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Antioxidant effect of rosemary (*Rosmarinus officinalis* L.) extract in soybean lecithin-based semen extender following freeze–thawing process of ram sperm

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ABSTRACT

The aim of current study was to evaluate effect of rosemary aqueous extract on post-thawed ram sperm quality in a soybean lecithin-based (SL) extender. Ram semen samples were obtained, extended with SL extender and supplemented with 0% (SL-R0), 2% (SL-R2), 4% (SL-R4), 6% (SL-R6), and 8% (SL-R8) rosemary aqueous extract. Following equilibration, the straws were frozen, and then plunged into the liquid nitrogen. After thawing, sperm motility and velocity parameters, plasma membrane functionality, viability, acrosomal and capacitation status were evaluated. Membrane lipid peroxidation was also analyzed through the malondialdehyde (MDA) concentration. Our results showed that SL-R4 and SL-R6 groups resulted in higher ($p < 0.05$) percentages of total motility, progressive motility, and plasma membrane functionality, as compared with other groups. Highest ($p < 0.05$) viable and lowest ($p < 0.05$) dead spermatozoa were observed in SL-R6 group compared to the other groups. The acrosomal and capacitation status were not affected ($p > 0.05$) by different levels of rosemary aqueous extract. Lower ($p < 0.05$) MDA concentration has been observed in SL-R4 and SL-R6 groups. The results of this study demonstrate that supplementation of SL extender with rosemary aqueous extract influences post-thawed ram sperm quality in a dose dependent manner.

Introduction

It is well known that spermatozoa of mammalian species is highly sensitive to oxidative stress [9,16,26,39,41] due to the extrusion of its antioxidant-rich cytoplasm during maturation stages. The high concentration of polyunsaturated fatty acids in the plasma membrane of the spermatozoa is also considered as another key factor in this susceptibility to the oxidative stress. Furthermore, the process of semen cryopreservation produces significant amounts of reactive oxygen species (ROS) which may lead to impairment of sperm morphology, function, and ultwimately male fertility [5,40].

On the basis of these, several studies [1,4,23,28,31,35,43,45] including our own work [44] have shown the beneficial effect of antioxidant therapy on oxidative stress in mammalian spermatozoa. During recent years, use of herbal antioxidants, has been gaining attention from several researchers [2,10,25,29]. In this regards, it is of great interest to note that two-thirds of the world's plant species have medicinal value; in particular, many medicinal plants have great antioxidant potential [22]. For instance, the plant species such as sage [19], oregano [38], and rosemary [19] have been tested for development of the natural antioxidant formulations in the areas of medicine and nutrition. Among these species, rosemary (*Rosmarinus officinalis* L.) aqueous extract with its powerful antioxidant activity is often the first choice for processed foods and widely used in the food industry [34].

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Caffeic acid and its derivatives such as diterpenes, triterpenes, flavonoids, and polyphenols are among the main characteristic components of rosemary, which have antioxidant properties [11,18]. Rosemary aqueous extract has been utilized as a preserving and treating agent against oxidative stress in several previous reports [14,17,20,21], However, until now, such a positive effect on sperm processing and semen cryopreservation has been reported only in a few studies [23,44,24] including our previous study [44].

In light of the above information, it can be hypothesized that rosemary aqueous extract may acts as an appropriate antioxidant against cryopreservation-mediated decrease in sperm viability and motility after freeze–thawing cycles. Evaluation of the rosemary extract antioxidant activity would be quite interesting in a ram model since spermatozoa of this species contain a high content of poly unsaturated fatty acid in the membrane phospholipids. In addition the seminal plasma of the ram were found to have a lower potential of antioxidant activities compared to other species [32].

Our trial to introduce a plant-origin extender for ram semen in recent years, has revealed that exogenous phosphatidylcholine from soybean lecithin would be a perfect cryoprotectant to protect sperm against freeze–thawing damages [13,27,33,37]. Therefore, with the aim of evaluating a plant origin antioxidant substance for our plant-origin extender, a prospective study was conducted using rosemary as a natural antioxidant in semen extender. To accomplish this we added rosemary aqueous extract to a soybean lecithin-based extender in a dose dependent manner and after the freeze–thawing process we assessed sperm motility and velocity parameters using the sperm class analysis system. In addition, sperm plasma membrane functionality, total abnormality, capacitation status, and apoptosis features were evaluated.

Materials and methods

Ethics

All issues concerning the experimental setups and evaluation techniques have been approved by the local Scientific Ethical Committee, university of Tehran, Karaj, Iran.

Chemicals

Chemicals used in this study were obtained from Sigma Co (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Semen collection

Semen samples were obtained from four mature Chal rams (3–5 years old) of superior genetic merit and proven fertility. Ejaculates were collected twice a week using an artificial vagina during breeding season. Immediately after collection, the ejaculates were transferred to a water bath (37 °C) and then evaluated. Only ejaculates with the following criteria were used for experimentation: >0.75 mL in volume; $>3 \times 10^9$ spermatozoa/mL; >80% motile and <10% abnormal spermatozoa. To eliminate individual differences, semen were pooled and processed for extending.

Preparation of rosemary aqueous extract

For preparation of the rosemary aqueous extract 0.15 g of fresh rosemary leaves were added to 100 mL of boiling distilled water and maintained for 10 min. After cooling of the water (25 °C), the solution was filtered with a syringe filters with a pore diameter of 0.2 μm to remove the debris [23,44]. The resulting solution had a pH around 7 (final pH 6.8, the final osmolarity 425 mOsm/kg).

Extender preparation and cryopreservation

The pooled sample (obtained from four rams in each replicate) was split into five equal aliquots and diluted with the SL extender supplemented by different concentrations of rosemary aqueous extract: Extender containing no rosemary aqueous extract (SL-R0), extender containing 2% (v/v) rosemary aqueous extract (SL-R2), extender containing 4% (v/v) rosemary aqueous extract (SL-R4), extender containing 6% (v/v) rosemary aqueous extract (SL-R6) and extender containing 8% (v/v) rosemary aqueous extract (SL-R8). The SL extender consisted of basic buffer (27 g/l Tris, 10 g/l fructose, and 14 g/l citric acid) and 1% (w/v) soybean lecithin along with 7% (v/v) glycerol [13]. After dilution, semen samples were aspirated into 0.25 mL French straws (100×10^6 - spermatozoa/mL) and sealed with polyvinyl alcohol powder and equilibrated at 5 °C for 2 h [13]. After equilibration, the straws were frozen in liquid nitrogen vapor, 5 cm above the liquid nitrogen for 12 min, and then plunged into the liquid nitrogen for storage. The frozen straws were thawed individually (37 °C) for 30 s in a water bath for evaluation.

Sperm motility and velocity parameters

Sperm class analysis system (SCA, Version 5.1; Microptic, Barcelona, Spain) was used to analyze sperm motility and velocity parameters. The following motility values were recorded: TM (total motility, %), PM (progressive motility, %), VAP (average path velocity, μm/s), VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s), ALH (amplitude of later al head displacement, μm), BCF (beat/cross frequency, Hz); STR (straightness, %); LIN (linearity, %).

Sperm plasma membrane functionality

The hypoosmotic swelling (HOS) test was used to evaluate plasma membrane functionality of spermatozoa after freeze–thawing. The HOS test relies on the resistance of the membrane to loss of permeability barriers under stress condition of stretching in a hypo-osmotic medium. The assay was performed by mixing 30 μL of semen with a 300 μL hypo-osmotic solution (9 g/l fructose and 4.9 g/l sodium citrate, 100 mOsm/kg) (Revell and Mrode 1994). This mixture was incubated (37 °C) for 30 min, and then 0.2 mL of the mixture was placed on a microscope slide and mounted with a cover slip and immediately evaluated (400× magnification) under phase-contrast microscope (CKX41; Olympus, Tokyo, Japan). Two hundred spermatozoa with swollen and not-swollen tails were recorded.

Phosphatidylserine translocation assay

Commercial kit (IQP, Groningen, The Netherlands) was used for determination of viable, apoptotic and dead spermatozoa. Briefly, spermatozoa were washed in a calcium buffer and then, 10 μL Annexin V-FITC was added to 100 μL sperm suspension and incubated for 20 min on ice. Afterward, 10 μL PI was added to sperm suspension and incubated for 15 min at room temperature. Finally, each tube was analyzed by flow cytometry (Becton Dickinson, San Khosoz, CA, USA). For each sample, 10,000 events were collected. The spermatozoa were classified to three groups: The lower left quadrant contains viable non-apoptotic cells which are negative for Annexin-V and exclude PI staining (A^-/PI^-). The lower right quadrant shows early apoptotic cells which bind Annexin-V but exclude PI (A^+/PI^-) and upper left and right quadrants contain dead spermatozoa, stained with PI (PI^+) [44,13,27].

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