Original Article

Effect of Mito-TEMPO on Post-thawed Semen Quality in Goats

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ABSTRACT

Background: Although sperm cryopreservation seems to be an efficient technique for distributing competent sperm for artificial insemination, the process affects the quality of post-thawed sperm.

Objectives: This study was designed to see how the novel mitochondria-targeted antioxidant "Mito-TEMPO" affected buck sperm quality during cryopreservation.

Methods: After proper semen samples collection, they were diluted and divided into 5 equal groups and cryopreserved in liquid nitrogen with 0, 1, 10, 100, and 1000 µM Mito-TEMPO. Sperm motility, lipid peroxidation, abnormal morphology, acrosome integrity, membrane integrity, and viability were all evaluated after thawing.

Results: When the freezing extender was supplemented with 10 µM Mito-TEMPO, total motility, progressive motility, membrane integrity, acrosome integrity, and viability increased ($P \leq 0.05$), while lipid peroxidation decreased ($P \leq 0.05$).

Conclusion: Finally, the novel mitochondria-targeted antioxidant "Mito-TEMPO" could be introduced as an effective cryo-additive to improve buck semen quality parameters during cryopreservation.

Keywords: Buck, Cryopreservation, Extender, Mito-TEMPO, Sperm

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1. Introduction

Although cryopreservation of sperm cells protects the genetic pool and expands desirable reproductive qualities through reproductive programming, the freezing method reduces sperm potency by causing physical and chemical shocks (Sharafi et al., 2015a). Polyunsaturated fatty acids are rapidly peroxidized by reactive oxygen species (ROS) in the sperm membrane (Mohajer & Dadashpour Davachi, 2022). ROS disrupts sperm plasma membrane stability and reduces sperm fertility capacity (Yousef et al., 2022). Endogenous antioxidants in sperm neutralize ROS, but the amount of endogenous antioxidants in sperm is insufficient to neutralize ROS during the freezing process (Sharafi et al., 2015b), so exogenous antioxidants are required to overcome the side effects of ROS.

Mito-TEMPO is a new antioxidant targeting mitochondria and a potent ROS scavenger in cells. TEMPO is combined with triphenylphosphonium (TPP+) that is a membrane-permeable cation that mimics superoxide dismutase biological activity and catalyzes the dismutation of superoxide (Trnka et al., 2008). This combination generates a mitochondria-targeted component that efficiently removes superoxide. In certain human illnesses, Mito-TEMPO also prevents oxidative damage to cells (Dikalova et al., 2010; Choumar et al., 2011). During cryopreservation, Mito-TEMPO has also been proven to preserve the quality of thawed human spermatozoa (Lu et al., 2018).

This study was done to determine the influence of Mito-TEMPO supplementation of cryopreservation media on motility, lipid peroxidation, membrane integrity, aberrant morphogenesis, acrosome integrity, and viability of post-thawed buck semen in goats, as no studies have been conducted to date.

2. Materials and Methods

Chemicals

Chemicals were supplied by Merck (Darmstadt, Germany) and Sigma (St. Louis, Missouri, United States).

Samples collection

Five adult Saanen bucks (aged 3 to 4 years) were sampled twice weekly via artificial vagina in 6 repetitions to obtain sperm. Samples were examined and included in the experiment if the following conditions were met: $>3\times10^9$ spermatozoa/mL sperm concentration; >75% overall motility; and >85% normal morphology. To reduce individual disparities among men, certain samples were combined. The cryopreservation extender contained citric acid (1.64 g/100 mL), fructose (1.26 g/100 mL), Tris (3.07 g/100 mL), egg yolk (15% v/v), glycerol (5% v/v), streptomycin (1 mg), and penicillin (100 IU). The osmotic pressure was 425 mOsm/kg of water, and the pH was 6.8. The sperm samples were then diluted with the extenders MT0 (extender without Mito-TEMPO), MT1 (extender with 1 M Mito-TEMPO), MT10 (extender with 10 M Mito-TEMPO), MT100 (extender with 100 M Mito-TEMPO), and MT1000 (extender with 1,000 M Mito-TEMPO) (extender with 1000 M Mito-TEMPO). Before loading 50×10^6 spermatozoa per straw into 0.25 mL French straws (Biovet, L'Agile, France), the diluted sperm samples were chilled to 5°C over 120 minutes. The straws were then subjected to static nitrogen vapor (-70°C) for 10 minutes, immersed in liquid nitrogen (LN2), and stored in LN2 until thawing.

In vitro evaluation of the quality of thawed sperm samples

Employing sperm class analysis software, version 5.1; Microptic, Barcelona, Spain), the parameters of sperm motility were determined (Askarianzadeh et al., 2018). In this investigation, sperm samples were initially diluted in PBS buffer. Then, 5 µL of the diluted sample was placed on a chamber slide that had been preheated (38°C, Leja 4.2 m height, Luzernestra at B.V., Holland). At least 6 fields containing at least 400 spermatozoa were analyzed for each sample. The following parameters 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), linearity (LIN, %), the amplitude of lateral head displacement (ALH, m), and beat/cross frequency (BCF, Hz).

As an indication of lipid peroxidation, the malondialdehyde (MDA) concentration was measured using the reaction of thiobarbituric acid (Masoudi et al., 2020). The MDA content was determined using absorption in conjunction with a standard curve of MDA equivalent formed by the acid-catalyzed hydrolysis of 1, 1, 3, and 3-tetra methoxy propane. To precipitate protein, 1 mL of diluted sperm sample (400×10^6 sperm cells/mL) was combined with 1 mL of cold 20% (w/v) tricholoroacetic acid. One milliliter of the supernatant was incubated with 1 mL of 0.67% (w/v) thiobarbituric acid in a 100°C boiling water bath for 10 minutes. After cooling, the absorbance at 532 nm was measured using a spectropho-

tometer (UV-1200, Shimadzu, Japan). All MDA concentrations were given in nmol/mL.

Hancock solution was utilized to evaluate aberrant morphology in thawed sperm. Consequently, a drop of the thawed material was added to 1 mL of Hancock solution: 150 mL sodium saline solution, 500 mL double-distilled water, 62.5 mL formalin (37%) and 150 mL buffer solution in an Eppendorf tube (Shahverdi et al., 2015). The proportion of sperm cells with aberrant heads and or tails was then determined by counting 300 spermatozoa using a microscope.

Pisum sativum agglutinin (PSA) was utilized to evaluate sperm acrosome integrity (Feyzi et al., 2018). Two hundred spermatozoa were viewed on a slide, and sperm with and without green heads were categorized as undamaged versus disrupted/damaged acrosomes.

The hypoosmotic swelling test was used to capture membrane-integrated sperm samples (Zarei et al., 2021). The test relies on the resistance of the sperm membrane to stressful situations in a hypoosmotic medium. About 5 µL of sperm was incubated in 50 µL of hypoosmotic solution (57.6 mM fructose and 19.2 mM sodium citrate, 100 mOsm/L) for 20 minutes. Then, 300 spermatozoa were inspected under a phase-contrast microscope (400× magnification, CKX41, Olympus, Tokyo, Japan), and samples with swollen and non-swollen tails were documented as sperm cells with integrated and non-integrated membranes, respectively.

As previously described (Masoudi et al., 2021), using an annexin V-FITC kit (IQP, Groningen, and The Netherlands) and PI, we evaluated phosphatidyl serine externalization as an indicator of apoptotic-like alterations in spermatozoa. Flow cytometry was used to analyze, and 4 sperm subpopulations were identified: Live cells that are negative for both Annexin V and PI (A-/PI-), cells that are positive for Annexin V but negative for PI (A+/PI-), cells that are positive for both Annexin V and PI (A+/PI+), and necrotic cells that are negative for Annexin V but positive for PI (A-/PI+). Finally, viable sperm were determined by counting live and early apoptotic sperm.

Statistical analyses

The SAS software, version 9.1 program's Proc GLM procedures were used to analyze the data (SAS Institute, version 9.1, 2002, Cary, NC, USA). To determine statistical differences between groups, the Tukey test was used. The results are presented as Mean+SE.

3. Results

Motility and lipid peroxidation

The effect of Mito-TEMPO on motility parameters and lipid peroxidation in sperm samples is shown in Table 1. Compared to the other groups, the MT10 had higher TM and PM ($P \leq 0.05$). The PM and TM levels in the MT1 and MT100 groups were higher ($P \leq 0.05$) than in the MT0 and MT1000 groups. There was no significant difference ($P > 0.05$) between treatment groups for VAP, VSL, VCL, LIN, ALH, and BCF items.

MDA concentrations were lower ($P \leq 0.05$) in the MT1, MT10, and MT100 groups than in the MT0 and MT1000 groups. There were no statistically significant differences ($P > 0.05$) between the MT1, MT10, and MT100 groups.

Membrane integrity, abnormal morphology, acrosome integrity, and viability

The effect of Mito-TEMPO on membrane integrity, aberrant morphology, acrosome integrity, and viability of post-thawed buck spermatozoa is presented in Table 2. MT10 had greater membrane integrity ($P \leq 0.05$) than the other groups. The MT1 and MT100 groups exhibited greater membrane integrity ($P \leq 0.05$) than the MT0 and MT1000 groups.

The addition of Mito-TEMPO to the freezing extender had no influence on the aberrant morphology of deer sperm after thawing ($P > 0.05$).

The MT10 group had greater acrosome integrity than the MT0, MT1, and MT1000 groups ($P \leq 0.05$).

The MT100 group had higher acrosome integrity than the MT0 and MT1000 groups ($P \leq 0.05$).

The MT10 and MT100 groups had a greater viability rate ($P \leq 0.05$) than the MT0, MT1, and MT1000 groups. There were no differences ($P > 0.05$) between the MT0, MT1, and MT1000 groups.

4. Discussion

Adding antioxidants to the freezing extender efficiently preserves sperm quality and fertility in small ruminants during the cryopreservation procedure (Sharafi et al., 2015a). Polyunsaturated fatty acids in the sperm membrane render spermatozoa sensitive to cryo-damage by diminishing the reproductive capacity of thawed sperm cells (Askarianzadeh et al., 2018). It is reasonable to add an exogenous antioxidant to the freezing extender

Table 1. Effects of Mito-TEMPO on the motility parameters and MDA concentration of post-thawed Buck semen

Groups	MT0	MT1	MT10	MT100	MT1000	SEM
TM (%)	40.6^{c}	49.0^{b}	55.8^{a}	50.5^{b}	41.4^{c}	1.7
PM (%)	26.9^{c}	30.8^{b}	34.7^{a}	30.2^{b}	25.8^{c}	1.4
VAP (µm/s)	89.0	89.7	90.0	91.1	90.7	1.3
VSL (µm/s)	86.6	70.0	70.4	71.2	69.2	1.7
VCL (µm/s)	165.4	169.0	168.8	165.2	166.7	1.5
LIN (%)	41.4	41.4	41.7	43.0	41.5	1.3
ALH (µm)	7.2	7.5	7.7	8.0	7.0	0.6
BCF (Hz)	30.2	29.6	30.5	31.2	30.9	1.1
MDA (nmol/mL)	3.56^{b}	2.25^{a}	1.85^{a}	2.05^{a}	3.44^{b}	0.33

Abbreviations: MT: Mito-TEMPO; TM: Total motility; PM: Progressive motility; VAP: Average path velocity; VSL: Straight-line velocity; VCL: Curvilinear velocity; LIN: Linearity; ALH: Amplitude; BCF: Beat/cross frequency; MDA: Malondialdehyde.

$^{a, b, c}$Significant differences among the groups (P≤0.05).

to solve this issue and protect spermatozoa from cold shocks during freeze-thaw.

This research aimed to determine how the mitochondria-targeted antioxidant Mito-TEMPO influenced buck sperm quality measures like motility, lipid peroxidation, membrane integrity, aberrant morphology, acrosome integrity, and viability during the freeze-thaw cycle. Mito-TEMPO is a novel, effective, mitochondria-targeted antioxidant that selectively accumulates in the mitochondrial matrix due to its positive charge. It has a focused antioxidant effect by reducing or eliminating lipid peroxidation and forming free radicals in the mitochondria. It can also regulate cell antioxidant enzyme activity (Du et al., 2017). Mito-TEMPO therapy enhanced the TM, PM, membrane integrity, acrosome integrity, and survival of sperm cells after thawing while lowering lipid peroxidation. The findings are consistent with prior studies (Bateni et al., 2014) on the ROS scavenging capabilities of TEMPO and Mito-TEMPO (Bateni et al., 2014; Lu et al., 2018).

Mitochondria provide spermatozoa with energy via oxidative phosphorylation and ATP synthase (Ruiz-Pesini et al., 2007); however, heat shocks and ROS reduce mitochondrial activity, resulting in ATP transport impairment (Fang et al., 2014). An imbalance between creating and eliminating free radicals creates oxidative stress, resulting in DNA damage and apoptosis (Takahashi, 2012; Hamdan et al., 2016). Mitochondrial fission, aggregation, and malfunction are caused by intense oxidative stress (Pung et al., 2013; Chen et al., 2017). Most likely, increased mitochondrial fission decreases ATP generation, leading to mitochondrial-derived death (Chen et al., 2005). Mitochondrial failure causes nuclear translocation of apoptotic factors and endonuclease G, which enhances the creation of holes in the outer mitochondrial membrane and, consequently, mitochondrial permeability transition via matrix swelling (Bajt et al.,

Table 2. Effects of Mito-TEMPO on MI, AM, AI, and VI of post-thawed Buck semen

Groups	MT0	MT1	MT10	MT100	MT1000	SEM
MI (%)	42.0^{c}	51.5^{b}	56.4^{a}	52.2^{b}	40.3^{c}	1.8
AM (%)	15.9	18.2	16.3	17.5	16.8	1.6
AI (%)	54.7^{c}	56.5^{bc}	62.5^{a}	60.1^{ab}	55.4^{c}	2.0
VI (%)	44.7^{b}	45.7^{b}	53.3^{a}	50.6^{a}	45.0^{b}	1.5

$^{a, b, c, ab}$Significant differences among the groups (P≤0.05).

2008). Mito-TEMPO inhibits mitochondrial Bax translocation (Liang et al., 2010), indicating that it may be an effective method for protecting mitochondrial function and viability during freezing-thawing. Mito-TEMPO also protects mitochondrial activity and decreases stress-induced apoptosis and necrosis by reducing superoxide (Park et al., 2015; Gómez-Torres et al., 2017). DNA integrity and mitochondrial activity are related to cell quality metrics and the ability of post-thawed sperm cells to fertilize. The structure of mito-hydroxylamine-like TEMPO avoids the excessive production and overflow of oxygen free radicals during the freezing-thawing cycle. Producing nitroxide radicals preserves the electron transport chain and the stability of the phospholipid bilayer membrane (Du et al., 2017; Yang et al., 2018).

Due to the relationship between sperm morphology and spermatogenesis, Mito-TEMPO did not affect the proportion of sperm cells with normal morphology. The results of the morphology evaluation are consistent with findings from earlier research indicating sperm morphology is independent of the freezing-thawing process (Masoudi et al., 2021).

5. Conclusion

In this work, adding Mito-TEMPO to the buck-freezing extender maintained the quality metrics of sperm cells after thawing. Therefore, adding Mito-TEMPO to the cryopreservation solution effectively retains sperm quality in reproductive programs after thawing.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Animal Science Institute of Iran (ASRI-2020-980284)

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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مقاله پژوهشی

اثر میتوتمپو بر کیفیت منی بز پس از یخ‌گشایی

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چکیده

زمینه مطالعه: انجماد اسپرم روشی مؤثر برای توزیع اسپرم با کیفیت با هدف تلقیح مصنوعی است، اما فرآیند انجماد باعث کاهش کیفیت اسپرم پس از یخ‌گشایی می‌شود.

هدف: هدف از ارزیابی اثر آنتی‌اکسیدان هدف‌مند میتوکندریایی میتوتمپو بر کیفیت اسپرم بز بعد از ذخیره سرمایی بوده است.

روش کار: نمونه‌های اسپرم پس از جمع‌آوری و رقیق‌سازی به ۵ قسمت تقسیم شدند. مقادیر ۰، ۱، ۱۰، ۱۰۰ و ۱۰۰۰ میکرو مولار میتوتمپو را دریافت کردند. پارامترهای جنبایی، پراکسیداسیون لیپیدها، مورفولوژی غیرنرمال، سلامت غشا، سلامت آکروزوم و زنده‌مانی پس از یخ‌گشایی مورد ارزیابی قرار گرفتند.

نتایج: زمانی که مقدار ۱۰ میکرومولار میتوتمپو به رقیق‌کننده انجماد اسپرم افزوده شد، پارامترهای جنبایی کل، جنبایی پیشرونده، سلامت غشا، سلامت آکروزوم و زنده‌مانی افزایش یافت ($P\leq 0/05$)، درحالی‌که پراکسیداسیون لیپیدهای غشایی نسبت به سایر گروه‌ها کاهش یافت ($P>0/05$).

نتیجه‌گیری نهایی: درنتیجه، استفاده از آنتی‌اکسیدان هدف‌مند میتوکندریایی میتوتمپو می‌تواند به‌عنوان یک افزودنی مناسب برای بهبود کیفیت اسپرم بز در هنگام انجام فرایند انجماد-یخ‌گشایی باشد.

کلیدواژه‌ها: بز، ذخیره سرمایی، رقیق‌کننده، میتوتمپو، اسپرم

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