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Effects of CoQ10 on the quality of ram sperm during cryopreservation in plant and animal based extenders

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ABSTRACT

The purpose of this study was to evaluate the effect of different concentrations of CoQ10 in soybean lecithin (SL) or egg yolk (EY) extenders on ram semen cryopreservation. Semen samples were collected from five rams, twice a week, then diluted in the extenders (SL and EY) containing different concentrations of CoQ10 as follows: extender containing SL: 0 μM (control, SL/Q0), 1 μM (SL/Q1), 2 μM (SL/Q2), 5 μM (SL/Q5) and 10 μM (SL/Q10) CoQ10; extender containing EY: 0 μM (control, EY/Q0), 1 μM (EY/Q1), 2 μM (EY/Q2), 5 μM (EY/Q5) and 10 μM (EY/Q10) CoQ10. Sperm motion characteristics, membrane integrity, abnormal morphology, viability, apoptotic-like changes, mitochondria active potential, acrosome integrity and lipid peroxidation were evaluated after freeze-thaw process. The SL/Q1, SL/Q2, EY/Q1 and EY/Q2 resulted in greater ($P \leq 0.05$) sperm total motility, progressive motility, membrane integrity and mitochondria active potential compared to the other groups. Acrosome integrity in the SL/Q0, SL/Q1, SL/Q2, EY/Q0, EY/Q1 and EY/Q2 groups was greater ($P \leq 0.05$) than in the SL/Q5, SL/Q10, EY/Q5 and EY/Q10 groups. The SL/Q2 and EY/Q2 treatment groups had greater ($P \leq 0.05$) sperm viability rates and less apoptotic-like changes and lipid peroxidation. The CoQ10 compound could be explored as a novel potential antioxidant for cryopreservation of ram semen because with used of this compound in the present study there was an improved post-thawed sperm quality.

1. Introduction

There have been efforts towards optimization of ram semen cryopreservation to improve reproductive performance (Başpinar et al., 2011; Silva et al., 2011). The use of frozen-thawed ram semen is useful for distribution of genetic material in this species. Lesser than desirable pregnancy rates after artificial insemination, however, are a significant problem, that has limited the use of this technique (Salamon and Maxwell, 1995). Semen freezing-thawing processes induce structural and biochemical stress on sperm, thereby reducing post-thawing sperm quality (Evans, 1991; Grossfeld et al., 2008; Sharafi et al., 2009).

Reactive oxygen species (ROS), which are products of lipid peroxidation (LPO) during cryopreservation, suppress sperm viability and decrease the fertility capacity of frozen-thawed sperm by reducing sperm motility and fertilizing capacity (Agarwal et al., 2014; Aitken et al., 2014; Moghbeli et al., 2016).

Antioxidants are useful additives for mammalian semen cryopreservation (Bucak et al., 2009; El-Sheshtawy et al., 2015). The

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endogenous antioxidants, however, are not in adequate concentrations to counteract the effects of LPO (Aurich et al., 1997), hence, providing an auxiliary protection system could be helpful for ram sperm cryopreservation.

Coenzyme Q10 (CoQ10) is a component of the mitochondrial respiratory chain which has an important function in the energy metabolism and also functions as an antioxidant in cell membranes and for lipoproteins (Ernster and Forsmark-Andrée, 1993). There is a considerable amount of CoQ10 biosynthesis in the testis (Kalen et al., 1990). Ubiquinol, a reduced form of CoQ10, which is present in sperm (Mancini et al., 1998) and has a protective function as an antioxidant. The CoQ10 compound is also used as a motility enhancer in human sperm processing due to its capacity to control LPO and DNA fragmentation (Talevi et al., 2013). Furthermore, in farm animals, the use of CoQ10 improves the cryo-resistance of sperm cells of buffalo bulls (Saeed et al., 2016), stallions (Yousefian et al., 2014), boars (Pindaru et al., 2015), billy goats (Yousefian et al., 2018) and roosters (Masoudi et al., 2018).

Extender components can be modified most effectively in freezing processes for affecting sperm viability (Forouzanfar et al., 2010). Soybean lecithin (SL) has recently been effectively utilized as an extracellular cryoprotectant for semen freezing in humans (Reed et al., 2009), buffalo (Akhter et al., 2012), cattle (Aires, 2003), goats (Salmani et al., 2013) and sheep (Forouzanfar et al., 2010; Sharafi et al., 2015a). The SL is a replacement for egg yolk (EY) due to an increase in the risk of microbial contamination with use of egg yolk-based extenders (Sharafi et al., 2015a). In addition, EY increases the agglutination and early acrosome reaction of sperm, which have detrimental effects on fertilizing capacity of sperm (Salmani et al., 2014). Furthermore, there is considerable variation of EY composition that make it difficult to standardize extenders with use of EY (Sharafi et al., 2009).

The aim of the present study, therefore, was to evaluate the effect of different concentrations of CoQ10 with use of SL or EY extenders on post-thawed ram semen quality. For this purpose, sperm motion characteristics, membrane integrity, abnormal morphology, mitochondria active potential, acrosome integrity, viability, apoptotic like changes, as well as LPO were assessed after imposing freezing-thawing processes.

2. Materials and methods

Chemicals used in this study were obtained from Sigma Co (St. Louis, MO, USA) and Merck (Darmstadt, Germany) unless otherwise indicated. There was approval for the present study by the Research and Ethics Committees of University of Tehran and Iran National Science Foundation with the authorization code of 95007081.

2.1. Extender preparation

The components of the basic buffer were 62.8 mM Tris (Merck, Darmstadt, Germany), 55.5 mM fructose (Merck), 72.8 mM citric acid (Merck), 7% glycerol (v/v), 100 IU penicillin and 1 mg streptomycin. The osmolality and pH were controlled to be 320 mOsm/kg water and 7.2, respectively. The SL extender was made of basic buffer supplemented with 1% (w/v) soybean lecithin (from phosphatidyl choline). The EY extender was composed of the basic buffer supplemented with 20% (v/v) egg yolk.

2.2. Semen collection and processing

Semen was collected using artificial vagina twice a week from five mature Zell rams. A total of 30 ejaculates were collected for 3 consecutive weeks (twice a week), with six collections (technical replicates) from each ram. Semen was evaluated and accepted for this experiment if the following variables were assessed: volume ranging between 1 to 2 ml; sperm concentration of 3×10^9 sperm/ml, and sperm total motility greater than 70%. To eliminate individual differences, whole semen samples from each ram were pooled with the final concentration being 4×10^9 spermatozoa/ml. Pooled samples were aliquoted in two equal volumes and were diluted using the two different extenders (SL and EY). Different quantities of CoQ10 were subsequently added to each type of extenders as follows: extender containing SL: 0 μM (control, SL/Q0), 1 μM (SL/Q1), 2 μM (SL/Q2), 5 μM (SL/Q5) and 10 μM (SL/Q10) CoQ10; extender containing EY: 0 μM (control, EY/Q0), 1 μM (EY/Q1), 2 μM (EY/Q2), 5 μM (EY/Q5) and 10 μM (EY/Q10) CoQ10. A fresh semen group was also used as technical control group.

To obtain the concentration of 50×10^6 spermatozoa/straw, samples were diluted with the extenders at a ratio of 1:20. The diluted semen was gradually cooled to 4 °C for 90 min and subsequently loaded in 0.25 ml French straws (Biovet, L'Agile France).

The straws were subsequently positioned so there was exposure to static nitrogen vapor (−70 °C) for 10 min, plunged into liquid nitrogen (LN_2) and stored until thawed (at water bath in 37 °C for 40 s) and used for evaluation of sperm variables.

2.3. Gross evaluation of semen variables after thawing

2.3.1. Motility and velocity

Sperm class analysis software (Version 5.1; Microptic, Barcelona, Spain) was used to analyze sperm motion characteristics. For this purpose, semen samples were diluted to 20×10^6 sperm/ml with PBS buffer, then 10 μl of semen was placed onto a pre-warmed chamber slide (38 °C, Leja 4; 20 mm height; Leja Products, Luzernestraat B.V., Holland), and sperm motion characteristics were determined. At least six fields that contained a minimum of 400 sperm cells were evaluated for each sample at a 5-second average time to read each sample. The values for the following variables were recorded: total motility (TM, %); progressive motility (PM, %); average path velocity (VAP, m/s); straight-line velocity (VSL, m/s); curvilinear velocity (VCL, m/s); amplitude of lateral head displacement (ALH, m); beat/cross frequency (BCF, Hz) and linearity (LIN, %) (Reed et al., 2009).

2.3.2. Membrane integrity

The hypoosmotic swelling test (HOST) was performed using the Revell and Mrode methodologies to evaluate sperm cells with an intact acrosome membrane (Revell and Mrode, 1994). Semen (20 µl) was added to 200 µl of the hypo osmotic solution (100 mOsm/l, 55.5 mM fructose and 19.2 mM sodium citrate). The sperm cells were evaluated using an inverted light microscope (×400 magnification) after 45 min incubation at room temperature. Spermatozoa (n = 200) were recorded in five different microscopic fields. Afterwards, the number of sperm cells with swollen and non-swollen tails were recorded as having intact and damaged membranes, respectively.

2.3.3. Abnormal morphology

Sperm morphology was evaluated using the Hancock solution (Schaäfer and Holzmann, 2000). Each sample (50 µl) was added to Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 150 ml sodium saline solution composed of 9.01 g NACL in 500 ml double-distilled water and 150 ml PBS buffer). A mixture (10 µl) was placed on a slide and covered with a cover slip. The percentage of abnormal acrosomes (acrosome and cap abnormalities, tail defects, abnormal mid-pieces and detached heads) was recorded by assessing 200 sperm cells using a phase-contrast microscope (×1000 magnification, oil immersion).

2.3.4. LPO

The malondialdehyde (MDA), an index of sperm LPO, was measured using thiobarbituric-acid reaction (Esterbauer and Cheeseman, 1990). Briefly, 1 ml of the diluted semen (250×10^6 sperm/ml) was mixed with 1 ml of cold 20% (w/v) tricholoro acetic acid to precipitate protein. The precipitate was pelleted by centrifuging (960 × 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 using a spectrophotometer (UV-1200, Shima- dzu, Japan) at 532 nm. All MDA concentrations were expressed as nmol/ml.

2.4. Flow cytometry of semen variables after thawing

2.4.1. Mitochondria active potential

Rhodamine-123 (R123; Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI) were used for evaluating mitochondria active potential in sperm (Fattah et al., 2017). The R123 solution (10 µl; 0.01 µg/ml) was added to 300 µl of diluted semen and incubated for 20 min in the dark room. Sperm suspension was centrifuged at 500 × g for 3 min, and again re-suspended in 500 µl Tris buffer. After addition of 10 µl of PI (1 µg/ml) to the sperm suspension, the sample was analyzed using a flow cytometer. For each sample, 10,000 cells were assessed. Sperm with active mitochondria was detected as a positive signal for R123 and as a negative signal for PI.

2.4.2. Phosphatidyl serine externalization as an indicator of apoptotic-like changes

An Annexin V kit and PI were used for evaluation of phosphatidyl serine externalization as an index of apoptotic-like changes in the sperm cells (Masoudi et al., 2019a). Semen samples were washed in calcium buffer and adjusted to the concentration of 1×10^6 sperm/ml followed by the addition of 10 µl Annexin V–FITC (0.01 µg/ml) to 100 µl of the sperm suspension, which was then incubated for 20 min on ice. Afterwards, 10 µl of propidium iodide (PI; 1 µg/ml) was added to the sperm suspension and incubated for at least 10 min on ice. This suspension was subsequently evaluated by flow cytometer. After flow cytometry procedure, sperm subpopulations were classified into four groups: 1) live non-apoptotic cells that were negative for both Annexin-V and PI (A^-/PI^-); 2) early apoptotic cells that were positive for Annexin-V but negative for PI (A^+/PI^-); 3) late apoptotic cells that were positive for both Annexin-V and PI (A^+/PI^+); and 4) necrotic cells that were negative for Annexin-V but positive for PI (A^-/PI^+).

2.4.3. Flow cytometry procedure

Evaluation of mitochondria active potential and apoptotic-like changes was performed using the FACSCalibur (Becton Dickinson, San Khosoz, CA, USA) flow cytometer equipped with standard optics. A minimum of 10,000 sperm cells were examined for each assay at a flow rate of 100 cells/s. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 250 mW. Annexin V fluorescence and R123 fluorescence were detected on detector FL1 with a green filter and PI fluorescence was detected on detector FL2 with a red filter (Fig. 1). FL1 and FL2 were measured using 527/25 and 585/42 nm filters, respectively (Lee et al., 2008). The analysis of flow cytometry data was performed using FlowJo software (Treestar, Inc., San Carlos, CA).

2.5. Fluorescent analysis of acrosome integrity after thawing

Sperm acrosome integrity was assessed using Pisum sativum agglutinin (PSA) according to the method of Thys et al. (2009). The semen sample (5 µl) was added to 100 µl ethanol. After 20 min, 10 µl of the suspended sample were mixed with 30 µl of PSA (50 µg/ml) on a glass slide (Thys et al., 2009). Afterwards, 200 spermatozoa were observed on the slide by fluorescent microscope (BX51, Olympus) possessing fluorescence illumination and a FITC filter (×400 magnification). Sperm cells with green heads were detected as having an intact acrosome while those lacking green heads were recorded as having a disrupted or damaged acrosome.

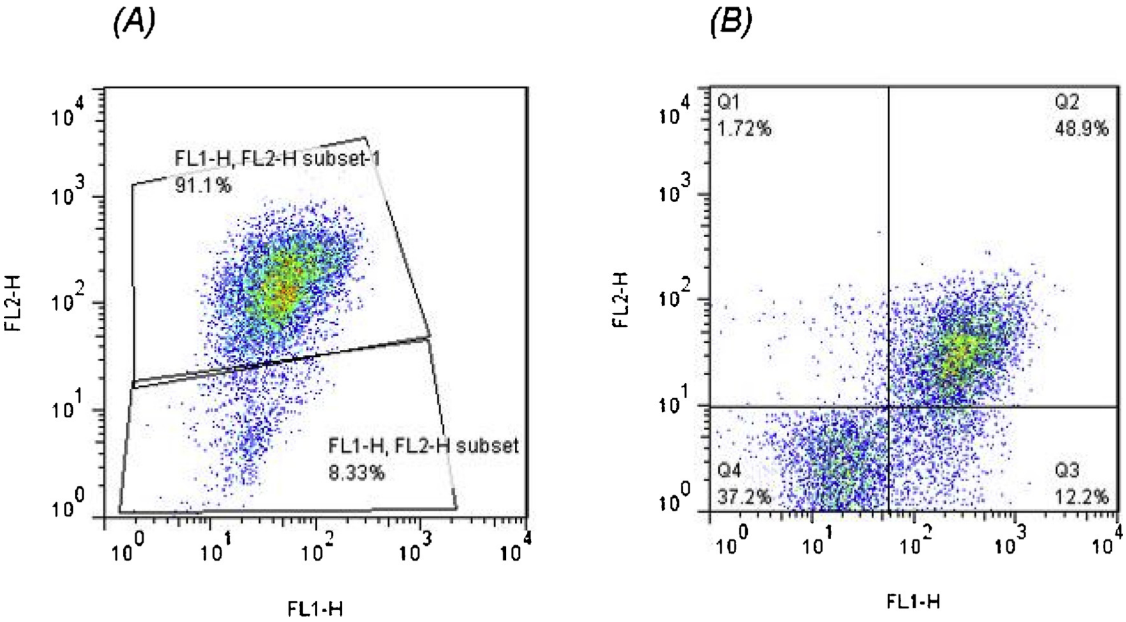

Fig. 1. A) Dot-plots staining with R123 and PI (FL1: R123 fluorescence, FL2: PI fluorescence): sperm cells with active mitochondria were identified as a positive signal for R123 and as a negative signal for PI. B) Dot-plots of staining with Annexin-V and PI (FL1: Annexin-V fluorescence, FL2: PI fluorescence) in thawed sperm; Q4 contains viable non-apoptotic cells negative for both Annexin-V and propidium iodide (PI) (A-/PI-); Q3 contains early apoptotic cells positive for Annexin-V but negative for PI (A+/PI-); Q2 represents late apoptotic cells bound to both Annexin-V and PI (A+/PI+); Q1 represents necrotic cells negative for Annexin-V but positive for PI (A-/PI+).

2.6. Statistical analysis

The levene's and Kolmogorov–Smirnov and Shapiro–Wilk tests were used to examine the data for equality of variances and normality, respectively. Six replicates of semen were used for evaluation of sperm variables. The data were analyzed using Proc GLM of SAS 9.1 (SAS Institute, version 9.1, 2002, Cary, NC, USA). The experimental design of the study was factorial (extender × antioxidant). The interaction effect was not significant; therefore, data were expressed as completely random design. The Tukey's test was used to determine statistical differences among the various groups. The P values ≤ 0.05 were considered to be statistically significant. Results were presented as Mean ± SE.

3. Results

3.1. Motion and velocity

The values for effects of extenders and different concentrations of CoQ10 on the motility and velocity variables of frozen-thawed ram sperm are included in Table 1. In fresh semen group, TM, PM, VAP and VSL was greater ($P \leq 0.05$) than in other groups. Sperm TM was greater ($P \leq 0.05$) in SL/Q1, SL/Q2, EY/Q1 and EY/Q2 (44.1 ± 1.5%, 43.0 ± 1.5%, 40.5 ± 1.5% and 42.7 ± 1.5%, respectively) compared with the other groups. Total sperm motility in the SL/Q0 and EY/Q0 (36.5 ± 1.5% and 36.2 ± 1.5%, respectively) was greater ($P \leq 0.05$) than in SL/Q5, SL/Q10, EY/Q5 and EY/Q10 (31.2 ± 1.5%, 30.8 ± 1.5%, 30.3 ± 1.5% and 29.5 ± 1.5%, respectively).

Sperm PM in the SL/Q1, SL/Q2, EY/Q1 and EY/Q2 (21.0 ± 1.5%, 22.1 ± 1.5%, 20.9 ± 1.5% and 21.8 ± 1.5%, respectively) groups was greater ($P \leq 0.05$) than in other groups. There was no difference among SL/Q0, SL/Q5, SL/Q10, EY/Q0, EY/Q5 and EY/Q10 groups for PM. For VAP, VSL, VCL, ALH, BCF and LIN, there was no difference among values for the different groups.

3.2. Membrane integrity and abnormal morphology

Effects of extenders and different concentrations of CoQ10 on ram sperm membrane integrity and abnormal morphology are depicted in Fig. 2. In the fresh semen group, membrane integrity was greater ($P \leq 0.05$) and abnormal morphology rate was less ($P \leq 0.05$) than in the other groups. The percentage of sperm cells with an intact membrane after freeze-thaw processing was greater ($P \leq 0.05$) in SL/Q1, SL/Q2, EY/Q1 and EY/Q2 (42.2 ± 1.6%, 43.6 ± 1.6%, 41.3 ± 1.6% and 44.0 ± 1.6%, respectively) compared with the other groups. Furthermore, membrane integrity in the SL/Q0 and EY/Q0 groups (37.0 ± 1.6% and 37.4 ± 1.6%, respectively) was greater ($P \leq 0.05$) than in SL/Q5, SL/Q10, EY/Q5 and EY/Q10 (32.5 ± 1.6%, 24.2 ± 1.6%, 31.6 ± 1.6% and 26.8 ± 1.6%, group respectively). Supplementation of different freezing media with various quantities of CoQ10 had no effect on the

Table 1
Effects of different concentration of CoQ10 (μM) on the values for sperm motion variables of frozen-thawed ram semen preserved in the extenders containing SL and EY.

Extenders	fresh			SL					EY		
Q10	–	control	1	2	5	10	control	1	2	5	10
TM (%)	91.1 ± 1.5 [a]	36.5 ± 1.5 [c]	44.1 ± 1.5 [b]	43.0 ± 1.5 [b]	31.2 ± 1.5 [d]	30.8 ± 1.5 [d]	36.2 ± 1.5 [c]	40.5 ± 1.5 [b]	42.7 ± 1.5 [b]	30.3 ± 1.5 [d]	29.5 ± 1.5 [d]
PM (%)	67.5 ± 1.5 [a]	17.4 ± 1.5 [c]	21.0 ± 1.5 [b]	22.1 ± 1.5 [b]	16.5 ± 1.5 [c]	15.3 ± 1.5 [c]	16.8 ± 1.5 [c]	20.9 ± 1.5 [b]	21.8 ± 1.5 [b]	16.2 ± 1.5 [c]	14.3 ± 1.5 [c]
VAP (μm/s)	113.2 ± 4.5 [a]	90.6 ± 4.5 [b]	92.1 ± 4.5 [b]	91.8 ± 4.5 [b]	92.9 ± 4.5 [b]	90.4 ± 4.5 [b]	91.9 ± 4.5 [b]	92.0 ± 4.5 [b]	93.1 ± 4.5 [b]	91.7 ± 4.5 [b]	89.2 ± 4.5 [b]
VSL (μm/s)	81.4 ± 1.7 [a]	76.1 ± 1.7 [b]	75.8 ± 1.7 [b]	77.0 ± 1.7 [b]	75.9 ± 1.7 [b]	76.2 ± 1.7 [b]	75.5 ± 1.7 [b]	76.8 ± 1.7 [b]	76.9 ± 1.7 [b]	76.7 ± 1.7 [b]	75.3 ± 1.7 [b]
VCL (μm/s)	167.1 ± 2.9	164.2 ± 2.9	165.3 ± 2.9	166.1 ± 2.9	163.4 ± 2.9	162.7 ± 2.9	163.7 ± 2.9	166.4 ± 2.9	165.8 ± 2.9	161.5 ± 2.9	162.9 ± 2.9
LIN (%)	48.7 ± 1.8	46.3 ± 1.8	45.8 ± 1.8	46.3 ± 1.8	46.4 ± 1.8	46.8 ± 1.8	46.1 ± 1.8	46.1 ± 1.8	46.3 ± 1.8	47.4 ± 1.8	46.2 ± 1.8
ALH (μm)	6.9 ± 0.6	6.6 ± 0.6	6.7 ± 0.6	6.5 ± 0.6	6.6 ± 0.6	6.4 ± 0.6	6.8 ± 0.6	6.7 ± 0.6	6.8 ± 0.6	6.9 ± 0.6	6.3 ± 0.6
BCF (Hz)	29.6 ± 0.8	29.1 ± 0.8	28.4 ± 0.8	29.4 ± 0.8	28.7 ± 0.8	27.9 ± 0.8	28.8 ± 0.8	29.0 ± 0.8	28.5 ± 0.8	27.8 ± 0.8	28.0 ± 0.8

Means ± SEM of total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), amplitude of lateral head displacement (ALH) and beat/cross frequency (BCF) were assessed after thawing; Different letters indicate differences $P \leq 0.05$ between groups; Soybean lecithin (SL); egg yolk (EY); CoQ10 (Q10).

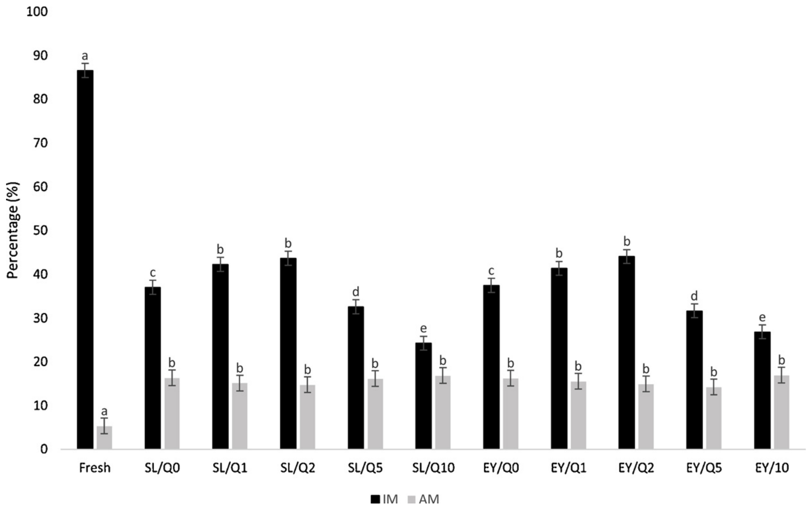

Fig. 2. Percentage of sperm with intact membrane (IM, SEM = 1.6) and abnormal morphology (AM, SEM = 1.8) of ram sperm after freezing-thawing processing in the SL and EY extenders containing different concentrations of CoQ10 (μM); Different letters indicate differences $P \leq 0.05$ among groups; Soybean lecithin (SL); egg yolk (EY); CoQ10 (Q).

morphology of frozen-thawed sperm ($P > 0.05$).

3.3. Mitochondria active potential and acrosome integrity

Fig. 3 depicts the effects of extenders and different concentrations of CoQ10 on mitochondria active potential and acrosome integrity of frozen-thawed semen. In fresh semen group, mitochondria active potential and acrosome integrity were greater ($P \leq 0.05$) than other groups. After freeze-thaw processing, the percentage of mitochondria active potential was greater ($P \leq 0.05$) in SL/Q1, SL/Q2, EY/Q1 and EY/Q2 groups (50.5 ± 2.2%, 52.6 ± 2.2%, 49.3 ± 2.2% and 51.8 ± 2.2%, respectively) compared with the other groups. Furthermore, mitochondria active potential in the SL/Q0 and EY/Q0 groups (43.8 ± 2.2% and 44.4 ± 2.2%, respectively) was greater ($P \leq 0.05$) than in SL/Q5, SL/Q10, EY/Q5 and EY/Q10 groups (37.5 ± 2.2%, 35.6 ± 2.2%, 38.0 ± 2.2% and 36.7 ± 2.2%, respectively).

For acrosome integrity, the SL/Q0, SL/Q1, SL/Q2, EY/Q0, EY/Q1 and EY/Q2 (57.4 ± 2.4%, 57.1 ± 2.4%, 56.9 ± 2.4%, 59.0 ± 2.4%, 58.1 ± 2.4% and 58.6 ± 2.4%, respectively) had greater integrity percentages ($P \leq 0.05$) compared to SL/Q5, SL/Q10, EY/Q5 and EY/Q10 groups (50.9 ± 2.4%, 51.2 ± 2.4%, 52.4 ± 2.4% and 50.3 ± 2.4%, respectively).

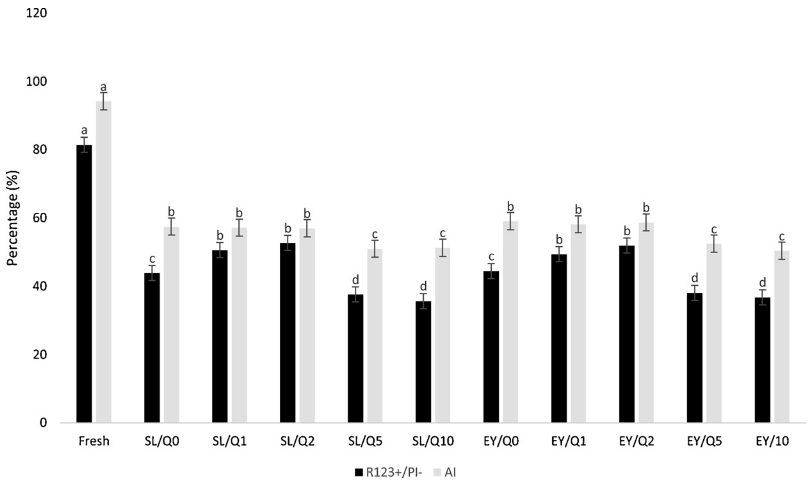

Fig. 3. Percentage of mitochondria with intact membrane (R123+/PI-, SEM = 2.2) and acrosome integrity (AI, SEM = 2.5) of ram sperm after freezing-thawing processing in the SL and EY extenders containing different concentrations of CoQ10 (μM) Different letters indicate differences $P \leq 0.05$ among groups; Soybean lecithin (SL); egg yolk (EY); CoQ10 (Q).

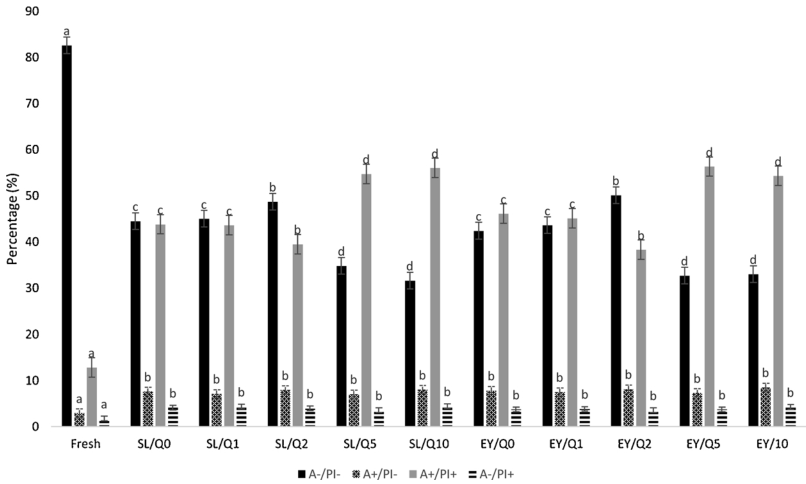

Fig. 4. Viability and apoptotic like changes of ram sperm after freezing-thawing processing using the SL and EY extenders that contained different concentrations of CoQ10; Different letters indicate differences $P \leq 0.05$ among groups; Soybean lecithin (SL); egg yolk (EY); CoQ10 (Q), viable (A-/PI-, SEM= 1.8), early apoptotic (A+/PI-, SEM = 0.9), late apoptotic (A+/PI+, SEM = 2.1), necrotic (A-/PI+, SEM = 0.5).

3.4. Viability and apoptotic-like changes

The data for Annexin V/PI analysis are depicted in Fig. 4. The fresh semen group had the greatest viability percentage and the least apoptotic-like changes. The percentage viable sperm cells was greater ($P \leq 0.05$) in the SL/Q2 and EY/Q2 groups (48.7 ± 1.8% and 50.1 ± 1.8%, respectively) compared to other groups. The percentage of viable sperm cells in SL/Q0, SL/Q1, EY/Q0 and EY Q1 groups (44.5 ± 1.8%, 45.0 ± 1.8%, 42.4 ± 1.8% and 43.6 ± 1.8%, respectively) was greater ($P \leq 0.05$) than that of the SL/Q5, SL/Q10, EY/Q5 and EY/Q10 groups (34.8 ± 1.8%, 31.6 ± 1.8%, 32.7 ± 1.8% and 33.0 ± 1.8%, respectively). When considering early apoptotic and necrotic sperm cells, there was no difference among groups. The percentage of late apoptotic sperm cells was greater ($P \leq 0.05$) in the SL/Q5, SL/Q10, EY/Q5 and EY/Q10 groups (54.7 ± 2.1%, 56.0 ± 2.1%, 56.3 ± 2.1% and 54.3 ± 2.1%, respectively) compared to the other groups. The least percentage of late apoptotic sperm cells was in the SL/Q2 and EY/Q2 groups (39.5 ± 2.1% and 38.3 ± 2.1%, respectively).

3.5. LPO

The MDA concentrations, as an indication of LPO in the frozen-thawed sperm are depicted in Fig. 5. Although the fresh semen

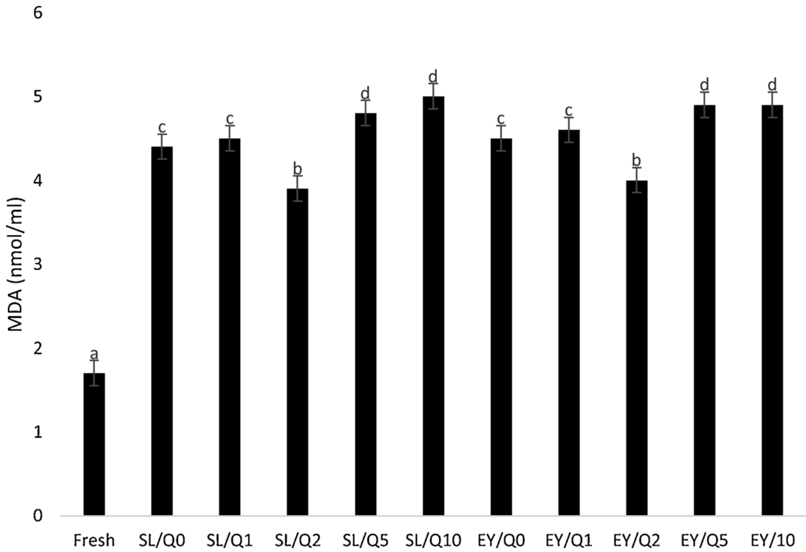

Fig. 5. Effect of different concentrations of CoQ10 (μM) in SL and EY extenders on MDA production of ram sperm after freezing-thawing processing; Different letters indicate differences $P \leq 0.05$ among groups; Soybean lecithin (SL); egg yolk (EY); malondialdehyde (MDA); CoQ10 (Q). SEM = 0.15.

group had the least ($P \leq 0.05$) LPO percentage, MDA concentrations in SL/Q2 and EY/Q2 groups (3.9 ± 0.15 nmol/ml and 4.0 ± 0.15 nmol/ml, respectively) were less ($P \leq 0.05$) than in the other groups. Furthermore, the SL/Q0, SL/Q1, EY/Q0 and EY/Q1 groups (4.4 ± 0.15 nmol/ml, 4.5 ± 0.15 nmol/ml, 4.5 ± 0.15 nmol/ml and 4.6 ± 0.15 nmol/ml, respectively) produced less ($P \leq 0.05$) MDA compared to the SL/Q5, SL/Q10, EY/Q5 and EY/Q10 groups (4.8 ± 0.15 nmol/ml, 5.0 ± 0.15 nmol/ml, 4.9 ± 0.15 nmol/ml and 4.9 ± 0.15 nmol/ml, respectively).

4. Discussion

Addition of antioxidants to the extenders is a common beneficial strategy to protect mammalian sperm during cryopreservation (Bucak et al., 2008, 2009; Motemani et al., 2017). Polyunsaturated fatty acids (PUFAs), which are present in the sperm plasma membrane, make membranes sensitive to LPO, resulting in a decreased sperm viability. Using antioxidants in freezing medium, therefore, could prevent LPO and improve the viability of sperm after cryopreservation and thawing (Naijian et al., 2013). Some characteristics of CoQ10 make it an effective supplement to be examined as an additive for ram sperm freezing media. The hypothesis in the present study was that CoQ10 addition, as a supplement in freezing extenders, could be effective in reducing oxidative damage during cryopreservation.

Using extenders based on SL has increased due to several problems with use of animal-origin extenders (Forouzanfar et al., 2010). Two types of freezing extenders based on SL and EY were used, therefore, in the present study.

In the present study, fresh semen was compared to frozen semen treated with different quantities of CoQ10 and the quality of frozen semen in all experimental groups were less compared to sperm in fresh semen. Thermal deleterious changes, osmotic shock and formation of intracellular and extracellular ice crystals may occur during cryopreservation (Sharafi et al., 2015b), all of which can induce ROS production (Emamverdi et al., 2015). The ROS production can consequently lead to apoptosis and reduce fertility potential of spermatozoa (Dodaran et al., 2015) that would subsequently lead to sperm with a lesser quality after imposing freezing-thawing processes as compared to sperm in fresh semen.

In the experimental groups of the present study, the effects of different concentrations of CoQ10 in extenders containing SL and EY were evaluated. Several sperm quality variables for ram sperm were assessed after cryopreservation and thawing and the use of SL and EY extenders with optimized doses (2 μM) of CoQ10 resulted in ram sperm with a greater viability as indicated by values for sperm quality variables after imposing the freezing-thawing processes.

Total sperm motility, PM, membrane integrity, acrosome integrity and mitochondria active potential were greater when semen samples were cryopreserved with quantities of 1 and 2 μM of CoQ10 in SL and EY extenders. The results of the present study are consistent with those of other studies where beneficial effects of CoQ10 on sperm viability have been reported (Yousefian et al., 2014; Masoudi et al., 2018; Yousefian et al., 2018).

There is biosynthesis of CoQ10 in the testis (Kalen et al., 1990), while a large amount of this component is present in the seminal plasma (Mancini et al., 1998), which indicates it has beneficial effects on sperm. Results of different studies indicate there is a reduction of CoQ10 concentrations and its reduced form in the seminal plasma and spermatozoa of infertile men with idiopathic and varicocele associated asthenospermia (Balercia et al., 2002). Exogenous administration of CoQ10 improved sperm motility in men (Balercia et al., 2009) and there is a positive correlation between concentration of coenzyme Q10 and TM of sperm cells (Mancini et al., 1994). Furthermore, CoQ10 administration resulted in improvement values for semen variables in men with sperm pathologies (Balercia et al., 2009; Safarinejad, 2009) and in bulls (Gualtieri et al., 2014), stallions (Yousefian et al., 2014), billy goats (Yousefian et al., 2018) and roosters (Masoudi et al., 2018, 2019b).

Reduction of susceptibility of sperm to peroxidation preserves membrane integrity. The supplementations with CoQ10 can lead to protection of the membrane sperm membrane integrity by inhibiting LPO (Littarru and Tiano, 2007). The CoQ10 compound can also inhibit the formation of hydroperoxides and thus protect the plasma membrane against oxidation (Turunen et al., 2004).

In the mitochondria, CoQ10 has an energy transfer function (Littarru and Tiano, 2007) as a result of synthesis of adenosine triphosphate (ATP) and energy transfer are related to the availability of CoQ10 (Lewin and Lavon, 1997). The functions of CoQ10 can lead of modulation of mitochondrial permeability of transition pores (Turunen et al., 2004), and can deactivate mitochondria membrane potential depolarization, decrease concentration of ATP, and decrease caspase-9 activity as a consequence of this action (Turunen et al., 2004). In the present study, therefore, the greater percentage of mitochondria activity combined with the lesser percentage of apoptotic-like changes are probably due to these functions of CoQ10.

Phosphatidyl serine (PS) translocation, as an indicator of apoptotic-like changes was detected using Annexin-V/PI assessment in the present study. Data indicate that externalizations of phosphatidyl serine increased during cryopreservation. The CoQ10 functioned to decrease the apoptotic-like changes because of the beneficial characteristics of CoQ10 in stabilization of the plasma membrane (Masoudi et al., 2018). Also, the presence of 2 μM CoQ10 in SL and EY extenders resulted in greater percentage of viable sperm and lesser percentage of dead sperm, which are consistent with the findings of Gualtieri et al. (2014), where there was a lesser percentage of apoptosis in blastocysts of cattle when there was treatment with CoQ10 (Gualtieri et al., 2014).

The ROS are the main products of oxidative stress during cryopreservation that can have actions at the plasma membrane leading to peroxidation. The MDA is the index that is generally used as an indicator of LPO that was assessed in the present study because it was hypothesized that there would be a reduction in LPO as a result of CoQ10 supplementation. The MDA concentrations in the present study were less in the group where there was supplementation with 2 μM CoQ10 which was evidence for accepting the hypothesis for the present and also in other studies (Lewin and Lavon, 1997; Turunen et al., 2004; Yousefian et al., 2014, 2018) where the potential effects of CoQ10 on LPO were assessed.

The percentage of abnormal sperm morphology was not affected by the different concentrations of CoQ10 in the experimental groups of the present study; however, this percentage was greater in the cryopreservation-thawing treatment groups as compared with the fresh semen group. This observation may be attributed to the fact that primary abnormalities of sperm develop during spermatogenesis and, therefore, would not be affected by these supplementations during freezing-thawing processes (Chenoweth, 2005). Results in the present study are consistent with those of Sharafi et al. (2015b) where it was reported that extenders do not have any effects on bull sperm morphology (Sharafi et al., 2015b). The results, however, are not consistent with those of Yousefian et al. (2018) where there was a greater percentage of normal morphology of post thawed goat sperm.

Furthermore, there was a lesser TM, membrane integrity, mitochondria active potential and acrosome integrity, while LPO and percentage dead sperm were greater in groups where there was supplementation with 5 and 10 μM CoQ10, indicating that relatively greater concentrations of antioxidants at concentrations greater than a specific threshold concentration can be toxic and lead to stimulation of oxidative actions (Nohl et al., 1998; Breininger et al., 2005). It was postulated that greater concentrations of CoQ10 may convert to its toxic redox form, semiquinone, which can have markedly detrimental effects on spermatozoa (Ernster and Dallner, 1995).

In the present study, there was use of two types of extenders for cryopreservation of ram semen. In previous studies (Sharafi et al., 2009; Forouzanfar et al., 2010), the use of SL had several beneficial effects in preventing cryo-damage because constituents of this extender can adhere to the sperm plasma membrane and facilitate preservation of sperm cells by inhibiting formation of ROS (Sharafi et al., 2009). The use of EY-based extenders can lead to several problems such as agglutination, standardization and contamination of sperm, which in turn leads to a desire for replacement of EY in extenders (Forouzanfar et al., 2010). In various studies, the greater protective effects of SL compared with EY have been reported (Emamverdi et al., 2013), while in other studies, the outcomes with use of SL and EY were similar (Sharafi et al., 2009). For this reason, in the present study there was use of different concentrations of CoQ10 in both SL and EY extenders to evaluate effects on ram semen cryopreservation. Values for all sperm variables assessed indicate the semen quality preservation effects were similar with use of the two extenders (SL compared with EY). It, therefore, is recommended that SL be used in cryopreservation ram semen due to some of the problems that occur with use of EY.

5. Conclusion

There were greater percentages for TM, PM, membrane integrity and mitochondria activity with supplementation of SL and EY freezing extenders with 1 and 2 μM CoQ10. There were greater percentage for sperm viability and lesser LPO after imposing freezing-thawing processes when there was supplementation of extenders with 2 μM CoQ10. The type of extenders (SL and EY) had no effect on the frozen-thawed sperm quality. Due to the problems that often occur with use of EY, SL is suggested as a more desirable freezing cryo-protectant. Further investigations could be helpful in elucidating the protective mechanisms of CoQ10 during ram sperm cryopreservation and thawing.

References

Agarwal, A., Virk, G., Ong, C., du Plessis, S.S., 2014. Effect of oxidative stress on male reproduction. World J. Men's Health 32, 1–17.

Aires, V., 2003. In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. Theriogenology 60, 269–279.

Aitken, R.J., Smith, T.B., Jobling, M.S., Baker, M.A., De Iuliis, G.N., 2014. Oxidative stress and male reproductive health. Asian J. Androl. 16, 31–38.

Akhter, S., Ansari, M.S., Andrabi, S.M.H., Rakha, B.A., Ullah, N., Khalid, M., 2012. Soya-lecithin in extender improves the freezability and fertility of buffalo (bubalus bubalis) bull spermatozoa. Reprod. Domest. Anim. 47, 815–819.

Aurich, J.E., Schönherr, U., Hoppe, H., Aurich, C., 1997. Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen. Theriogenology 48, 185–192.

Balercia, G., Arnaldi, G., Fazioli, F., Serresi, M., Alleva, R., Mancini, A., Mosca, F., Lamonica, G.R., Mantero, F., Littarru, G.P., 2002. Coenzyme Q10 levels in idiopathic and varicocele-associated asthenozoospermia. Andrologia 34, 107–111.

Balercia, G., Buldreghini, E., Vignini, A., Tiano, L., Paggi, F., Amoroso, S., Ricciardo-Lamonica, G., Boscaro, M., Lenzi, A., Littarru, G., 2009. Coenzyme Q10 treatment in infertile men with idiopathic asthenozoospermia: a placebo-controlled, double-blind randomized trial. Fertil. Steril. 91, 1785–1792.

Başpinar, N., Çoyan, K., Bucak, M.N., Tuncer, P.B., 2011. Effects of dithioerythritol on ram semen after the freeze-thawing process. Cryobiology 63, 152–156.

Breininger, E., Beorlegui, N.B., O'Flaherty, C.M., Beconi, M.T., 2005. Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. Theriogenology 63, 2126–2135.

Bucak, M.N., Ateşşahin, A., Yüce, A., 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. Small Rumin. Res. 75, 128–134.

Bucak, M.N., Sariözkan, S., Tuncer, P.B., Ulutaş, P.A., Akçadağ, H.I., 2009. Effect of antioxidants on microscopic semen parameters, lipid peroxidation and antioxidant activities in Angora goat semen following cryopreservation. Small Rumin. Res. 81, 90–95.

Chenoweth, P.J., 2005. Genetic sperm defects. Theriogenology 64, 457–468.

Dodaran, H.V., Zhandi, M., Sharafi, M., Nejati-Amiri, E., Nejati-Javaremi, A., Mohammadi-Sangcheshmeh, A., Shehab-El-Deen, M.A.M.M., Shakeri, M., 2015. Effect of ethanol induced mild stress on post-thawed bull sperm quality. Cryobiology 71, 12–17.

El-Sheshtawy, R.I., Sisy, G.A., El-Nattat, W.S., 2015. Effects of different concentrations of sucrose or trehalose on the post-thawing quality of cattle bull semen. Asian Pac. J. Reprod. 4, 26–31.

Emamverdi, M., Zhandi, M., Zare Shahneh, A., Sharafi, M., Akbari-Sharif, A., 2013. Optimization of ram semen cryopreservation using a chemically defined soybean lecithin-based extender. Reprod. Domest. Anim. 48, 899–904.

Emamverdi, M., Zhandi, M., Zare Shahneh, A., Sharafi, M., Akhlaghi, A., Khodaei Motlagh, M., Dadkhah, F., Dadashpour Davachi, N., 2015. Flow cytometric and microscopic evaluation of post-thawed ram semen cryopreserved in chemically defined home-made or commercial extenders. Anim. Prod. Sci. 55, 551–558.

Ernster, L., Dallner, G., 1995. Biochemical, physiological and medical aspects of ubiquinone function. BBA - Mol. Basis Dis. 1271, 195–204.

Ernster, L., Forsmark-Andrée, P., 1993. Ubiquinol: an endogenous antioxidant in aerobic organisms. Clin. Investig. (Lond.) 71, 60–65.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. 186, 407–421.

Evans, G., 1991. Salamon's artificial insemination of sheep and goats. Trop. Anim. Health Prod. 23, 114.

Fattah, A., Sharafi, M., Masoudi, R., Shahverdi, A., Esmaeili, V., 2017. L-carnitine is a survival factor for chilled storage of rooster semen for a long time. Cryobiology 74, 13–18.

Forouzanfar, M., Sharafi, M., Hosseini, S.M., Ostadhosseini, S., Hajian, M., Hosseini, L., Abedi, P., Nili, N., Rahmani, H.R., Nasr-Esfahani, M.H., 2010. In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. Theriogenology 73, 480–487.
Grossfeld, R., Sieg, B., Struckmann, C., Frenzel, A., Maxwell, W.M.C., Rath, D., 2008. New aspects of boar semen freezing strategies. Theriogenology 70, 1225–1233.
Gualtieri, R., Barbato, V., Fiorentino, I., Braun, S., Rizos, D., Longobardi, S., Talevi, R., 2014. Treatment with zinc, d-aspartate, and coenzyme Q10 protects bull sperm against damage and improves their ability to support embryo development. Theriogenology 82, 592–598.
Kalen, A., Appelkvist, E.L., Chojnacki, T., Dallner, G., 1990. Nonaprenyl-4-hydroxybenzoate transferase, an enzyme involved in ubiquinone biosynthesis, in the endoplasmic reticulum-Golgi system of rat liver. J. Biol. Chem. 265, 1158–1164.
Lee, J.A., Spidlen, J., Boyce, K., Cai, J., Crosbie, N., Dalphin, M., et al., 2008. MIFlowCyt: the minimum information about a flow cytometry experiment. Cytometry Part A 73, 926–930.
Lewin, A., Lavon, H., 1997. The effect of coenzyme Q10 on sperm motility and function. Mol. Aspects Med. 18, 213–219.
Littarru, G.P., Tiano, L., 2007. Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Molecul. Biotech. 37, 31–37.
Mancini, A., Conte, G., Milardi, D., De Marinis, L., Littarru, G.P., 1998. Relationship between sperm cell ubiquinone and seminal parameters in subjects with and without varicocele. Andrologia 30, 1–4.
Mancini, A., De Marinis, L., Oradei, A., Hallgass, M.E., Conte, G., Pozza, D., Littarru, G.P., 1994. Coenzyme Q10 concentrations in normal and pathological human seminal fluid. J. Androl. 15, 591–594.
Masoudi, R., Sharafi, M., Shahneh, A.Z., Khodaei-Motlagh, M., 2019a. Effects of reduced glutathione on the quality of rooster sperm during cryopreservation. Theriogenology 128, 149–155.
Masoudi, R., Sharafi, M., Pourazadi, L., 2019b. Improvement of rooster semen quality using coenzyme Q10 during cooling storage in the Lake extender. Cryobiology 88, 87–91.
Masoudi, R., Sharafi, M., Zare Shahneh, A., Kohram, H., Nejati-Amiri, E., Karimi, H., Khodaei-Motlagh, M., Shahverdi, A., 2018. Supplementation of extender with coenzyme Q10 improves the function and fertility potential of rooster spermatozoa after cryopreservation. Anim. Reprod. Sci. 198, 193–201.
Moghbeli, M., Kohram, H., Zare-Shahaneh, A., Zhandi, M., Sharafi, M., Nabi, M.M., Zahedi, V., Sharideh, H., 2016. Are the optimum levels of the catalase and vitamin E in rooster semen extender after freezing-thawing influenced by sperm concentration? Cryobiology 72, 264–268.
Motemani, M., Chamani, M., Sharafi, M., Masoudi, R., 2017. Alpha-tocopherol improves frozen-thawed sperm quality by reducing hydrogen peroxide during cryo-preservation of bull semen. Span. J. Agric. Res. 15, 1–7.
Naijian, H.R., Kohram, H., Shahneh, A.Z., Sharafi, M., 2013. Effects of various concentrations of BSA on microscopic and oxidative parameters of Mahabadi goat semen following the freeze-thaw process. Small Rumin. Res. 113, 371–375.
Nohl, H., Gille, L., Kozlov, A.V., 1998. Antioxidant-derived prooxidant formation from ubiquinol. Free Radic. Biol. Med. 25, 666–675.
Pindaru, L.P., Cenariu, M., Pall, E., Groza, I.Ș., 2015. Effects of coenzyme Q10 on sperm viability during storage of boar semen at 17°C. Scientific Works. Series C. Vet. Med. 61, 32–36.
Reed, M.L., Ezeh, P.C., Hamic, A., Thompson, D.J., Caperton, C.L., 2009. Soy lecithin replaces egg yolk for cryopreservation of human sperm without adversely affecting postthaw motility, morphology, sperm DNA integrity, or sperm binding to hyaluronate. Fertil. Steril. 92, 1787–1790.
Revell, S.G., Mrode, R.A., 1994. An osmotic resistance test for bovine semen. Anim. Reprod. Sci. 36, 77–86.
Saeed, A.M., El-Nagar, H.A., Wafa, W.M., Hussein, Y.S., 2016. Effect of coenzyme Q10 as an antioxidant added to semen extender during cryopreservation of buffalo and cattle semen. Mansoura J. Anim. Poult. Prod. 7, 403–408.
Safarinejad, M.R., 2009. Efficacy of coenzyme Q10 on semen parameters, sperm function and reproductive hormones in infertile men. J. Urol. 182, 237–248.
Salamon, S., Maxwell, W.M.C., 1995. Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. Anim. Reprod. Sci. 37, 185–249.
Salmani, H., Nabi, M.M., Vaseghi-Dodaran, H., Rahman, M.B., Mohammadi-Sangcheshmeh, A., Shakeri, M., Towhidi, A., Shahneh, A.Z., Zhandi, M., 2013. Effect of glutathione in soybean lecithin-based semen extender on goat semen quality after freeze-thawing. Small Rumin. Res. 112, 123–127.
Salmani, H., Towhidi, A., Zhandi, M., Bahreini, M., Sharafi, M., 2014. In vitro assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. Cryobiology 68, 276–280.
Schaäfer, S., Holzmann, A., 2000. The use of transmigration and SpermacTMstain to evaluate epididymal cat spermatozoa. Anim. Reprod. Sci. 59, 201–211.
Sharafi, M., Forouzanfar, M., Hosseini, S.M., Hajian, M., Ostadhosseini, S., Hosseini, L., Abedi, P., Nili, N., Rahmani, H.R., Javaheri, A.R., Esfahani, M.H.N., 2009. In vitro comparison of soybean lecithin based-extender with commercially available extender for ram semen cryopreservation. Int. J. Fertil. Steril. 3, 149–152.
Sharafi, M., Zhandi, M., Akbari Sharif, A., 2015a. Supplementation of soybean lecithin-based semen extender by antioxidants: complementary flowcytometric study on post-thawed ram spermatozoa. Cell Tissue Bank 16, 261–269.
Sharafi, M., Zhandi, M., Shahverdi, A., Shakeri, M., 2015b. Beneficial effects of nitric oxide induced mild oxidative stress on post-thawed bull semen quality. Int. J. Fertil. Steril. 9, 230–237.
Silva, S.V., Soares, A.T., Batista, A.M., Almeida, F.C., Nunes, J.F., Peixoto, C.A., Guerra, M.M.P., 2011. In vitro and in vivo evaluation of ram sperm frozen in Tris egg-yolk and supplemented with superoxide dismutase and reduced glutathione. Reprod. Domest. Anim. 46, 874–881.
Talevi, R., Barbato, V., Fiorentino, I., Braun, S., Longobardi, S., Gualtieri, R., 2013. Protective effects of in vitro treatment with zinc, d-aspartate and coenzyme Q10 on human sperm motility, lipid peroxidation and DNA fragmentation. Reprod. Biol. Endocrinol. 11, 1–10.
Thys, M., Nauwynck, H., Maes, D., Hoogewijs, M., Vercauteren, D., Rijsselaere, T., Favoreel, H., Van Soom, A., 2009. Expression and putative function of fibronectin and its receptor (integrin α 5β 1) in male and female gametes during bovine fertilization in vitro. Reproduction 138, 471–482.
Turunen, M., Olsson, J., Dallner, G., 2004. Metabolism and function of coenzyme Q. Biochim. Biophys. Acta Biomembr. 1660, 171–199.
Yousefian, I., Emamverdi, M., Karamzadeh-Dehaghani, A., Sabzian-Melei, R., Zhandi, M., Zare-Shahneh, A., 2018. Attenuation of cryopreservation-induced oxidative stress by antioxidant: impact of Coenzyme Q10on the quality of post-thawed buck spermatozoa. Cryobiology 81, 88–93.
Yousefian, I., Zare-Shahneh, A., Zhandi, M., 2014. The effect of coenzyme Q10 and α-tocopherol in skim milk-based extender for preservation of caspian stallion semen in cool condition. J. Equine Vet. Sci. 34, 949–954.