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Royal jelly supplemented soybean lecithin-based extenders improve post-thaw quality and incubation resilience of goat spermatozoa

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ABSTRACT

The aim of the present study was to evaluate different concentrations of royal jelly (RJ) supplemented extenders for post-thaw quality and incubation resilience of goat spermatozoa. Semen samples were collected from five goats. Pooled semen were diluted with soybean lecithin-based extender without RJ (control) or supplemented with different concentrations (0.25, 0.5 and 0.75%) of RJ (RJ0.25, RJ0.5, RJ0.75 respectively), at a final concentration of 150 × 10^6 spermatozoon/mL. Semen samples were assessed for sperm motility, plasma membrane integrity using hypoosmotic swelling test (HOST) damaged acrosome using FITC-Pisum sativum agglutinin (PSA-FITC) and DNA integrity using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL).

The addition of RJ (0.5%, 0.75%) led to higher percentages of subjective motilities (55.33 ± 2.29%, 57.67 ± 2.58%) compared to control and RJ0.25 groups (49.00 ± 2,80%, 51.67 ± 3.09%) ($P < 0.05$) following the freeze–thawing process. RJ0.5 and RJ0.75 groups had higher plasma membrane functional integrities (66.40 ± 1.34%, 68.20 ± 2.05%) and lower defected acrosome rates (24.60 ± 3.36%, 23.80 ± 2.27%) compared to the other groups ($P < 0.05$). DNA damaged spermatozoa in all groups were not significant ($P > 0.05$).

In the end of incubation, motility and HOST rates of RJ0.5 (14.00 ± 3.87%, 31.20 ± 3.70%) and RJ0.75 (15.00 ± 3.27%, 29.20 ± 2.59%) groups were higher than control (8.00 ± 2.54%, 18.20 ± 3.11%) and RJ0.25 (9.00 ± 2.07%, 20.60 ± 2.88%) groups ($P < 0.05$). Also defected acrosome and DNA fragmation rates of RJ0.5 (32.20 ± 1.30%, 5.4 ± 0.55%) and RJ0.75 (29.20 ± 1.30%, 5.80 ± 0.45%) groups were significantly lower than control (38.80 ± 0.84%, 7.40 ± 1.34%) and RJ0.25 (39.80 ± 2.05%, 7.00 ± 1.58) groups. This study shows that RJ supplemented extenders have beneficial effect on goat sperm parameters at 0 h and 6 h of incubation.

1. Introduction

Artificial insemination (AI) with preserved semen is one of the most common reproductive techniques for production of domestic animals [30]. Semen preservation requires a depression or interruption of spermatozoa metabolism which is performed with cryopreservation [22,26].

Cryopreservation of semen is one of the most important technique in order to improve animal reproduction [3,7]. However, advanced reproductive efficiency of breeding with cryopreserved semen is possible with increasing the post-thaw sperm quality through improvements in extenders. Semen extenders with various additives have been tested to maintain fertilizing ability of semen for a long time. Efforts to improve the preservation of goat semen are focused on the modification of extenders [6,19]. For this reason, various component additions have been tested for the maintenance of sperm motility, fertilizing capacity and preserving sperm membrane integrity [23].

During freezing-thawing process, ice crystallization and lipid peroxidation cause functional and biochemical changes in spermatozoon that induce oxidative stress, which leads to the formation of reactive oxygen species (ROS) [27]. In addition, inappropriate ROS production leads to decrease in sperm motility, viability and fertilizing ability, stability of the sperm [9,21]. ROS level is quite important. In semen there is a natural antioxidant system, but this system is partly removed and severely altered during cryopreservation [27]. Therefore, the addition of antioxidants to the extender may have positive effects on semen

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cryopreservation in various species [9,10,21].

Royal jelly (RJ) is produced by hypo-pharyngeal and mandibular glands of worker bees using for feeding larvae and maintaining the queen bee. On dry matter basis, it contains proteins, lipids, sugars, vitamins and free amino acids particularly cysteine, lysine and arginine [1]. In addition, a successful effect of RJ supplementation on sperm fertilizing ability has been reported in lab model animals [1,25]. Similarly, a few studies have examined the effects of RJ on sperm quality of domestic animals [25]. However, the effect of RJ on cryopreservation and incubation resilience of goat semen has not been tested yet.

The protection of higher motility in sperm during post-thawing incubation reflects a greater probability of their survival in the female genital tract to undergo capacitation and fertilize ova. The duration of sperm motility and integrity of other parameters during post-thaw incubation is an indicator of the quality and usability of the semen [1,29]. The aim of this study was to evaluate RJ supplemented lecithin based extenders for the post-thaw quality and incubation resilience of goat spermatozoa using motility and plasma membrane, acrosome and DNA integrities.

2. Material and method

Scientific Ethical Committee (Uludag University, Bursa, Turkey, No: 2016-13/03) have approved all issues concerning the experimental setups and evaluation techniques.

Experimental design: We designed this study to evaluate the efficacy of RJ supplementation for goat semen cryopreservation. For this purpose we used various concentrations of RJ (0, 0.25, 0.5 or 0.75%) supplemented lecithin based extender for post-thaw goat semen quality in non-breeding season.

Semen extender preparation: Experimental groups were designed according to the amount of RJ supplementation such as RJ0.25 (with 0.25% RJ), RJ0.5 (with 0.5% RJ), RJ0.75 (with 0.75% RJ) and control (without RJ). Extenders consisted of 223.7 mmol/L Tris (Sigma, USA), 55.5 mmol/L fructose (Sigma), 66.6 mmol/L citric acid (Merck, Darmstadt, Germany), 100.4 mmol/L Trehalose, 4.03 mmol/L EDTA, 4 g/L penicillin G, 3 g/L dihydrostreptomycin, 1% lecithin in distilled water. Each extender group, except the control, was supplemented with relevant concentrations of RJ according to experimental design.

Semen collection and dilution: Between 3 and 5 years old five Saanen goats were used as the material of study during non-breeding season. Goats were maintained at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey. Electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) was used for semen collection five times in every other day. Immediately after collection, the ejaculates were transferred to a water bath (37 °C) and then evaluated for rapid wave motion and motility. Evaluation was performed using a phase-contrast microscope (Olympus BX51, Olympus Optical Co., Tokyo, Japan) with a warm slide (37 °C). Only ejaculates with rapid wave (>+3 on 0–5 scale), >75% motility and >1.0 × 10^9 spermatozoon/mL were used for cryopreservation. To eliminate individual differences, chosen ejaculates were pooled. Five pooled ejaculates were included in the study.

Briefly, pooled ejaculates were split into four equal aliquots. Each group was separately diluted to final concentration of approximately 150 × 10^6 (spermatozoon/mL) with control or RJ supplemented extenders. Diluted semen was gradually cooled to 4 °C within 1 h and equilibrated for further 2 h at 4 °C [6].

Semen freezing and thawing: After equilibration, cooled semen was placed into 0.25 mL French straws and frozen at 3 °C/min from +5 °C to −8 °C and at 15 °C/min from −8 °C to −120 °C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France). The straws were then plunged into liquid nitrogen at −196 °C where they were stored for at least one month. Three straws from each group were thawed at 37 °C for 30 s in a water bath and incubated in humidified air chamber with 5% CO_2 for 6 h at 39 °C to evaluate post-thaw semen characteristics [26].

Semen evaluation: We assessed sperm motility, plasma membrane integrity (hypoosmotic swelling test [HOST]), acrosome integrity (FITC conjugated Pisumsativum agglutinin [PSA-FITC]) and DNA integrity (using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL]) at two time points (post-thaw at). All processes and measurements were conducted by the same person during the study. Sperm motility was assessed subjectively using a phase-contrast microscope (400x) with a warm slide (37 °C).

The hypoosmotic swelling test (HOST): HOST was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails, and was performed by incubating 10 μL of semen with 100 μL of 100 mOsm hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37 °C for 60 min. After incubation, 20 μL of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm cells was evaluated under 1000x magnification with phase-contrast microscope. Sperms with swollen or coiled tails were recorded [11].

Fluorescein lectin staining assay: Acrosome integrity was assessed using PSA-FITC [5]. Briefly, 20 μL of diluted semen was resuspended in 500 μL PBS and centrifuged at 100 RCF (g) for 10 min; the supernatant was then discarded. The spermatozoa pellet was resuspended in 250 μL PBS and one drop from resuspended spermatozoa was smeared on a glass microscope slide and air dried. The air-dried slides were fixed with acetone at 4 °C for 10 min, and the slides were covered with FITC PSA solution (50 μg/mL in PBS solution) in the dark room for 30 min. Stained slides were rinsed with PBS solution, covered with glycerol, and examined under a fluorescence microscope. At least 200 spermatozoa per smear were evaluated for acrosome integrity.

TUNEL assay: For the TUNEL technique, we used the In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications. In brief, one drop of resuspended spermatozoa was smeared on a glass slide and fixed with 10% formaldehyde for 20 min at room temperature. The slides were washed in PBS and stored at 4 °C until use. Upon removal from storage, samples were washed again in PBS (three times for 5 min). They were then treated in a humidified chamber with proteinase K for 10 min at the room temperature, washed with PBS, treated with 3% H_2O_2 in distilled water for 10 min at the room temperature and washed again with PBS. The slides were permeabilized with 0.1% Triton X-100 for 5 min on ice. The permeabilized slides were incubated in the dark chamber at 37 °C for 1 h with the TUNEL reaction mixture which contained terminal deoxynucleotidyl transferase (TdT) plus dUTP label. After labeling, samples were washed with PBS and analyzed immediately via fluorescence microscopy. Negative (omitting TdT from the reaction mixture) and positive (using DNase I, 1 mg/mL, for 10 min at room temperature) controls were included in each trial. At least 200 sperm cells were evaluated to determine the percentage of TUNEL positive sperm cells. Each microscopic field was evaluated first under fluorescence microscopy (400 x magnification) to determine the number of reactive sperm and then under phase-contrast microscope to determine the total number of sperm per field [6].

Statistical analysis: All data obtained from study were analyzed using SPSS (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Data were represented as mean ± standard deviation. Shapiro Wilk test was used as normality test. Means of obtained semen parameters