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Mito-TEMPO Protects Buck's Semen Quality During Cooling Process

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Abstract

Background: Membrane lipids peroxidation is one of the most important chemical stresses in sperm, but utilization of antioxidants in semen diluent protect cells against oxidative damages and it could be a beneficial way to preserve semen quality during storage process.

Objectives: The current study was aimed to assess the effect of Mito-TEMPO on quality parameters of buck semen during cooling process.

Methods: In this experiment, 5 Saanen bucks were used and semen samples were collected twice a week. After semen collection and primary evaluation, semen samples were diluted and assigned into five equal aliquots. Then samples were added to extenders containing 0, 1, 10, 100 and 1000 µM Mito-TEMPO. The prepared samples were stored at 5 °C up to 60 h. Chilled sperm motility, progressive motility, viability, mitochondrial activity, membrane functionality and malondialdehyde concentration were analyzed during 0, 30 and 60 h.

Results: In results at time 0, no difference was observed among groups but at 30 and 60 h storage, higher ($P \leq 0.05$) sperm total motility, progressive motility, mitochondrial activity, membrane functionality, viability and lower lipid peroxidation were observed in 10 and 100 µM Mito-TEMPO groups than the others.

Conclusion: In conclusion, supplementation of buck cooling medium with Mito-TEMPO as a novel mitochondria targeted antioxidant could be an impressive method to conserve buck's semen quality during cooling storage process.

Keywords: Buck; Chilling; Mito-TEMPO; Spermatozoa

1. Introduction

Artificial insemination as one of the key stages of assisted reproductive techniques (ARTs) plays an important role in optimization of ruminant reproduction. Success in this method depends on semen quality which relies on the semen processing and ability of the extenders to maintain sperm characteristics (Zarei et al., 2022). Although semen liquid storage in 5 °C for a short time to artificial insemination prolonged lifespan of sperm via decreasing the metabolism rate of cell but on the other hand it reduces sperm quality and fertility (Zarei et al., 2022; Stefanov et al., 2015). Basically buck sperm membrane is rich in polyunsaturated fatty acids (PUFAs) that making its highly sensitive to lipid peroxidation by reactive oxygen species (ROS) which are produced during semen storage and reduces the functional life of spermatozoa (Aitken et al., 2014). It seems supplementation of extenders with exogenous antioxidants could be an efficient method to overcome ROS activities (Masoudi et al., 2022).

Mito-TEMPO (triphenylphosphonium chloride) as a novel mitochondria-targeted antioxidant has been used to reduce sperm storage-associated damages. Mito-TEMPO is a compound with superoxide dismutase like activity which scavenges superoxide anion (Dikalova et al., 2010). This substance is combined from piperidine nitroxide TEMPO with antioxidant properties and lipophilic cation triphenylphosphonium (TPP^{+}). The TPP accumulates several hundred folds within mitochondria driven by the membrane potential (Trnka et al., 2008). In this way, a large amount of TEMPO antioxidant which conjugated with TPP, accumulates at the mitochondria as main site of ROS production and protects cell against oxidative injury (Zarei et al., 2022). Supplementation of semen extender with Mito-TEMPO conserved sperm quality in human (Lu et al., 2018) and ram (Zarei et al., 2022).

Nowadays, soybean lecithin as an alternative for egg yolk used commercially in semen extenders (Sharafi et al., 2015). The effective components of soybean lecithin such as low density lipoproteins and phospholipids can prevent damage to the sperm plasma membrane during cooling and freezing process (Bergeron and Manjunath, 2006). This non-animal additive can decrease hygienic risks of diseases transmission in international semen transport (Chelucci et al., 2015). No study has yet reported on the effect of Mito-TEMPO on the quality of buck semen chilled in an extender containing soybean lecithin. Therefore, the objective of current study was

to determine the effects of different concentrations of Mito-TEMPO on total motility, progressive motility, viability, mitochondrial activity, membrane functionality and malondialdehyde concentration of buck's cooled semen.

2. Materials and methods

2.1. Ethics and used chemicals

Current study was approved by Research Ethics Committees of Animal Science Research Institute of Iran and all chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) companies.

2.2. Semen collection and chilling medium preparation

Five Saanen goats (3-4 years) were used in this study. Semen samples were collected during breading season and twice a week by an artificial vagina. Collected samples were transported to the laboratory in a water bath (38 °C) for quality evaluation. Only samples with the following parameters were selected for cooling storage: sperm concentration ≥ 3×10^9 sperm/ml, semen volume 1-2 ml, abnormal morphology ≤ 15% and total sperm motility ≥ 70%. After evaluation, selected samples were mixed to omit individual differences and diluted in the extender.

The chilling medium was composed of fructose (1.26 g/100ml), citric acid (1.64 g/100ml), soybean lecithin (1.5% w/v), tris (3.07 g/100ml), streptomycin (1 mg), penicillin (100 IU) and catalase (200 IU/ml). Osmolarity and pH were set at 425 mOsm/kg and 6.8 respectively. Then different concentrations of Mito-TEMPO were added to this extender and five experimental groups were prepared as follows: medium without Mito-TEMPO (MT0), medium containing 1 µM (MT1), 10 µM (MT10), 100 µM (MT100) and 1000 µM (MT1000) Mito-TEMPO. Sperm total motility (TM), progressive motility (PM), viability, membrane functionality, mitochondrial activity and malondialdehyde (MDA) content were assessed at 0 (start time), 30 and 60 h after cooling.

2.3. Chilled semen quality evaluation

Sperm class analysis software (SCA) (Version 5.1; Microptic, Barcelona, Spain) was used to assess sperm total motility (TM) and progressive motility (PM) during 0, 30 and 60 h chilling times. To determine sperm motility parameters, five µl of each semen samples were placed onto a pre-warmed chamber slide. Afterward, for each sample, 6 fields containing a minimum of 400 sperm were counted (Masoudi et al., 2022).

The hypo-osmotic swelling test (HOST) was used for membrane functionality evaluation. This method depends on the resistance of the sperm membrane to stressful conditions in a hypoosmotic medium (Zarei et al., 2022). In this assessment, five µl of buck's chilled semen samples were mixed with 50 µl of hypo-osmotic solution and incubated for 30 min. Then under a phase contrast microscope, 300 sperm cells were counted and the percentage of sperms with swollen and curved tails were recorded as intact membrane.

Rhodamine 123 (Rh123; Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI) as fluorescent dye were used to determine mitochondrial activity during a flow cytometry study. To assess mitochondrial activity 10 µl of Rh123 solution were added to 300 µl of chilled semen and incubated in the dark room (20 min). Then, semen sample was centrifuged (500 g for 3 min) and again resuspended in 500 µl tris buffer. Next, 10 µl of PI were added to the sperm suspension. Via FACSCalibur flow cytometer (Becton Dickinson, USA) 10,000 events were recorded. Sperm cells with negative signal for PI and positive signal for Rh123 ($Rh123^{+}/PI^{-}$) were recorded as sperm with active mitochondria (Heidari et al., 2022).

Lipid peroxidation in buck's chilled semen was assessed via measurement of malondialdehyde (MDA) concentration by reaction of thiobarbituric acid. The malondialdehyde

content was recorded by absorption with the standard curve of MDA equivalent generated by the acid catalyzed hydrolysis of 1, 1, 3, 3-tetramethoxypropane (Sharafi et al., 2015). Diluted semen sample in amount of 1 ml (250 $\times 10^6$ sperm cells/ml) was mixed with 1 ml of cold 20% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted via centrifuge (900 g for 15 min) and 1 ml of the supernatant was incubated with 1 ml of 0.67% (w/v) thiobarbituric in a water bath (100 °C for 10 min). After cooling, spectrophotometer (UV-1200, Shimadzu, Japan) recorded the absorbance at 532 nm and MDA concentration was expressed as nmol/ml.

Eosin–nigrosine staining was used to assess buck's chilled semen viability. semen sample was mixed with two drops of eosin–nigrosine dye (1.67 g eosin-Y, 10 g nigrosine and 2.9 g sodium citrate dissolved in 100 ml distilled water) on a warm slide. Then, this mixture was spread on a second slide to counting 200 sperm cells under a phase contrast microscope (×400 magnification). Sperm cells with unstained head were recorded as live cells and dead cells were stained head (Masoudi et al., 2022).

2.4. Statistical analysis

The Shapiro–Wilk test was used to examine the data for normality and Data analyzing was conducted by Proc GLM of SAS 9.1 (SAS Institute, version 9.1, 2002, USA) software. Statistical

differences between groups were determined by Tukey's test. Results are presented as Mean ± SE.

Results

3.1. Total motility and progressive motility

The effects of Mito-TEMPO on buck's chilled sperm motility parameter at 0, 30 and 60 h after cooling are shown in table 1. At the storage time of 0 no significant difference ($P>0.05$) was observed among groups but during 30 and 60 h cooling periods, total motility and progressive motility in groups received 10 and 100 µM Mito-TEMPO were higher ($P\leq0.05$) than other groups.

3.2. Viability and malondialdehyde concentration

Table 2 summarizes the effects of Mito-TEMPO on buck's chilled sperm viability and lipid peroxidation during cooling storage in media contained different doses of Mito-TEMPO. At first time (0 h), no significant difference ($P>0.05$) was observed between treatments for sperm viability and MDA concentration. At 30 and 60 h, 10 and 100 µM Mito-TEMPO presented lower ($P\leq0.05$) MDA concentration in contrast with the other groups, but the difference among other

three groups was not significant (P>0.05). In the case of viability rate, during 30 and 60 h cooling periods, 10 and 100 µM Mito-TEMPO presented higher (P≤0.05) viability compared to others.

3.3. Mitochondrial activity and membrane functionality

The data related to the effects of Mito-TEMPO on buck's chilled sperm mitochondrial activity and membrane functionality are shown in table 3. In both parameters there were no statistically difference (P>0.05) among groups at time 0 h storage. But during 30 and 60 h cooling periods mitochondrial activity and membrane functionality were greater (P≤0.05) in 10 and 100 µM Mito-TEMPO compared to other groups and the observed difference among other groups were not significant (P>0.05).

3. Discussion

Semen liquid storage is generally associated with biochemical, osmotic and thermal changes that lead to decrease in sperm characteristics and reduced fertility (Gibb et al., 2015: Zarei et al., 2022). Some of biochemical changes in sperm can be attributed to oxidative stress because sperm antioxidant enzymes do not have the capacity to sustain sperm quality against

ROS generation in excess (Kasimanickam et al., 2006). In this situation, using exogenous antioxidants as compounds which suppress the formation of ROS and oppose their actions (Bansal and Bilaspuri, 2011) could be an efficient method to preserve sperm against storage shocks and damages (Asl et al., 2018). ROS scavenger mitochondria-targeted antioxidants such as Mito-TEMPO with high efficiency, wide application and low toxicity are potential candidates to protect sperm from damages caused by storage (Oyewole and Birch-Machin, 2015). There is no information about the effects of Mito-TEMPO on buck' semen in medium containing soybean lecithin during liquid storage. Therefore, the current study was performed to evaluate the impact of this antioxidant on buck's chilled semen quality parameters during 60 h storage at 5 °C. Using Mito-TEMPO (10 and 100 µM) effectively protected semen quality and resulted in higher total motility, progressive motility, membrane functionality, mitochondrial activity, viability and lower malondialdehyde concentration, but efficiency of 60 h-stored semen samples was lower than 30 h-stored. Indeed with increasing of the time of storage, the sperm quality parameters were reduced. The results of this experiment are in agreement with our previous studies on cooling storage of ram (Zarei et al., 2022).

Mitochondria as an important intracellular organelle regulates bioenergetic metabolism and provides ATP via oxidative phosphorylation for sperm functions (Cabezas-Opazo et al., 2015).

This activity correlates with sperm quality parameters and semen storage potential (Chen et al., 2014). The accumulation of toxic products such as ROS to high concentrations that formed from the sperm's metabolism in cooling process leads to oxidative stress and mitochondrial inefficacy to supply required energy for spermatozoa (Agarwal and Prabakaran, 2005).

mitochondria-targeted antioxidant, Mito-TEMPO is comprised of lipophilic triphenylphosphonium (TPP) moiety and piperidine nitroxide TEMPO (Lu et al., 2018). This compound is a novel cell permeable antioxidant because TPP unit with its positive charge endows Mito-TEMPO with ability to pass through cell lipid bilayer membranes rapidly and accumulate 1000-fold in mitochondria matrix as the major site of ROS generation (Jiang et al., 2015). The TEMPO unit catalyze the dismutation of superoxide and to react directly with many radicals (Yang et al., 2018). TEMPO through its hydroxylamine-like structure inhibits the excessive generation and overflow of oxygen free radicals via convert superoxide anion into hydrogen peroxide and oxygen while the nitroxide radicals produced act as superoxide dismutase to maintain the stability of the phospholipid bilayer membrane and electron transport chain (Du et al., 2017). Therefore, this ROS scavenging property can reduce the rate of lipid peroxidation and helps sperm cells to be safe against oxidative stress (Lu et al., 2018). In the current study

concentrations of 10 and 100 µM Mito-TEMPO decreased malondialdehyde (MDA) content, as an indicator of lipid peroxidation.

Furthermore, Mito-TEMPO exerting a targeted antioxidant effect by as well as regulating antioxidant related enzyme activities (Trnka et al., 2009). Phosphohexose isomerase (GPI) as a ubiquitous cytosolic enzyme plays a critical role in provides ATP via glycolytic and glucogenic pathways and closely related to sperm quality. This soluble enzyme loosely bound to the sperm mitochondria (Jiang et al., 2015), so in chilling shocks releases easily into the extracellular that maybe induced reduction of sperm motility, membrane functionality and viability (Harrison and White, 1972). One of the possible mechanism of Mito-TEMPO on sperm could be attributed to the regulating the GPI activity which reduced during semen manipulation. But this reduction would be controlled by Mito-TEMPO which improvement of sperm energy metabolism and conserves sperm quality parameters during cooling process (Jiang et al., 2015). So according to our findings in this work, Mito-TEMPO can be relieving the decline in buck's sperm motility, viability, membrane functionality and mitochondrial activity during cooling process.

Conclusion

Supplementation of buck's semen extender with 10 and 100 µM Mito-TEMPO by maintenance of mitochondrial activity preserved the sperm quality parameters after cooling process. Therefore, it could be concluded, adding mitochondria targeted antioxidant Mito-TEMPO to the soybean lecithin-based chilling media is an efficient method to retention buck semen quality for artificial insemination.

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TABLES

Table 1. Effects of Mito-TEMPO on buck's chilled sperm total motility (TM) and progressive motility (PM) during 0, 30 and 60 h of storage at 5ºC.

Mito-TEMPO	TM (%)			PM (%)		
concentrations	0 h	30 h	60 h	0 h	30 h	60 h
MT0	85.0 ± 1.0	56.0 ± 1.3 [b]	18.1 ± 1.6 [b]	65.3 ± 0.9	19.4 ± 1.2 [b]	9.5 ± 1.5 [b]
MT1	85.0 ± 1.0	57.9 ± 1.3 [ab]	19.0 ± 1.6 [b]	64.8 ± 0.9	20.1 ± 1.2 [b]	10.0 ± 1.5 [b]
MT10	84.5 ± 1.0	59.0 ± 1.3 [a]	22.4 ± 1.6 [a]	65.1 ± 0.9	24.2 ± 1.2 [a]	14.1 ± 1.5 [a]
MT100	84.0 ± 1.0	60.5 ± 1.3 [a]	23.6 ± 1.6 [a]	65.0 ± 0.9	25.0 ± 1.2 [a]	14.5 ± 1.5 [a]
MT1000	86.0 ± 1.0	55.6 ± 1.3 [b]	16.4 ± 1.6 [b]	66.2 ± 0.9	18.5 ± 1.2 [b]	9.0 ± 1.5 [b]

Different letters within the same column show significant differences among the groups (P≤0.05).

Table 2. Effects of Mito-TEMPO on buck's chilled sperm viability and MDA concentration during 0, 30 and 60 h of storage at 5ºC.

Mito-TEMPO concentrations	Viability (%)			MDA concentration (nmol/ml)		
	0 h	30 h	60 h	0 h	30 h	60 h
MT0	85.8 ± 1.1	55.2 ± 1.5 [b]	16.0 ± 2.0 [b]	3.15 ± 0.25	5.80 ± 0.35 [b]	9.95 ± 0.57 [b]
MT1	86.5 ± 1.1	58.4 ± 1.5 [ab]	16.5 ± 2.0 [b]	3.10 ± 0.25	5.05 ± 0.35 [ab]	9.00 ± 0.57 [ab]
MT10	86.2 ± 1.1	60.1 ± 1.5 [a]	21.0 ± 2.0 [a]	3.45 ± 0.25	4.65 ± 0.35 [a]	8.55 ± 0.57 [a]
MT100	85.0 ± 1.1	61.3 ± 1.5 [a]	22.6 ± 2.0 [a]	3.30 ± 0.25	4.40 ± 0.35 [a]	8.00 ± 0.57 [a]
MT1000	87.0 ± 1.1	55.8 ± 1.5 [b]	14.6 ± 2.0 [b]	3.40 ± 0.25	6.00 ± 0.35 [b]	10.10 ± 0.57 [b]

Different letters within the same column show significant differences among the groups (P≤0.05).

Table 3. Effects of Mito-TEMPO on buck's chilled sperm mitochondrial activity and membrane functionality during 0, 30 and 60 h of storage at 5°C.

Mito-TEMPO concentrations	Mitochondrial activity (%)			Membrane functionality (%)		
	0 h	30 h	60 h	0 h	30 h	60 h
MT0	87.4 ± 1.1	56.2 ± 1.6 [b]	22.5 ± 1.4 [b]	85.0 ± 1.0	56.0 ± 1.8 [b]	21.5 ± 2.0 [b]
MT1	88.5 ± 1.1	58.1 ± 1.6 [ab]	25.0 ± 1.4 [ab]	86.0 ± 1.0	58.4 ± 1.8 [ab]	23.0 ± 2.0 [ab]
MT10	88.0 ± 1.1	60.0 ± 1.6 [a]	26.5 ± 1.4 [a]	86.0 ± 1.0	60.0 ± 1.8 [a]	25.9 ± 2.0 [a]
MT100	87.2 ± 1.1	60.5 ± 1.6 [a]	26.9 ± 1.4 [a]	85.5 ± 1.0	60.5 ± 1.8 [a]	26.8 ± 2.0 [a]
MT1000	89.0 ± 1.1	56.9 ± 1.6 [b]	23.4 ± 1.4 [b]	86.8 ± 1.0	55.5 ± 1.8 [b]	21.5 ± 2.0 [b]

Different letters within the same column show significant differences among the groups (P≤0.05).

میتوتمپو از کیفیت منی بز طی فرایند سردسازی محافظت می‌کند

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چکیده

زمینه مطالعه: پراکسیداسیون لیپیدهای غشایی یکی از مهمترین تنش‌های شیمیایی در اسپرم است. استفاده از آنتی‌اکسیدان‌ها در رقیق‌کننده منی، اسپرم را در برابر آسیب‌های اکسیداتیو محافظت نموده و می‌تواند روشی مفید برای حفظ کیفیت مایع منی در طول فرآیند ذخیره سازی باشد.

هدف: مطالعه حاضر به منظور بررسی اثر آنتی‌اکسیدان هدفمند میتوکندریایی میتوتمپو بر فراسنجه‌های کیفی منی بز در طول فرآیند سردسازی اجرا شد.

روش کار: در این آزمایش از پنج رأس بز سانن استفاده شد. نمونه‌های منی دو بار در هفته جمع‌آوری شد. پس از جمع‌آوری منی و ارزیابی اولیه، نمونه‌های منی رقیق شده و به پنج قسمت مساوی تقسیم شدند. سپس نمونه‌ها به رقیق‌کننده حاوی 0، 1، 10، 100 و 1000 میکرومولار میتوتمپو اضافه شدند. منی رقیق‌شده در دمای پنج درجه سانتی‌گراد به مدت 60 ساعت ذخیره شد. سپس فراسنجه‌های کیفی اسپرم شامل تحرک، تحرک پیش‌رونده، زنده‌مانی، فعالیت میتوکندری، عملکرد غشا و غلظت مالون‌دی‌آلدهید در 0، 30 و 60 ساعت سردسازی مورد ارزیابی قرار گرفت.

نتایج: نتایج فرایند سردسازی اسپرم نشان داد در زمان صفر تفاوتی بین تیمارها وجود نداشت. در زمان‌های 30 و 60 ساعت سردسازی استفاده از غلظت‌های 10 و 100 میکرومولار میتوتمپو سبب حفظ تحرک کل، تحرک پیش‌رونده، فعالیت میتوکندری، عملکرد غشا و زنده‌مانی اسپرم و کاهش میزان تولید مالون‌دی‌آلدهید در اسپرم بز شد ($P<0/05$).

نتیجه گیری نهایی: استفاده از میتوتمپو به عنوان یک آنتی‌اکسیدان هدفمند میتوکندریایی جدید می‌تواند راهکاری مؤثر برای حفظ کیفیت اسپرم بز در طول فرایند سردسازی باشد.

کلید واژه: اسپرم، بز، سردسازی، میتوتمپو