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The Plant-Based Extender Supplemented with MitoQ as a Mitochondrial-Targeted Antioxidant Enhances Buck Sperm Quality during Liquid Storage

Research Article

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ABSTRACT

One of the problems with the refrigerated storage of buck semen is the short shelf life. Reactive oxygen species (ROS) generated by sperm cells are a major obstacle to successful cooled semen storage. In this regard, the mitochondria are the most sensitive organelles to cooling damage in sperm. Consequently, this study was aimed to determine the impacts of Mito-quinol (MitoQ), a mitochondrial-targeted antioxidant, in a soy lecithin containing extender on the quality of goat semen during cooling and storage at 4 °C. Semen samples from Markhoz goats were collected and pooled. Then, samples were diluted in the extender and divided into five equal aliquots and supplemented with 0 (control), 1, 10, 100, and 1000 nM of MitoQ and stored at 4 °C for 60 h. Finally, total motility (TM) and progressive motility (PM), viability, membrane functionality, mitochondrial activity potential (MAP), and MDA concentration (lipid peroxidation) were measured at 0, 30, and 60 h of cooling period. According to the current results, higher ($P \leq 0.05$) rates of TM, PM, viability, membrane functionality, and MAP, as well as lower ($P \leq 0.05$) MDA concentration were observed in MQ10 and MQ100 groups than groups of MQ0, MQ1, and MQ1000 at 30 and 60 h cooling storage. However, MitoQ had no effect ($P > 0.05$) on the mentioned parameters at 0 h storage. Consequently, MitoQ can reduce oxidative stress by regulating mitochondrial function during cooling of the buck sperm and could be an effective additive in the cooling medium to protect sperm quality.

KEY WORDS buck, cooling, mitochondrial activity, MitoQ, sperm.

INTRODUCTION

It is common in buck to use cooled semen in the non-breeding season to inseminate females with its first 24 hours after being stored (Leboeuf *et al*. 2008). As opposed to frozen semen samples, cooled semen samples are easier to handle and transport, as well as having greater fertility rates (Borges-Silva *et al*. 2016). In addition, cooled doses require fewer spermatozoa per dose, resulting in a higher number of artificial insemination (AI) doses per ejaculate (Murphy *et al*. 2013). Also, cooled AI doses are less expensive than frozen AI doses in buck (Borges-Silva *et al*. 2016). However, due to present of a high unsaturated/saturated fatty acid ratio in buck sperm membranes, sperm cryo-resistance and viability are impaired in ROS-containing medium because its sensitive to lipid peroxidation (LPO). Moreover, oxidative stress reduces MAP in mitochondria as the major organelle of endogenous ROS production (Rezaei *et al*. 2023). Consequently, using mitochondria-targeted antioxidants, including Mito-quinol (MitoQ), in the cooling extender can be useful.

MitoQ, a mitochondrial-targeted antioxidant, contains triphenyl-lphosphonium (TPP^+) and coenzyme Q10, enabling it to accumulate inside mitochondria. MitoQ has an

excellent antioxidant capacity and improves mitochondrial dysfunction due to its mitochondria targeting (Zhang *et al*. 2019). By donating a hydrogen atom from one of its hydroxyl groups to peroxyl radicals in lipids, it acts as a chain-breaking antioxidant (Barfourooshi *et al*. 2023). Adding MitoQ to semen extenders preserved post-thawed semen quality in various species, including humans (Kumar *et al*. 2021) and rooters (Alipour-Jenaghard *et al*. 2023a).

On the other hand, a replacement for high molecular weight lipoproteins and phospholipids, soy lecithin (SL) reduces hygienic risks in diluents. During cooling, it prevents damage to sperm plasma membranes (Sun *et al*. 2020). Some promising results have been reported on the post-thaw quality of goat semen using SL as the primary source of lipoproteins (Alcay *et al*. 2019; Nadri *et al*. 2019; Sun *et al*. 2020).

According to our knowledge, although the protective effect of MitoQ has been published in recent studies, no study has reported the impact of MitoQ in a SL containing extender on buck cooled sperm quality. Consequently, the aim of this research was to detect the impacts of various levels of MitoQ, as a mitochondrial-targeted antioxidant, in a SL containing extender on rates of TM and PM, viability, membrane functionality, MAP, and LPO in buck's chilled sperm.

MATERIALS AND METHODS

The chemical materials were purchased from the companies Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA). Animal Science Research Institute of Iran Research Ethics Committee approved the current study.

Semen samples collection, primary evaluation and cooling process

Five mature Markhoz bucks between 3-4 years old were used in this study. Semen samples (n=30) were collected using artificial vagina twice a week in six replicates. For quality evaluation, collected samples were transported to the laboratory in a water bath (37 °C) during 5 min after ejaculation. Then, samples with semen volume 1-2 mL, sperm concentration >300 × 10^6 spermatozoa/mL, ≥75% motility, ≥85% normal morphology were selected, and pooled them to eliminate individual differences at 37 °C (Heidari *et al*. 2022).

Extender supplementation with antioxidant

The cooling extender was composed of [citric acid (1.64 g/100 mL), fructose (1.26 g/100 mL), Tris (3.07 g/100 mL), soybean lecithin (1.5% w/v)]. The pH was 6.8 and the osmolality without glycerol was 425 mOsm/kg water. The samples assigned into five aliquots as follows: MQ0 (no additives, control), MQ1 (1 nM MitoQ), MQ10 (10 nM MitoQ), MQ100 (100 nM MitoQ), and MQ1000 (1000 nM MitoQ). Semen samples were diluted in the extender and the concentration of 4 × 10^8 spermatozoa/ml was gained. Next, French straws (0.25 mL, IMV, L'Aigle, France) were filled with diluted semen to achieve 1 × 10^8 sperm cells/straws. Then, straws were sealed with polyvinyl alcohol powder, and balanced at 4 °C up to 60 h. Rates of TM, PM, viability, membrane functionality, MAP, and MDA level were assessed during times 0 (start time), 30 and 60 h of cooled-storage.

Sperm quality traits

Sperm class analysis software (Version 5.1; Microptic, Barcelona, Spain) evaluated sperm motility. For this aim, after diluting sperm samples with PBS buffer (1:7), they were incubated for 5 min at 35 °C. In the next step, 5 µL of diluted sample was placed on a pre-warmed chamber slide (38 °C, Leja 4, 20 µm height, Leja Products, Luzernestraat B.V., Holland) and covered immediately with a 24 × 24-mm coverslip. Using a phase contrast microscope (Nikon, Eclipse E200, Japan) at 37 °C and magnification ×400, each sample was examined under at least six fields that contained a minimum of 400 spermatozoa (Khodaei-Motlagh *et al*. 2022). Percentages of TM and PM were recorded.

The viability of spermatozoa cells was evaluated using eosine-nigrosin staining (1.67 g eosin-Y, 10 g nigrosin, and 2.9 g sodium citrate, 100 mL distilled water). 10 mL sperm sample with 20 mL of eosine-nigrosine stain on a warm slide was blended. Next, the blend was spread on a second slide and under a phase contrast microscope (magnification ×400), 300 spermatozoa cells were counted to determine unstained heads (viable) or stained/partial stained heads (dead) (Hatami *et al*. 2023).

Hypo osmotic swelling test (HOST) was used to detect spermatozoa with functional membranes (Revell and Mrode, 1994). Approximately 10 µL of semen samples were diluted in 100 µL of hypoosmotic solution (57.6 mM fructose and 19.2 mM sodium citrate 100 mOsm) and incubated at 37 °C for 20-30 min. Afterward, under a phase-contrast microscope (magnification 400×), 300 spermatozoa were analyzed, and samples with non-swollen and swollen tails were identified as cells with non-functional and functional membranes, respectively.

To evaluate MAP, 300 mL of diluted semen with 10 mL of Rhodamin123 solution (Rh123, 0.01 mg/mL; Invitrogen TM, Eugene, OR, USA) were incubated in the dark for 20 min. Following centrifugation at 500 g for 3 min, sperm suspension was resuspended in 500 mL Tris-buffer. Then, sperm suspension was mixed with 10 mL of propidium iodide (PI, 1.0 mg/mL). In the end, 10000 events via FAC-

SCalibur flow cytometer (Becton Dickinson, USA) were recorded. Positive R123 and negative PI samples were recorded as active mitochondria. An argon-ion 488 nm laser excited fluorescent probes (R123 and PI). Probes of R123 and PI were measured in the channels of FL1 and FL2, respectively. Finally, FlowJo software (Treestar, Inc., USA) analyzed the received data (Asadzadeh *et al*. 2021).

Analysis of malondialdehyde (MDA) concentration was performed using thiobarbituric acid as an indicator of LPO (Esterbauer and Cheeseman, 1990). In brief, 1 mL of the diluted semen sample (200 $\times 10^6$ sperm/mL) with 1 mL of cold 20% (w/v) trichloroacetic acid was mixed to precipitate protein. Then, the solution was mixed with 0.5 mL of butylated hydroxytoluene solution (2% ethanol) and 0.5 mL of EDTA. The precipitate was centrifuged (1200 g for 15 min) and incubated 1 mL of the supernatant with 1 mL of 0.67% thiobarbituric acid (w/v) for 20 min at 100 °C. Measuring the absorbance at 532 nm with a spectrophotometer (UV-1200, Shimadzu, Japan), the absorbance was measured after cooling.

Data analysis

In this study was used six replicates. To examine the data for equality of variances and normality were used the levene's and Kolmogorov–Smirnov tests, respectively. Proc GLM of SAS 9.1 (SAS, 2003) was applicated to data analyzing. Tukey's test was used to determine statistical differences among the various groups. Results were presented as Mean±SE.

RESULTS AND DISCUSSION

The impact of MitoQ on percentages of TM and PM in buck cooled semen during storage time are shown in Table 1. There was no difference (P>0.05) between the experimental groups at time 0 h of liquid storage. The higher (P≤0.05) rates of TM and PM were demonstrated in MQ10 and MQ100 compared to control and the other groups at times 30 and 60 h of storage. Other groups (MQ0, MQ1, and MQ1000) demonstrated no significant difference (P>0.05) for TM and PM.

Table 2 presents the data related to effect of MitoQ on rates of viability and membrane functionality in buck cooled semen during storage times. At the starting time of storage (0 h) the impact of various levels of MitoQ on the rates of viability and membrane functionality was not significant (P>0.05). MQ10 and MQ100 groups had the greater (P≤0.05) rates of viability and membrane functionality than MQ0, MQ1 and MQ1000 at 30 and 60 h of storage. However, the among of groups MQ0, MQ1 and MQ1000 were not observed significant difference for mentioned parameters (P>0.05).

The statistical analysis related to the effect of MitoQ various concentrations on MAP of buck semen stored at 4 °C is reported in Figure 1. Adding MitoQ to the cooling extender had no significant (P>0.05) effect on the MAP rate at the start time of storage (0 h). the percentage of MAP in groups of MQ10 and MQ100 were raised (P≤0.05) compared to control and the other groups at times 30 and 60 h of the short-term storage and no significant difference (P>0.05) was observed among other groups.

Figure 2 indicated the impact of adding MitoQ to cooling extender on MDA concentration in buck semen at times of liquid storage. In case of LPO, there was no remarkable difference (P>0.05) at the begin time (0 h). MQ10 and MQ100 illustrated lower (P≤0.05) MDA levels compared to control and the other groups in times 30 and 60 h after storage in 4 °C and there was no difference (P>0.05) among MQ0, MQ1, and MQ1000.

Preserving sperm successfully requires the appropriate extender. Physical and chemical disruptions occur in sperm preserved either in cooled or frozen conditions (Hatami *et al*. 2023), which decreases its fertility potential in addition to deteriorating its quality parameters. In recent years, a wide range of cryoprotectants, extenders, and antioxidants have been used to improve semen cooling outcomes (Fadl *et al*. 2022). The cooling and freezing adversely affect sperm quality first by disrupting plasma membranes, and then by impairing mitochondria (Peris-Frau *et al*. 2020). Mitochondrial dysfunction caused by cooling is likely responsible for the loss of sperm health and function (Gualtieri *et al*. 2021). In this regard, mitochondria-targeted antioxidants, including MitoQ, has been very helpful in semen extenders of various species such as goats (Rezaei *et al*. 2023), roosters (Alipour-Jenaghard *et al*. 2023b), and rams (Barfourooshi *et al*. 2023).

In addition, other researches have presented that SL could be an appropriate replacement to egg yolk in buck semen cooling and cryopreservation (Salmani *et al*. 2014; Chelucci *et al*. 2015), related to better preserve from membrane damage by cold shock. For successful fertilization, the sperm must protect plasma membrane and acrosomal integrity; the phospholipids and cholesterol from SL can maintain the plasma membrane and acrosome to protection sperm motility at low temperature (Thun *et al*. 2002). Furthermore, SL has the physiological effect of reducing the cooling point and lowering the alternative of plasmalogens, thereby limiting the possible mechanical injuries of the sperm membrane during semen cooling and cryopreservation (Layek *et al*. 2016).

In the current study, groups of MQ10 and MQ100 presented higher percentages of TM, PM, viability, membrane functionally, and MAP, as well as lower MDA concentration.

Table 1 The effects of Mito-quinol (MitoQ) on the motility of cooled semen in buck

Treatments	TM (%)			PM (%)		
	0 h	30 h	60 h	0 h	30 h	60 h
MQ0	84.5	54.2^{b}	15.5^{c}	64.0	18.6^{c}	10.0^{c}
MQ1	85.2	55.5^{b}	19.5^{b}	63.5	21.3^{b}	12.5^{b}
MQ10	85.0	58.1^{a}	24.0^{a}	63.0	24.0^{a}	15.0^{a}
MQ100	84.5	58.8^{a}	24.9^{a}	64.2	24.1^{a}	15.7^{a}
MQ1000	85.5	54.8^{b}	16.0^{c}	64.5	18.9^{c}	9.5^{c}
SEM	0.8	1.0	1.6	1.0	1.1	1.2

TM: total motility and PM: progressive motility.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 2 The effects of Mito-quinol (MitoQ) on the viability and membrane functionality of cooled semen in buck

Treatments	Viability (%)			Membrane functionality (%)		
	0 h	30 h	60 h	0 h	30 h	60 h
MQ0	88.0	53.0^{c}	16.5^{c}	87.1	52.0^{b}	17.2^{b}
MQ1	87.9	55.5^{b}	20.5^{b}	87.6	53.8^{b}	20.0^{b}
MQ10	88.5	58.5^{a}	25.0^{a}	89.0	56.3^{a}	24.5^{a}
MQ100	86.8	60.0^{a}	25.9^{a}	88.4	57.2^{a}	25.0^{a}
MQ1000	87.5	53.2^{c}	15.8^{c}	88.5	52.4^{b}	17.8^{b}
SEM	1.3	1.1	1.5	1.2	1.0	1.7

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

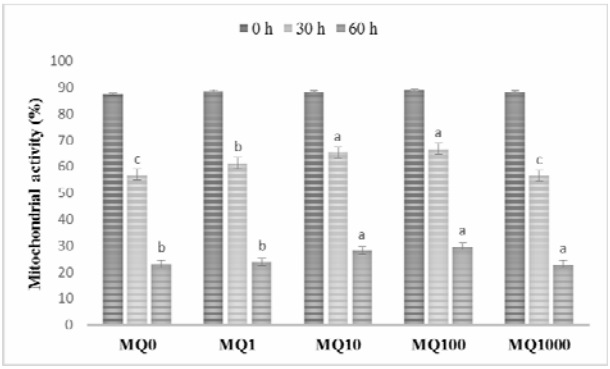

Figure 1 The effects of Mito-quinol (MitoQ) on mitochondrial activity rate (%) of cooled semen in buck

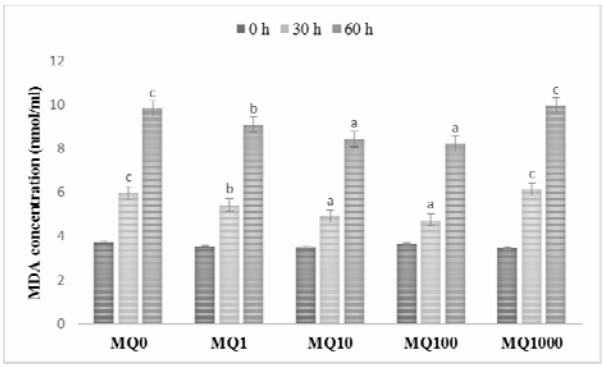

Figure 2 The effects of Mito-quinol (MitoQ) on mitochondrial concentration (nmol/mL) of cooled semen in buck

In agreement with this results, the mitochondrial activity of rooster cooled semen was preserved by supplementing the cooling medium with 100 nM MitoQ (Alipour-Jenaghard *et al*. 2023b). In addition, Mito-TEMPO, as a mitochondrial-targeted antioxidant, conserved the quality parameters of rooster cooled-stored semen by conserving MAP (Masoudi *et al*. 2020). Adding 200 nM MitoQ alone or in combination with 150 mM Trehalose to buck semen extender increased antioxidant enzyme defense, MAP, and reduced DNA fragmentation (Rezaei *et al*. 2023). Barfourooshi *et al*. (2023) stated MitoQ is can protect quality parameters and fertility characteristics of ram semen after thawing.

Based on the results in the present study, the mechanism for increasing sperm motility may be as follows: when MitoQ is added to semen extender, due to the conjugation of a lipophilic triphenyl-phosphonium cation, it easily crosses the mitochondrial inner membrane and accumulates in the matrix (Arjun *et al*. 2022). After detaching from TPP+, ubiquinone adsorbed onto mitochondrial membranes (Ross *et al*. 2005). An electron-donating component of the respiratory chain, ubiquinone can take electrons from complex I or II and reduce them to ubiquinol, which is then donated to complex III. Thus, ubiquinones increase sperm motility by transferring electrons through the electron transport chain and enhancing mitochondrial ATP production. In response to ubiquinones supplements, antioxidant ubiquinols are

produced (Arjun *et al*. 2022). As a result, MitoQ reduces LPO by scavenging ROS and inhibiting oxidative stress (Tiwari *et al*. 2021). CoQ10, a vital component of the electron transport chain, is made more bioavailable by MitoQ in the cell, preventing the formation of hydrogen peroxides and reduces LPO (Ross *et al*. 2008).

Sperm activity depends on mitochondrial function and integrity. Mitochondrial membrane potential (MMP), as an electrostatic potential, is produced by creating an electrochemical gradient across the intermembrane space of mitochondria. As a consequence, MMP can be used as an indicator of mitochondrial function based on mitochondrial energy status, permeability transitions, and respiratory chain activity (Escada-Rebelo *et al*. 2022). During cooling and freezing, mitochondrial permeability increases, leading to the release of proapoptotic factors and decreasing sperm viability (Rezaei *et al*. 2023). In our study, MitoQ improved sperm MAP and viability by 10 and 100 nM MitoQ, which is consistent with other studies that found MitoQ improved sperm MAP (Kumar *et al*. 2021; Barfourooshi *et al*. 2023). Additionally, MitoQ reduces ROS by enhancing SOD, GPX, and GSH in mitochondria (Ibrahim *et al*. 2019). Since mitochondria play a major role in cells, mitochondria-targeted antioxidants can help preserve the fertility potential of sperm cells during cooling.

CONCLUSION

In conclusion, adding MitoQ, as a mitochondria-targeting antioxidant, to soy lecithin containing extender preserves the quality parameters of buck cooled-stored semen by preserving mitochondrial activity. Thus, application of 10 or 100 nM MitoQ in a cooled extender containing soy lecithin could be an effective method for preserving buck cooled semen quality during transportation for artificial insemination.

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