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Soybean lecithin-based extender as an alternative for goat sperm cryopreservation

Andréa Helena Vidal, André Mariano Batista, Ellen Cordeiro Bento da Silva, Wilton Arruda Gomes, Marina Arruda Pelinca, Sildivane Valcácia Silva, Maria Madalena Pessoa Guerra *

Andrology Laboratory (ANDROLAB), Veterinary Medicine Department – UFRPE, Dom Manuel de Medeiros Street, s/n, CEP 52171-900, Recife, PE, Brazil

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ABSTRACT

The aim of this study was to evaluate the effect of different concentrations of soybean lecithin (SL) in extenders for sperm goat cryopreservation. Sexually mature male Saanen goats ($n = 4$) were used, and the ejaculates were obtained using an artificial vagina method. The semen samples were pooled and diluted in a skim milk-based extender (control group; CG) or Tris extender supplemented with SL at different concentrations (G1 = 0.04%, SL G2 = 0.08% SL and G3 = 0.16%) for a final concentration of 240×10^6 spermatozoa/mL. The semen samples were packed in straws (0.25 mL), frozen using an automated system and stored in liquid nitrogen (−196 °C). After thawing (37 °C/30 s), the samples were evaluated for sperm quality parameters, including sperm motility, membrane integrity, acrosome integrity and mitochondrial activity. No significant difference was observed among the experimental and control groups for all of the parameters ($P > 0.05$). However, even though the control group presented a significantly lower mitochondrial membrane potential compared to fresh semen ($P < 0.05$), the same did not occur for the extender supplemented with soybean lecithin, that is, it did not differ from fresh sperm ($P > 0.05$). The extender containing soybean lecithin at different concentrations preserved the sperm quality parameters in a manner similar to the conventional skim milk-based extender. Thus, it is concluded that an extender containing soybean lecithin as the lipoprotein source can be used for freezing goat semen.

1. Introduction

The use of cryopreserved semen in artificial insemination (AI) has numerous advantages for the animal husbandry, especially when used in breeding programs (Salamon and Maxwell, 1995). The use of frozen semen in AI protects the animals from the stress caused by transportation for mating and the risk of disease transmission during copulation, in addition to favoring the preservation of high-value genetic material (Silva et al., 2000). However, for some animals, the cold preservation of sperm is a problem (Ortega et al., 2003), especially during the freezing step, which results in biological and functional changes to the sperm cells (Oliveira, 2002; Ortega et al., 2003).

The extenders used for semen cryopreservation protect the sperm against thermal shock, preserving both motility and fertility by promoting the stabilization of the plasma membrane and providing energy substrates. These attributes reduce the deleterious effects of changes in the pH and osmolarity, prevent the growth of bacteria and protect the sperm cells from the damage caused by refrigeration, freezing and thawing (Futino et al., 2010).

The extenders commonly used for freezing goat semen are based on animal products, such as egg yolk and/or

* Corresponding author. Tel.: +55 81 3320 6414.
E-mail address: mmpguerra@pq.cnpq.br (M.M.P. Guerra).

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milk. The low-density lipoproteins (LDLs) of egg yolk protect the sperm against damage during storage, cooling, and freezing. Milk caseins decreased the binding of seminal plasma proteins to sperm and reduced sperm lipid loss, while maintaining sperm motility and viability during storage (Bergeron et al., 2007). In addition, it is believed that the lecithin present in these components protects the plasma membrane by restoring the phospholipids lost during heat shock (Farstad, 1996; Futino et al., 2010). Several extender formulations containing different amounts of these compounds have been studied for cryopreserved goat semen (Bittencourt et al., 2008).

However, despite the good fertility rates observed when using extenders containing egg yolk and/or milk, these components represent a risk of contamination if microorganisms, such as bacteria and fungi, are present in the fresh product. Such contamination can release endotoxins that reduce the fertilization capacity of sperm (Bousseau et al., 1998; Bittencourt et al., 2008). Accordingly, extenders free of animal protein have been tested in recent years (Bousseau et al., 1998).

A viable alternative to replace the components of animal origin in extenders for freezing semen is soybean lecithin, a phospholipid that is the main component of the phosphate fraction of egg yolk and soybean (Campbell and Farrel, 2007). Therefore, the aim of this study was to evaluate the effect of soybean lecithin (SL) at different concentrations in extenders for sperm goat cryopreservation.

2. Materials and methods

2.1. Chemicals

The chemicals used in this study were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA).

2.2. Animals and semen collection

Four Saanen bucks, previously approved in clinical–andrological evaluations, were maintained under an artificial vagina semen collection regimen that included a female induced into estrus as a stimulus. The animals were raised in a confinement system with natural light at Federal Rural University of Pernambuco (8.0314S, 34.5252W). A total of six replicates were performed at intervals of 48 h; there were two samples from each buck for each replication.

2.3. Analyses and semen freezing

Initially, the semen samples were analyzed for the parameters of wave motion, progressive motility and vigor using a phase contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan). A sperm aliquot of 10 μL was placed on a prewarmed slide (37 °C) for the evaluation of the wave motion, and deposition of the drop on a 20 mm × 20 mm coverslip was used for evaluating the motility and vigor. The sperm concentration was obtained using a Neubauer Chamber at a dilution of 1:400 in formol saline. A humid chamber was used for the analysis of the sperm morphology (Mies Filho, 1987).

The semen of each buck was assessed separately, and the semen was pooled when the following were observed: wave motion ≥3; motility ≥70%; vigor ≥3; sperm concentration $\geq 1.0 \times 10^9$/mL and sperm pathology ≤20%.

For the control group (CG), the pool of semen was diluted in a skim milk-based extender (10 g skim milk, 0.0194 g glucose, 100 mL ultrapure water and 7% glycerol; 1238 mMol/kg). For the experimental groups (G1 = 0.04% SL, 394 mMol/kg; G2 = 0.08% SL, 399 mMol/kg and G3 = 0.16% SL, 400 mM/kg), different concentrations of soybean lecithin (SL) were added to a Tris-based extender (250 mM Tris, 88.5 mM citric acid, 69.38 mM glucose, 100 mL pure water and 7% glycerol; G1: 1378 mMol/kg; G2: 1383 mMol/kg; G3: 1384 mMol/kg) for a final concentration of 240×10^6 sperm/mL.

After filling mini straws (0.25 mL) with the mixtures, the straws were frozen using a programmable freezer (TK-3000®, TK Tecnologia em congelação LTDA, Uberaba, Brazil) using a fast freezing curve (−0.25 °C/min, from 25 °C to 5 °C, and −20 °C/min, from 5 °C to −120 °C) that starting at 28 °C (room temperature). After reaching a temperature of 5 °C (approximately 80 min), the straws were subjected to an equilibration time for 120 min. The freezing curve was implemented immediately after the equilibration time and was sustained until the temperature reached −120 °C. The straws were then placed in liquid nitrogen and stored in cryobiological container (−196 °C).

2.4. Thawing and in vitro sperm analysis

The frozen samples were thawed (37 °C by 30 s) after 48 h of storage and analyzed for progressive motility, plasma membrane and acrosome integrity, and mitochondrial membrane potential, according to the above methods used for the fresh semen samples.

2.4.1. Acrosomal integrity

For detection of the sperm acrosomal integrity, the sperm cells were stained with Fluorescein Isothiocyanate conjugated to Peanut Agglutinin (FITC-PNA) using the method described by Silva et al. (2011). Aliquots of 5 μL of semen from each treatment were placed on microscope slides and air-dried. Twenty microliters of FITC-PNA working solution (100 μg/mL) was spread over the slides, followed by incubation at 4 °C in a humidity chamber for 15–20 min in the absence of light. The slides were then immersed in PBS at 4 °C twice and dried naturally in the absence of light. At the time of evaluation, 5 μL of a solution composed of 4.5 mL glycerol, 0.5 mL PBS and 5 mg phenylenediamine was placed on the slide, and the sample was covered with a slip cover and subjected to an epifluorescence analysis (Carl Zeiss, Göttingen, Germany) using BP 450–490 nm excitation and LP 515 nm emission filters. Two hundred cells were examined at a magnification of 1000×. The sperm cells were classified as having in intact acrosome (iAC) when the acrosome region was stained fluorescent green and as having a reacted acrosome when the green fluorescence was absent from the head region or when appearing in the equatorial region of the sperm head.

2.4.2. Membrane integrity

Integrity of the sperm membrane was assessed using the combination of propidium iodide (PI) and carboxyfluorescein diacetate (CFDA), as described by Harrison and Vickers (1990) and modified by Câmara et al. (2011). Aliquots of 50 μL of each sample were diluted in 150 μL Tris containing 20 μL PI (0.5 mg/mL in PBS) and 5 μL CFDA (0.46 mg/mL in DMSO). Two hundred cells from each sample were assessed under epifluorescence microscopy (Carl Zeiss, Göttingen, Germany) using DBP 485/20 nm excitation and DBP 580–630 nm emission filters at a magnification of 400×. Green fluorescence indicated an intact membrane, and red fluorescence indicated an injured membrane.

2.4.3. Mitochondrial membrane potential

Aliquots of 50 μL of semen from each sample were diluted in 150 μL Tris containing 5 μL lipophilic cationic JC-1 (0.15 mM in DMSO), incubated for 10 min, fixed with gluteraldehyde and subjected to epifluorescence microscopy (Carl Zeiss, Göttingen, Germany) using BP 450–490 nm excitation and LP 515 nm emission filters. Two hundred cells from each sample were examined at a magnification of 400×. The cells were classified as having a high mitochondrial membrane potential when emitting orange fluorescence in the region of the midpiece and as having a low mitochondrial membrane potential when emitting green fluorescence (Silva et al., 2012).

2.5. Statistical analysis

The parameters of progressive motility, plasma membrane and acrosome integrity, and mitochondrial membrane potential were evaluated by ANOVA after the arcsine transformation (arcsine $\sqrt{P/100}$) of the percentage values; when a difference was observed, the Tukey–Kramer multiple comparison test in SPSS version 11.0 for Windows was used, with P-values <0.05 being considered statistically significant. All of the data are expressed as non-transformed means ± the standard deviation.