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

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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

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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.

Table 1
Percentage (mean ± standard deviation) of the quality sperm parameters on pools of goat semen, fresh or frozen in a skim milk-based extender or Tris supplemented with different concentrations of soy lecithin.

Sperm parameters	Fresh	Frozen semen			
		Skim milk	Tris + LS 0.04%	Tris + LS 0.08%	Tris + LS 0.16%
PM	75.00 ± 4.47[a]	38.33 ± 4.08[b]	38.33 ± 7.53[b]	36.68 ± 5.16[b]	38.33 ± 4.08[b]
Acros. Integ.	70.25 ± 14.98	56.75 ± 14.21	62.25 ± 14.12	63.00 ± 17.42	61.33 ± 15.72
Memb. Integ.	65.75 ± 2.89[a]	45.17 ± 14.74[ab]	39.67 ± 16.72[b]	43.58 ± 14.77[ab]	38.08 ± 19.31[b]
Mit. Pot.	64.50 ± 6.02[a]	41.00 ± 20.35[b]	51.68 ± 10.56[ab]	58.00 ± 9.75[ab]	57.42 ± 11.65[ab]

PM, progressive membrane; Acros. Integ., acrosome integrity; Membr. Integ., plasma membrane integrity; Mit. Pot., high mitochondrial membrane potential. Different letters in the same row denote $P < 0.05$.

3. Results

The skim milk-based extender was used as the control group because the Tris-based extender, without the addition of an extracellular cryoprotectant (such as egg yolk or milk), did not protect the goat sperm cells, as observed in previous study (data not shown). The values of the sperm parameters observed in the pool of goat semen samples, either fresh or frozen in the skim milk-based extender (control group) or Tris supplemented with different concentrations of soybean lecithin, are shown in Table 1. After thawing, it was observed that the sperm motility values of the frozen were lower ($P < 0.05$) than those of the fresh semen; however, the motility did not differ ($P > 0.05$) between the samples frozen with skim milk or soy lecithin (0.04%, 0.08% or 0.16%).

The percentages of sperm with intact acrosomes did not differ ($P > 0.05$) between the samples of fresh or frozen semen (Fig. 1A). Our analysis of the plasma membrane showed that the semen samples frozen with the skim milk-based extender or Tris plus soy lecithin at 0.08% did not differ ($P > 0.05$) from the fresh semen samples. However, semen samples frozen with Tris plus soy lecithin at 0.04% or 0.016% presented lower ($P < 0.05$) percentages of gametes with intact plasma membranes in comparison with the fresh samples but did not differ ($P > 0.05$) from those samples frozen with skimmed milk (Fig. 1B).

The analysis of the mitochondrial membranes showed that the semen samples frozen with the skim milk-based extender had lower ($P < 0.05$) percentages of gametes with high mitochondrial membrane potentials than those of the fresh semen (Fig. 1C). However, this parameter did not differ from those frozen with Tris plus lecithin soybean (0.04%, 0.08% or 0.16%) compared with fresh semen.

4. Discussion

As expected, the results of this study showed that the sperm motility decreased after the freezing/thawing process when compared to the fresh samples because the sperm plasma membrane is the first structure to suffer damage during cryopreservation (Watson, 1995). When improperly performed, cryopreservation causes thermal shock as well as the stresses induced by ice crystal formation are mainly associated with the accompanying osmotic pressure changes in the unfrozen fraction (Watson, 2000), resulting in irreversible damage to the sperm cells, which is characterized by abnormal movement patterns, the rapid

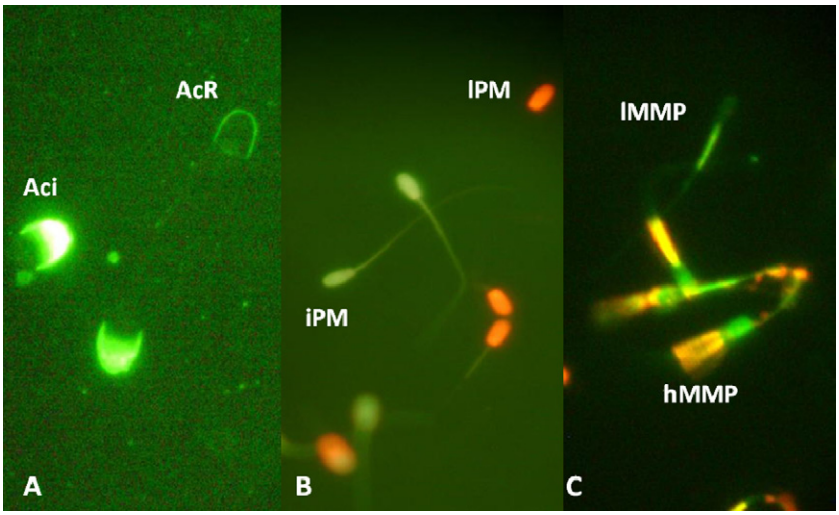

Fig. 1. Sperm assessment by fluorescent probes. (A) Acrosome evaluation; AcR, acrosome-reacted, and Aci, acrosome intact with green fluorescence. (B) Plasma membrane assessment; lPM, plasma membrane lesion, with red fluorescence by IP action and iPM, plasma membrane intact with green fluorescence, by DCF binding. (C) Mitochondrial activity; lMMP, low mitochondrial membrane potential, with green fluorescence and hMMP, high mitochondrial membrane potential, with orange fluorescence by *J*-aggregate formation.

loss of motility, the reduction of metabolic activity, the loss of intracellular components, and injury to the plasma membrane, acrosome, mitochondrial sheath and axoneme (Melo et al., 2007; Ortega et al., 2003).

No difference was observed among groups for acrosome integrity. Acrosomal injuries observed are generally caused by the formation of large ice crystals within the cell, damaging the internal structures and/or plasma membrane (Watson, 1995; Oliveira, 2002; Melo et al., 2007; Futino et al., 2010). Although the mechanism by which soybean lecithin protects sperm during freezing/thawing remains unknown, there are two hypotheses to explain the phenomenon. Phospholipids, being the major component of membranes, play important physiological functions in reducing the freezing point, thus avoiding the formation of large ice crystals, and in minimizing the replacement of plasmalogens to reduce the possible mechanical damage to the sperm membrane (Graham and Foote, 1987; Giraud et al., 2000; Waterhouse et al., 2006).

Therefore, the exogenous phospholipids presents in extenders can replace some of the sperm membrane phospholipids to maintain plasma membrane structure and function (Graham and Foote, 1987; Trimeche et al., 1997; Zhang et al., 2009). Another possibility, also widely accepted by many researchers, is that the egg yolk phospholipids or soy lecithin do not enter the membrane to alter the phospholipids concentration but may form a protective film around the cell to prevent the formation of intracellular ice crystals and to protect the sperm membrane from mechanical damage during freezing/thawing (Quinn et al., 1980; Simpson et al., 1987; Zhang et al., 2009).

About membrane integrity, the results suggest that the higher SL concentrations may have been toxic to the integrity of the membrane, whereas the lower concentration would be insufficient to provide the necessary protection. Futino et al. (2010) reported that, despite having a protective effect, soy lecithin (and other substances) at high concentrations can become harmful to sperm due to their potential toxicity, thus reducing the fertilization capacity. We emphasize that the concentration of soy lecithin used in this experiment was based on the studies of Beccaglia et al. (2009) in which lecithin was used in the refrigeration of canine semen. As cooling is not as deleterious to the structure of the sperm plasma membrane as the freezing process, which causes more significant rearrangements in the structure of the plasma membrane (Barbas and Mascarenhas, 2009), we chose to use the soy lecithin at higher concentrations.

The fact that the mitochondrial membrane potentials of the control group were lower than those of the fresh semen may suggest that: (1) the component of animal origin (skim milk) provided little protection to the goat sperm that was not subject to seminal plasma removal, with the soybean lecithin providing better protection under our conditions (Zhang et al., 2009); (2) the increased viscosity and increased presence of residual particles in the extenders containing egg yolk and/or milk may be factors that affect sperm parameters, thus the lecithin may have played a protective role during cryopreservation due to its low viscosity and the lower presence of debris (Vishwanath and Shannon, 2000; Zhang et al., 2009) and (3) an extender based on soy lecithin improves the kinematics of sperm cells compared to the extenders commonly used (Zhang et al., 2009).

In practice, however, contradictory results have been obtained by other researchers. Bittencourt et al. (2008) compared two extenders for freezing goat semen, lecithin (Bioexcell®) and another based on Tris-yolk, supplemented or not with Equex MTS® detergent. These authors reported that the mean values of sperm motility provided by the frozen/thawed semen diluted with Bioexcell® were below the minimum recommended for post-thawed goat semen (30%), as per the Brazilian College of Animal Reproduction (1998). The Bioexcell® and control extender resulted in higher ($P < 0.05$) rates of acrosomal defects when compared to the fresh semen. These findings differ from those obtained in the present study in which the extender based on soya lecithin at various concentrations was equally effective as skim milk in the preservation of the sperm parameters. Thus, it can be assumed that, despite using the same procedure of diluting and freezing, the extender used in our study had a different composition than the lecithin-based commercial extender; variability among the animals might also generate different results (Rodríguez-Martínez, 2007).

5. Conclusion

Based on the results, it can be concluded that an extender containing soybean lecithin as the lipid/lipoprotein source can be an alternative to animal products in extender preparation for freezing goat semen. However, studies are necessary to determine the effects of using this extender on the fertility of the cryopreserved goat semen.

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