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Soybean lecithin–based extender preserves spermatozoa membrane integrity and fertilizing potential during goat semen cryopreservation

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ABSTRACT

Soybean lecithin may represent a suitable alternative to egg yolk for semen cryopreservation in livestock species. However, additional studies are needed to elucidate its effects on spermatozoa functional properties. Semen collected from five Sarda bucks was cryopreserved in Tris-based extender and glycerol (4% v:v) with different supplementations. In a preliminary experiment, different soybean lecithin concentrations were tested (1%–6% wt/vol) and results in terms of viability, percentages of progressive motile and rapid spermatozoa, and DNA integrity after thawing showed that the most effective concentration was 1%. In the second experiment, semen was frozen in a Tris-based extender with no supplementation (EXT), with 1% lecithin (EXT LC), and 20% egg yolk (EXT EY). The effectiveness of these extenders was also compared with a commercial extender. The EXT EY led to the highest viability and motility parameters after freezing and thawing ($P < 0.0001$). No significant differences were observed in intracellular ATP concentrations. Additional molecular features revealed that sperm functionality was affected in EXT EY, as demonstrated by lower DNA and acrosome integrity ($P < 0.05$), and higher lipid peroxidation compared with spermatozoa cryopreserved in EXT LC ($P < 0.0001$). Results obtained in the heterologous *in vitro* fertilization test showed that EXT LC better preserved spermatozoa functionality, as demonstrated by the higher fertilization rates compared with the other media (66.2 ± 4.5% for EXT LC vs. 32.7 ± 4.5%, 38.7 ± 4.5%, 39.6 ± 5.2% for EXT, EXT EY, and commercial extender; $P < 0.01$). The present study demonstrated that lecithin can be considered as a suitable alternative to egg yolk in goat semen cryopreservation, because it ensures higher fertilization rates and a better protection from membrane damage by cold shock.

1. Introduction

Long-term storage of spermatozoa is one the most important tool to improve the reproductive technologies in the field of animal and human medicine [1]. However, semen cryopreservation leads to biological and functional changes in sperm cells, which impair their fertilizing ability. Over the past few years, there has been little progress in techniques to freeze sperm [2,3]. One of the features that most directly affect spermatozoa functionality is membrane integrity. Sperm cells have three membranes: plasma and mitochondrial membranes,

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which are involved not only in sperm viability and motility, respectively, but also in the process of capacitation, and the acrosome membrane for their final mission of penetrating the oocyte [4]. These membranes contain a high concentration of polyunsaturated fatty acids, and therefore are susceptible to oxidative stress, especially during the freezing and thawing procedures [5].

Extender solutions are normally supplemented with avian egg yolk. The main disadvantage in having an animal-derived material in the constitution of the cryopreservation media is represented by the sanitary risk of diseases transmission. This has raised boundaries in international semen transport legislations of many countries because of biosecurity issues [6]. Therefore, research efforts are focusing in finding a well-defined egg yolk substitute of nonanimal origin to be used in the constitution of semen cryopreservation media.

Soybean lecithin may represent a suitable alternative to egg yolk for semen cryopreservation in livestock species. The precise mechanism by which lecithin exerts its effects on spermatozoa plasma membrane during freezing/thawing procedures is not clear. It has been suggested that the lecithin protection mechanism is because of the replacement of sperm membrane phospholipids, with the reduction in the freezing point. Alternatively, it may form a protective film around the spermatozoon preventing the formation of intracellular ice crystals and avoiding the mechanical damage on the sperm membranes [7].

Soybean lecithin has been used successfully to supplement sperm cryopreservation media in several species such as human [8,9], bovine [10,11], equine [12,13], ovine [14–16], buffalo [17,18], canine [19,20], and goat [21,22]. However, its effects on spermatozoa functional properties still need to be fully characterized.

This study was designed to determine the proper concentration of soybean lecithin to be added to a Tris-based extender for buck semen cryopreservation, and to assess its effects on the acrosome, mitochondria, and plasma membrane functions on DNA integrity and IVF potential. To carry out the present experiments, we used Sarda bucks as a model, because semen freezing and thawing procedures are well established for this specie in our laboratory.

2. Materials and methods

2.1. Chemicals

All chemicals in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise.

2.2. Animals and semen collection

All experimental procedures were carried out during goat nonbreeding season (May-June) at the experimental facilities of the Department of Veterinary Medicine at the University of Sassari, Italy (latitude 40°43′ N). These facilities meet the requirements of the European Union for Scientific Procedure Establishments (EU Directive 2010/63/EU). This study followed ethical guidelines for care and use of agricultural animals for research. Ejaculates were obtained by artificial vagina from five adult Sarda bucks aged 4 to 5 years, maintained in an outdoor environment and fed a live-weight maintenance ration. Bucks were kept isolated in separated pens, but with visual contact between each others. A total of 25 ejaculates (five ejaculates from each buck) were collected twice a week (two ejaculates a week from all bucks and on same days) and were used for this study. Semen was transported to the laboratory at a controlled temperature of 25 °C within 5 minutes after collection, and it was immediately processed. Volume, concentration, and total sperm output were recorded for each ejaculate collected. Ejaculates meeting the following criteria: volume of 1.8 mL or greater, wave motion of 3 or greater, and sperm concentration of 1.0×10^9 spermatozoa/mL or greater, were pooled and processed as described subsequently.

2.3. Semen cryopreservation

After being washed in a Tris-based extender (Tris 375 mM, citric acid 124 mM, glucose 41 mM, plus an antibiotic solution of streptomycin-penicillin, 50 μg/mL-50 IU/mL) by two consecutive centrifugations (1500× *g* for 20 minutes), semen was diluted up to 400×10^6 sperm/mL in a Tris-based extender with different supplementations, as described in the experimental design, and glycerol (4%). It was cooled to 4 °C over a period of 2 hours and equilibrated for 20 minutes before freezing. Finally, semen was frozen in pellet form (0.25 mL) on dry ice with a freezing velocity of −0.7 °C/s. In particular, semen drops passed from a temperature of +4 °C to −80 °C in 2 minutes, and then were plunged into liquid nitrogen and stored in colour-coded goblets. Thawing was carried out by plunging a sterilized glass falcon tube containing the pellet in a 39 °C water bath for 20 seconds.

The content of the Falcon tube was then emptied into a conical tube containing 3 mL of Tris-based extender (Tris 375 mM; citric acid 124 mM; glucose 41 mM, pH 7; osmolality, 385 mOsm/kg). Semen was centrifuged at 900× *g* for 3 minutes maintaining a constant temperature (35 °C) to remove the freezing medium, the sperm pellet was then resuspended in a fresh Tris-based extender, and aliquots immediately used for all the experimental procedures.

2.4. Experimental design

To study the effects of the supplementation of soybean lecithin to the extender used for goat semen cryopreservation we carried out two consecutive experiments.

In a preliminary experiment, we determined the best concentration of soybean lecithin to be added to the extender by comparing the following concentrations: 1%, 2%, 3%, 4%, 5%, and 6% (wt/vol). The parameters evaluated included sperm viability, percentage of progressive motile and rapid spermatozoa before and after thawing, and DNA integrity after thawing using a Tris-based extender as control.

In the second experiment, having found 1% as the best soybean lecithin concentration, different supplementations to a Tris-based extender were compared: no supplementation (EXT), 1% lecithin (EXT lecithin), and egg yolk 20%