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Comparison of two different cryopreservation protocols for freezing goat semen

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ABSTRACT

In this study, two different semen cryopreservation protocols were compared to freeze goat semen. The ejaculates ($n = 12$) were collected by using electro-ejaculator from six mature bucks (two ejaculates per each buck). Each ejaculate was divided into two groups as Protocol 1 (P1) and Protocol 2 (P2). In P1, semen was diluted directly in an extender containing 15% egg yolk, 300 mM Tris, 28 mM glucose, 95 mM citric acid 5% glycerol to a concentration of 200×10^6 sperm/mL. In P2, after the removal of seminal plasma by centrifugation, the semen sample was diluted with the first portion of milk extender consist of 100 mg/mL skimmed milk powder and 27.75 mM glucose (without glycerol) to a concentration of 400×10^6 sperm/mL. The second portion of the milk extender containing 14% glycerol was added to semen gradually in order to achieve sperm concentration 200×10^6 sperm/mL and 7% glycerol level in the final volume. Extended semen was loaded in 0.25 mL straws, held for 2 h at 4 °C, frozen in nitrogen vapor and stored in liquid nitrogen. Post-thaw motility and live sperm rate (mean ± SEM) were significantly lower ($P < 0.05$) in P1 as compared to P2 (47.50 ± 1.23% vs. 55.63 ± 1.72%; 80.04 ± 1.29% vs. 84.04 ± 1.08%, respectively). However, live intact, total intact, abnormal, reacted acrosome and DNA damaged sperm rates were similar ($P > 0.05$) in both protocols. It was concluded that both protocols used in this study provided reasonable post-thaw parameters; however, P2 yielded better motility and live sperm rate compared to P1.

Introduction

Development of techniques for the successful freezing of spermatozoa has progressively evolved over the past 60 years. Various extenders and freezing procedures have been described in different animal species, especially in bull [24], goat [20,21] and ram [3] to minimize detrimental effects of cryopreservation on sperm motility, viability, morphology [17], plasma membrane [30], acrosome [9] and DNA [39] integrity.

The response of the spermatozoa to the cryopreservation varies among individual males of the same species as well as in different species. In general, the spermatozoa of small ruminants are extremely sensitive to cryopreservation compared to other species. Moreover, goat semen cryopreservation is very challenging [31] since it includes high concentration of lipase which may interact with the most common components of cryopreservation diluents such as egg yolk [34] and milk [28]. Due to this peculiar composition of goat semen it requires unique attention to maximize the post-thawing sperm parameters [31]. Thus, present study was designed to compare two semen cryopreservation protocols for freezing goat semen based on post thaw sperm parameters including motility, viability, abnormal sperm rate, and plasma membrane, acrosome and DNA integrity.

Materials and methods

Animals and semen collection

The ethical approval was obtained in the beginning of the experiments from the local ethical committee of Adnan Menderes University. Six regular semen donor Saanen bucks (1.5–4 years of age and 51–69 kg of body weight) were used in this study. They were maintained at the experimental animal shed, Department of Reproduction and AI, Adnan Menderes University under optimum feeding and managemental conditions. Semen was collected using electro-ejaculator and a total of 12 ejaculates (2 ejaculates per each buck) were collected throughout the study period.

Cryopreservation of semen

Two different semen cryopreservation protocols were used in this study. The ejaculates ($n = 12$), with a progressive motility

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higher than 70% and abnormal sperm rate lower than 20%, were used for cryopreservation. After initial evaluation each ejaculate was divided into two groups as protocol 1 (P1) and protocol 2 (P2). In P1, semen was diluted directly in the extender consisted of 15% egg yolk, 300 mM Tris, 28 mM glucose, 95 mM citric acid and 5% glycerol to a concentration of 200×10^6 sperm/mL [16]. In Protocol 2, semen plasma was removed by centrifugation (300×g for 5 min) in Tris-citric acid–glucose solution. The sperm pellet was then diluted with the first portion of milk extender consist of 100 mg/mL skimmed milk powder and 27.75 mM glucose (without glycerol) to a concentration of 400×10^6 sperm/mL [23]. The second portion of the milk extender containing 14% glycerol was added to semen gradually in order to achieve the sperm concentration of 200×10^6 sperm/mL and 7% glycerol level in the final volume. Extended ejaculates in both protocols were loaded into 0.25 mL straws and cooled gradually to 5 °C in 2 h. Upon reaching 5 °C, samples were frozen in static liquid nitrogen vapor, 4 cm above the liquid nitrogen. After freezing for 7 min, the straws were plunged into liquid nitrogen and stored at −196 °C for at least 7 days prior to thawing.

Evaluation of post-thaw sperm quality

Two straws from each cryopreserved ejaculate were used to determine the post-thaw sperm quality. Thawing of the straws was carried out in a water bath at 37 °C for 30 s. Sperm quality parameters were evaluated immediately after thawing.

Motility, viability and morphological assessment

Sperm motility was assessed subjectively by examining a uniform drop of sperm sample from each protocol on pre-warmed glass slide under a coverslip by using a phase contrast microscope equipped with warm stage adjusted at 37 °C. At least five widely-spaced fields were observed to estimate of the percentage of motile cells. Sperm viability was assessed as described before Ahmad et al. [1]. Briefly a small drop of sperm sample from each protocol was placed on a pre-warmed glass slide and mixed with an equal drop of the eosin-nigrosin stain (1% eosin, 3% nigrosin, 3% sodium citrate, 100 mL distilled water) by an applicator stick, and a thin, uniform smear was made. After air-drying the smear, 200 sperm from each slide were observed for unstained (live) and stained (dead) sperm heads under bright field microscopy at 400× magnification. The percentage of morphologically abnormal sperm was determined by fixing a 10 μL portion of sperm sample from each protocol in 1 mL of Hancock's solution [18]. A total of 200 sperm from each slide were observed for morphological abnormalities using wet-mount slide method under a phase-contrast microscope at 400× magnification.

Determination of plasma membrane integrity

Plasma membrane integrity of sperm was determined by using hypoosmotic swelling test in combination with eosin staining (HE-test) [5,15]. Briefly a 25 μL sperm sample from each protocol into 475 μL of fructose solution adjusted to 100 mOsm/L [4]. The sperm samples were incubated at 35 °C for 15 min. After incubation sperm plasma membrane integrity was estimated. A total of 400 sperm from each replication were counted for live-intact (unstained heads of sperm with coiled tails) and total intact (stained and unstained heads of sperm with coiled tails) under bright field microscope at 400× magnification.

Determination of acrosome reacted spermatozoa

Percentage of acrosome reacted spermatozoa was determined through Coomassie Blue G-250 staining procedure as reported earlier [22,2]. A total of 200 sperm from each slide of each replicate were counted for intact or reacted acrosome under bright field microscope at 400× magnification. Sperm heads with visible dense apical ridge were considered to have intact acrosome and sperm heads without dense apical ridge were considered as with reacted acrosome.

Comet assay

Validation of the technique

The alkaline comet assay procedure employed for ram spermatozoa in our laboratory (unpublished data) was validated for goat spermatozoa in the preliminary experiments. Simply, intact (negative control) and DNA damaged (positive control) goat semen samples were utilized in the comet assay procedure to observe and compare morphology of the sperm DNA mass after electrophoresis. The DNA damage in sperm cells in the positive control was induced by 3 h incubation in the water bath adjusted to 44 °C. The observation of the comet shaped DNA with different tail length in the positive control groups (Fig. 1A), while round shaped DNA mass in the negative control (Fig. 1B), was regarded as the indication of the reliable test to assess the level of DNA damages in goat spermatozoa. The preliminary experiments to validate the method were replicated several times with different ejaculates of goats until consistent results are obtained.

Preparation of slides, lysis buffer and electrophoresis

Agarose, 1% (w/v), and agarose low gelling temperature (LMA), 0.75% (w/v), was dissolved in phosphate buffered saline (PBS).

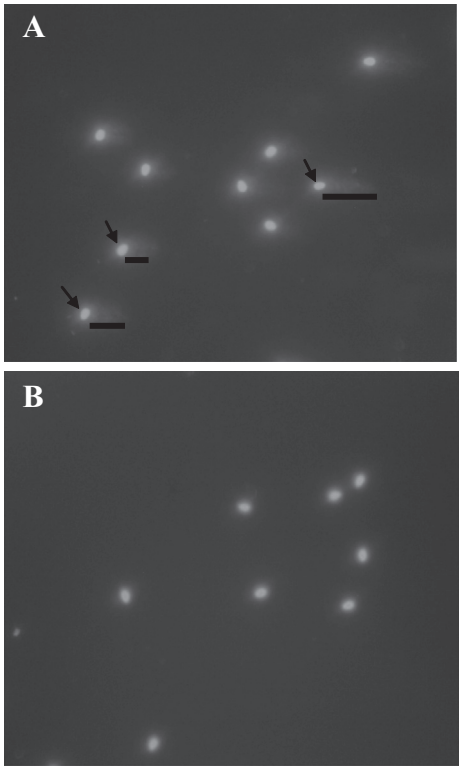

Fig. 1. Typical images of goat spermatozoa obtained from comet assay. In positive control group (A) the bright round circles "comet heads (\)" are nuclei of sperm whereas diffused areas "comet tails (—)" along with comet heads show different degree of DNA damage. In negative control group (B) the bright circles without comet show intact DNA.