showing a white post-acrosomal region and a purple acrosomal region were classified as viable spermatozoa with intact acrosomes. Capacitation status was assessed by induction of acrosome reaction using calcium ionophore A23187 (Sigma–Aldrich, St. Louis) at 5 μM for 15 minutes at 37 °C [24] and double stain technique [23]. Percentages of capacitated sperm were determined as the difference between the percentages of viable acrosome-reacted sperm after and before calcium ionophore treatment. Lipid peroxidation was determined by the thiobarbituric acid (TBA) assay (TBA test) [25]. Briefly, sperm samples (20×10^6 cells in 0.5 mL) were incubated for 60 minutes at 37 °C in the presence of 0.25 mL of ferrous sulfate (0.2 mM) and 0.25 mL of sodium ascorbate (1 mM). After samples were placed into an ice bath for 15 minutes, 0.5 mL of PBS and 0.5 mL of 40% trichloroacetic acid were added and centrifuged at 2500 *g* for 10 minutes at 4 °C. One milliliter of the supernatant was then added to 0.25 mL of 2% TBA cleared with NaOH (5 M). These reaction mixtures were placed in water bath at 90 °C for 10 minutes, cooled at room temperature for 30 minutes, and the absorbance was determined at 532 nm in a spectrophotometer (UVmini-1240; Shimadzu, Italy).

2.3. Experimental design

Experiment 1 was designed to evaluate ROS levels during different steps of the cryopreservation process. Ten semen samples were used for ROS measurements at 35, 25, 15, and 5 °C and after freezing and/or thawing. In addition, to simulate the equilibrium period, ROS were also measured after semen was kept at 5 °C for 0, 30, and 60 minutes.

Experiment 2 was designed to evaluate the effect of the timing of antioxidant addition during the cooling process. Two antioxidants were used: 2,2,6,6 tetramethyl-1-piperidinyloxyl (Tempo, 214000; Sigma–Aldrich; St Louis) and 4-hydroxy 2,2,6,6 tetramethyl-1-pip1eridinyloxyl

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