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Does rosemary aqueous extract improve buck semen cryopreservation?

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ABSTRACT

This study was conducted to investigate the effect of rosemary aqueous extract for protecting buck spermatozoa from freeze–thawing damages. In this study, a total of 32 ejaculates were collected from four Kordian bucks (8 ejaculates for each buck). On the day of semen collection, four ejaculates (one ejaculate for each buck) were pooled and diluted with egg yolk Tris-based extenders containing 0 (E0), 2 (E2), 4 (E4), and 6 (E6) percent of rosemary aqueous extract. After thawing, sperm motility (computer-assisted sperm motility analysis (CASA)), membrane integrity (eosin/nigrosin) and functionality (Hypoosmotic swelling (HOS) test), abnormal morphology, lipid peroxidation (malondialdehyde (MDA) production), capacitation status (Chlortetracycline (CTC)), mitochondrial activity (rhodamine-123) and apoptotic features (Annexin V/propidium iodide) were assessed. The results showed that E4 extender significantly improved total (45.71 ± 1.49) and progressive (24.86 ± 0.84) motility, functional membrane (47.18 ± 1.37), and integrity (49.25 ± 1.17) of post-thawed buck spermatozoa when compared to E0 (36.43 ± 1.49, 18.43 ± 0.84, 39.42 ± 1.37 and 41.32 ± 1.17, respectively) and E6 (30.43 ± 1.49, 16.29 ± 0.84, 34.54 ± 1.37 and 36.39 ± 1.17, respectively) extenders. Moreover, spermatozoa with abnormal morphology were significantly increased in E6 (22.5 ± 1.07) compared to E2 (17.29 ± 1.07) and E4 (16.11 ± 1.07) extenders. The results showed that only E2 (1.69 ± 0.08) extender significantly decreased the level of MDA formation when compared to E0 (2.04 ± 0.08) and E4 (2.03 ± 0.08) extender. In this study, different level of rosemary aqueous extract failed to alter the CTC staining patterns and the mitochondrial activity of post-thawed buck spermatozoa. Moreover, E4 extender significantly increased live (55.92 ± 2.97) and decreased dead (22.64 ± 3.37) spermatozoa compared to other extenders. In conclusion, addition of rosemary aqueous extract at level of 4% can improve post-thawed buck spermatozoa quality. More studies are required to reveal the active components in rosemary extract.

1. Introduction

Semen freezing is a technique to maintain sperm for long time and using them for artificial insemination (AI) and in vitro fertilization (IVF) especially in farm animals (Demianowicz and Strzezek, 1995). It has been found that freezing process has detrimental effects on DNA, mitochondria, plasma membrane structure and function of

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spermatozoa by means of some stressors such as osmotic and oxidative stresses (Aitken et al., 1998; Meyers, 2005; Purdy, 2006; Sariozkan et al., 2009). On the other hand, it has been well defined that mammalian sperm are prone to membrane damages mainly due to a high amount of unsaturated fatty acids in their plasma membrane (Alvarez and Storey, 1995). During sperm freezing the endogenous antioxidant defense cannot defend properly the sperm membrane against the increased amount of reactive oxygen species (ROS) during freezing (Aurich et al., 1997; Kasimanickam et al., 2006), therefore, sperm organelles become more susceptible to any oxidative damage.

It has been shown that sperm quality during freeze–thawing process can be better maintained by addition of various antioxidants to semen extender compared to control (Bilodeau et al., 2001). There are limited document studying the effect of medicinal plant extracts as a pool of antioxidants on sperm quality. One of the well known medicinal plants is rosemary (*Rosmarinus officinalis*) that has several biologically active components (Hammerstedt, 1993). Carnosic components and rosmaric acids are two major components of the rosemary which their antioxidant property has been previously reported (Moreno et al., 2006; Frankel et al., 1996; Richheimer et al., 1996; Cuvelier et al., 1996). More recently, it has been shown that semen freezing extenders supplemented with rosemary aqueous extract can improve post-thawed sperm quality in porcine (Malo et al., 2010), canine (Gonzalez et al., 2010) and ovine (Gil et al., 2010).

Therefore, the present study was conducted to determine the effects of rosemary aqueous extract in semen freezing extender on post-thawed buck sperm quality.

2. Materials and methods

2.1. Chemical

Chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Rosemary sources were natural herbs.

2.2. Media

A Tris-based extender [Tris buffer (Tris 30.7 g/l, citric acid 16.8 g/l, fructose 12.6 g/l), egg yolk 15% (v/v) + glycerol 5% (v/v)] was used in this study. Experimental treatments included four extenders supplemented with different levels (0, 2, 4, and 6%) of rosemary aqueous extract. Fresh rosemary was gathered in December for extraction [Karaj, Iran (37°.47 N, 50°.55 E)]. Rosemary aqueous extract was provided by soaking fresh leaves of rosemary in water. One hundred milliliters of water was heated to 100 °C and during boiling 0.15 g of fresh rosemary leaves were added to this water and maintained (10 min). Once the water had cooled off to 25 °C, the solution was filtered with a syringe filters with a pore diameter of 0.2 μm to remove the debris (Malo et al., 2010). The resulting solution had a pH around 7 (final pH 6.8, the final osmolarity 425 mOsm).

2.3. Animals and semen collection

In this study, semen samples were taken from four Kordian bucks (3 and 4 years of age). The Kordian bucks were housed at the Education and Research Farm of Animal Science Department, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. Ejaculates were taken twice a week using artificial vagina during the seasonal breeding (early autumn to early winter). After semen collection, the samples were kept at 33 °C in a water bath until primary evaluation in the laboratory.

2.4. Semen extending, freezing and thawing

In this study, only ejaculates with >0.75 ml in volume, >70% progressive motile spermatozoa and >2.5×10^9 spermatozoa/ml were used. A total of 32 ejaculates (8 ejaculates for each buck) were collected from the bucks using artificial vagina twice a week (two ejaculates from all bucks during 1 week and on same days). On the day of semen collection, four ejaculates (one ejaculate for each buck) were pooled and split into four equal aliquots and diluted at 37 °C with different extenders with a final concentration of 240×10^6 spermatozoa/ml. Semen extending was done by one step procedure. Diluted semen samples were aspirated into 0.25 ml French straws (IMV, L'Aigle, France), sealed with hematocrit pulp and equilibrated at 5 °C for 2 h. Afterward, the straws were horizontally frozen in liquid nitrogen (LN) vapor (4 cm above the LN, for 7 min), and plunged into LN for storage (Salmani et al., 2013). The frozen straws were thawed individually at 37 °C for 30 s in a water bath for different evaluations.

2.5. Semen evaluation

2.5.1. Sperm motility

A computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to evaluate sperm motility and motion parameters. Semen sample was placed in a chamber and the loaded chamber placed on the warm stage of the microscope (37 °C). Three randomly selected microscopic fields were scanned five times each. The mean of these 15 scans was used for statistical analysis (Salmani et al., 2013). The following variables were analyzed: total motility (TM, %); progressive motility (PM, %); average path velocity (VAP, μm/s); straight-line velocity (VSL, μm/s); curvilinear velocity (VCL, μm/s); amplitude of lateral head displacement (ALH, μm); beat/cross frequency (BCF, Hz); straightness (STR, %); linearity (LIN, %).

2.5.2. Sperm viability

Viable and non-viable spermatozoa were determined by the eosin–nigrosin staining (Barth and Oko, 1989). Twenty microliters of both semen suspension and eosin–nigrosin stain were mixed and spread on a warm slide. The viable and non-viable spermatozoa were determined by counting 200 sperm under phase-contrast microscopy (CKX41; Olympus, Tokyo, Japan) at 400×.

2.5.3. Sperm morphology

Spermatozoa with abnormal morphology were assessed by the Hancock's solution (HS) (HS: 62.5 ml formalin, 150 ml sodium saline solution, 150 ml buffer solution, and 500 ml double-distilled water) (Schafer and Holzmann, 2000). For this assessment, three drops of semen suspension were mixed with 1 ml HS. Afterward, one drop of this mixture was placed on a slide and mounted with a coverslip. The percent of abnormal spermatozoa was recorded by counting 200 spermatozoa under phase contrast microscopy at 1000×.

2.5.4. Hypo-osmotic swelling test

The hypo-osmotic swelling (HOS) test was used to evaluate the functional plasma membrane of spermatozoa as described by Revell and Mrode (1994). Briefly, 10 μl semen were incubated with 100 μl hypo-osmotic solution (9 g/l fructose and 4.9 g/l sodium citrate, 100 mOsm/kg) at 37 °C for 60 min. Afterward, 0.1 ml of the mixture was placed on a microscope slide and mounted with a coverslip. A total of 400 spermatozoa were evaluated and sperm with swollen and coiled tails were determined in each sample under phase-contrast microscopy at 400×.

2.5.5. Malondialdehyde (MDA) concentration

Malondialdehyde concentrations, as an index for lipid peroxidation (LPO) in the semen samples, were measured using the thiobarbituric-acid reaction (Esterbauer and Cheeseman, 1990). Briefly, 1 ml of the diluted semen (250×10^6 spermatozoa/ml) was mixed with 1 ml of cold 20% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifuging ($960 \times g$ for 15 min), and 1 ml of the supernatant was incubated with 1 ml of 0.67% (w/v) thiobarbituric acid in a boiling water bath at 100 °C for 10 min. After cooling, the absorbance was determined by a spectrophotometer at 532 nm. All MDA concentrations were expressed as nmol/ml.