Cryobiology 78 (2017) 34–40

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen

Mahdieh Mehdipour [a], Hossein Daghigh Kia [a, *], Maryam Nazari [b], Abouzar Najafi [a]

[a] Department of Animal Science, College of Agriculture, University of Tabriz, Tabriz, Iran
[b] Department of Food Science and Technology, College of Agriculture, University of Tabriz, Tabriz, Iran

ARTICLE INFO

Article history:
Received 1 January 2017
Received in revised form
28 May 2017
Accepted 13 July 2017
Available online 14 July 2017

Keywords:
Lecithin nanoliposome
Soybean lecithin
Pomegranate extract
Ram sperm
Cryopreservation

ABSTRACT

This investigation was carried out to study the effect of soybean lecithin 1.5% (wt/vol) (0, 2.5, 5 and 7.5 mg l^{-1} pomegranate extract (PE)) or PE-loaded lecithin nanoliposome (0, 2.5, 5 and 7.5 mg l^{-1}) to Tris-based extender. Sperm motility (CASA), viability, membrane integrity (HOS test), abnormalities, mitochondrial activity, apoptosis status, lipid peroxidation, total antioxidant capacity (TAC)) and antioxidant activities (GPX, SOD) were investigated following freeze-thawing. No significant differences were detected in motility parameters, viability, membrane integrity, and mitochondria activity after thawing sperm between soybean lecithin and lecithin nanoliposomes. It was shown that PE5 significantly improved sperm total and progressive motility, membrane integrity, viability, mitochondria activity, TAC and reduced lipid peroxidation (malondialdehyde concentration). Moreover, the percentage of apoptotic sperm in PE5 extenders was significantly the lowest among other treatments. Sperm abnormalities, SOD and GPX were not affected by the antioxidant supplements. For apoptotic status, no differences were observed between soybean lecithin and lecithin nanoliposome. We showed that lecithin nanoliposome extender can be a beneficial alternative extender to protect ram sperm during cryopreservation without any adverse effects. It was also observed that regarding pomegranate concentration, PE5 can improve the quality of ram semen after thawing.

1. Introduction

Sperm cryopreservation offers various advantages to the animal fertility by worldwide distribution of beneficial genetic material by means of artificial insemination [20]. Sperm cells can be injured by reactive oxygen species (ROS) during freeze-thawing. It is shown that ROS can cause various deleterious changes to the sperm, such as decreasing sperm motility, disrupting membrane integrity and causing DNA damage [40]. In this regard, production of high amount of ROS decreases mitochondria activity and sperm motility which results in a decrease in sperm membrane fluidity, which is necessary in sperm-oocyte conjugation [10]. Nevertheless, cell cytoplasm contains various protective antioxidant systems which are not sufficiently effective to prevent LPO of sperm cells during freeze–thaw process [25]. So, adding antioxidant to the semen extender may have favorable effects on ROS removal.

During recent years, using herbal antioxidants, has been gaining attention from several researchers [18,29]. Pomegranate (*Punica granatum*) belongs to a widely known group of edible plants, which has shown promising effects on semen quality without having undesirable side effects [17]. Pomegranates include large amount of polyphenolic compounds such as flavonoids, condensed tannins, and hydrolysable tannins (ellagitannins [ETs] and gallotannins) having antioxidant activities which reduces ROS [7]. Punicalagin is the most numerous ET and is considered as the largest polyphenol among the pomegranate ellagitannins which is responsible for most of the antioxidant activities of PE [41].

Effective delivery of an antioxidant into sperm in required levels is a valuable challenge. A distinguished advance for appropriate freezing is using antioxidant loaded liposomes in the extender. Liposomes are biodegradable and biocompatible carriers which can be obtained from lipids with adjustable physico-chemical characteristics and loaded with various lipophilic-hydrophilic

* Corresponding author.
E-mail addresses: Hdk6955@gmail.com, daghighkia@tabrizu.ac.ir (H. Daghigh Kia).

http://dx.doi.org/10.1016/j.cryobiol.2017.07.005
0011-2240/

compounds. For preparing liposomes, optimal composition and concentration of phospholipids, the active component of the egg-yolk that guarantee the cell protection during cryopreservation [8], are needed. The addition of liposomes to extenders is of great relevance because of its precisely defined structure and composition which can be improved by adjusting their phospholipids composition or their size [38]. Thus, liposomes would be a satisfactory alternative to improve the semen extender. The main goals include encapsulating and releasing antioxidant components in a controlled manner, as well as increasing their bioavailability and stability. Furthermore, the profitable effect of a nutrient is directly related to the rate and quantity at which the intact nutrient reaches the cell [42].

The purpose of this study was to carry an efficient amount of pomegranate extract to sperm via lecithin nanoliposome. In this research, we verified that the nano encapsulation improved the antioxidant effect of pomegranate by lecithin nanoliposome compared to the supplementation of soybean lecithin based semen extender by pomegranate. This study was therefore designed to prepare pomegranate loaded lecithin nanoliposome to protect sperm against lipid peroxidation.

2. Materials and methods

2.1. Pomegranate extract

Pomegranates were juiced and then shake with deionized water for 24 h. Clear extraction was achieved by filtering the mixture and condensing the extract under vacuum on a rotary evaporator. Finally, the condensed pomegranate extract was dried using a freeze dryer and kept refrigerated until further use [23].

2.2. Animals and semen collection

The experiment was done on four mature Ghezel rams (3–4 years old) known to have superior genetic merit and good fertility which the semen samples were obtained from. The Ghezel rams were housed at Livestock Research Station at Tabriz University (Iran, Tabriz) with uniform nutritional conditions. Semen collection was accomplished by the same people under the same condition by artificial vagina twice a week. The ejaculates with the following favorite criteria were accepted in the study: volume between 0.75 and 2 mL, sperm concentration $>3 \times 10^9$ sperm ml^{-1}, sperm motility higher than 80%, and less than 10% abnormal sperm.

2.3. Preparation of PE liposomes

PE liposome was achieved by reverse-phase evaporation procedure. Weighed amount of soy bean lecithin was added to the corresponding amount of pomegranate extract, then was dissolved with 6 mL of ethanol. In order to evaporate off the solvent, a rotary evaporator was used at 50 °C under vacuum and a thin lipid film was produced. The lipid film was gradually hydrated with 0.5 mL glycerol mixed with 9.5 mL of phosphate buffered saline. A water-in-oil (W/O) emulsion was produced by shaking for 30 min at 30 °C. This emulsion was dispersed for liposome formation. Liposomal size decreased with treated homogenizer for 20 min at 50–60 °C, the emulsion was dispersed by probe sonication for 10 min in amplitude of 70% to obtain a nanoliposome containing PE extract and was stored at 4 °C until future use in the semen extender.

2.4. Experimental design

The basic hand-made cryopreservation medium was composed of 223.71 mM Tris, 55.50 mM fructose, 72.87 mM citric acid (320 mOsm kg, pH 7.2) and glycerol 7% (v/v). The base extenders were supplemented with soybean lecithin 1.5% (wt/vol) (0, 2.5, 5 and 7.5 mg l^{-1} PE) or PE-loaded lecithin nanoliposome (0, 2.5, 5 and 7.5 mg l^{-1}) to Tris-based extender.

2.5. Semen dilution, cryopreservation and thawing

Semen samples were divided into eight aliquots and diluted in cryopreservation media in their groups to get concentration of 4×10^8 sperm ml^{-1}. The sperm samples were slowly equilibrated to 4 °C for a period of 2 h. After equilibration, they were aspirated into 0.25-ml French straws (IMV, L'Agile France). Then the straws were cryopreserved to static nitrogen vapor 4 cm above the liquid nitrogen (LN_2) level for 7 min, plunged LN_2 and stored in LN2 until thawed.

2.6. Sperm motility and motion parameters

For motility and velocity parameters, a computer-assisted sperm motility analysis (CASA; 12.3 CEROS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to assess the following motility parameters: sperm motility (TM), progressive motile sperm (PM) and Kinematic parameters for each spermatozoa included the curvilinear velocity (VCL, μm/s), the straightline velocity (VSL, μm/s), the average path velocity (VAP, μm/s), the linearity index [LIN %, (VSL/VCL) x 100], the straightness index [STR %,(VSL/VAP) x 100], mean amplitude of lateral head displacement (ALH, μm) and mean of the beat cross frequency (BCF, Hz). Five μl of sperm was placed onto a pre-warmed chamber slide (37 °C) for analyzing the sperm parameters.

2.7. Sperm viability

Sperm viability was evaluated by the nigrosin–eosin staining [30]. Semen suspension smears were prepared by mixing 20 μL of semen sample with 20 μL of stain on a warm slide and uniform smear (spreading the stain with a second slide). After drying, 200 Sperm cells were microscopically evaluated at × 400 magnification (CKX41; Olympus, Tokyo, Japan).

2.8. Membrane integrity

For membrane integrity, hypo-osmotic swelling (HOS) test was used based on curled and swollen tails of sperm. The assay was performed by mixing 10 μL of semen with a 100 μL of hypoosmotic solution (19.2 mM sodium citrate and 57.6 mM fructose, 100 mOsm kg^{-1}) [27]. After incubation, 200 sperm were counted in different fields under phase-contrast microscope (CKX41; Olympus, Tokyo, Japan) at × 400, and the percentage of sperm with curled and swollen tails were determined.

2.9. Sperm abnormalities

For the assessment of total abnormalities in the spermatozoa, three drops (10 μL) of semen were homogenized with 1 mL Hancock's solution [15]. Hancock solution consisted 62.5 mL formalin (37% formaldehyde), 150 mL of sodium saline solution [4.5 g NaCl in 250 mL double distilled water], 150 mL buffer solution [(1) 10.85 g Na_2HPO_4 in 250 mL double distilled water; (2) 11.127 g KH_2PO_4 in 250 mL double distilled water; %71.43 of (1) and 28.57% of (2) were mixed]. To detect total abnormalities in the spermatozoa, 15 μL of processed sperm was placed on a glass slide and covered with a coverslip. The percentage of total abnormalities in the sperm was assessed by counting a total of two hundred sperm under a phase-contrast microscope (× 1000).