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Effect of Mito-TEMPO on Post-Thawed Semen Quality in Goat

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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 the samples were diluted and divided into five 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 were all increased ($P \leq 0.05$), while lipid peroxidation was decreased ($P \leq 0.05$).

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

KEYWORDS: Buck; Cryopreservation; Extender, Mito-TEMPO; Sperm.

Introduction

Although cryopreservation of sperm cells protects 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 and Davachi, 2022). ROS disrupt sperm plasma membrane stability and reduces sperm fertility potential (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 that targets mitochondria and is a potent ROS scavenger in cells. Tempo is combined with triphenylphosphonium (TPP+). TPP 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 goat, as no studies have been conducted to date.

Materials and methods

Chemicals and Ethics

Chemicals were supplied by Merck (Darmstadt, Germany) and Sigma (St. Louis, Missouri, United States). The Animal Science Research Institute of Iran's Research Ethics Committees accepted the current work.

Samples collection

Five adult Saanen bucks (aged 3 to 4 years) were sampled twice weekly via artificial vagina in six repetitions to obtain sperm. Samples were examined and included in the experiment if the following conditions were met: >3 10^9 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), soybean lecithin (1.5% w/v), glycerol (5 % v/v), streptomycin (1 mg), and penicillin (100

IU). The osmotic pressure was 425 mOsm per kilogram 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 a period of 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 the time of 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. 5 µl of the diluted sample was then placed on a chamber slide that had been preheated (38 °C, Leja 4, 20 m height, Luzernestra at B.V., Holland). At least six fields containing at least 400 spermatozoa were analyzed for each sample, and 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. 1 ml 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 spectrophotometer (UV-1200, Shimadzu, Japan). All MDA concentrations were given in nmol/ml units.

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). 200 spermatozoa were viewed on a slide, and sperm with and without green heads were categorized as undamaged acrosomes versus disrupted/damaged acrosomes.

The hypoosmotic swelling test was used to capture membrane-integrated sperm samples (Zarei *et al.*, 2021). HOST relies on the resistance of the sperm membrane to stressful situations in a hypoosmotic medium. 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. 300 spermatozoa were then 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 four sperm subpopulations were identified: (1) live cells that are negative for both Annexin V and PI (A-/PI-); (2) cells that are positive for Annexin V but negative for PI (A+/PI-); (3) cells that are positive for both Annexin V and PI (A+/PI+); and (4) necrotic cells that are negative for Annexin V but positive for PI (A-/PI+). Finally, viable sperm were determined by counting both live and early apoptotic sperm.

Statistical investigation

The SAS 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.

Results

Motility and lipid peroxidation

The effect of Mito-TEMPO on motility parameters and lipid peroxidation in sperm samples is shown in Table 1. When 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 was no statistically significant difference($P > 0.05$) 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 was no difference ($P > 0.05$) between the MT0, MT1, and MT1000 groups.

Discussion

Adding antioxidants to the freezing extender is an efficient method of preserving 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). In order to solve this issue and protect spermatozoa from cold shocks during freeze-thaw, it is appropriate to add an exogenous antioxidant to the freezing extender.

This research aimed to determine how the mitochondria-targeted antioxidant Mito-TEMPO influenced buck sperm quality measures like as 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 accumulates selectively in the mitochondrial matrix due to its positive charge. By reducing or eliminating lipid peroxidation and the formation of free radicals in the mitochondria, it has a focused antioxidant effect. It can also regulate antioxidant enzyme activity in cells (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 study (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 reactive oxygen species

(ROS) reduce mitochondrial activity, resulting in ATP transport impairment (Fang *et al.*, 2014). An imbalance between the creation and elimination of free radicals creates oxidative stress, which results 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.*, 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 the freezing-thawing process. 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, it 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 had no effect on 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).

Conclusion

In this work, the addition of Mito-TEMPO to the buck freezing extender maintained the quality metrics of sperm cells after thawing. Therefore, adding Mito-TEMPO to the cryopreservation solution is an effective method for retaining sperm quality in reproductive programs after thawing.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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TABLES

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

Different letters within the same row show significant differences among the groups ($P \leq 0.05$).

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325 **Table 2.** Effects of Mito-TEMPO on membrane integrity (MI), abnormal morphology (AM), acrosome integrity (AI) and viability (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

Different letters within the same row show significant differences among the groups ($P \leq 0.05$).

اثر میتوتمپو بر کیفیت منی بز پس از یخگشایی

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چکیده

زمینه مطالعه: اگرچه انجماد اسپرم روشی موثر برای توزیع اسپرم با کیفیت با هدف تلقیح مصنوعی است، فرآیند انجماد باعث کاهش کیفیت اسپرم پس از یخگشایی می گردد.

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

روش کار: نمونه های اسپرم پس جمع آوری و رقیق سازی به پنج قسمت تقسیم شده و مقادیر 0، 1، 10، 100 و 1000 میکرو مولار میتوتمپو را دریافت نمودند. پارامترهای جنبایی، پراکسیداسیون لیپیدها، مورفولوژی غیرنرمال، سلامت غشا، سلامت آکروزوم و زنده مانی پس از یخگشایی مورد ارزیابی قرار گرفتند.

نتایج: وقتی مقدار 10 میکرومولار میتوتمپو به رقیق کننده انجماد اسپرم افزوده شد پارامترهای جنبایی کل، جنبایی پیشرونده، سلامت غشا، سلامت آکروزوم و زنده مانی افزایش یافت ($P\leq0.05$) درحالیکه پراکسیداسیون لیپیدهای غشایی نسبت به سایر گروهها کاهش یافت ($P>0.05$).

نتیجه‌گیری نهایی: در نتیجه، استفاده از آنتی اکسیدان هدفمند میتوکندریایی میتوتمپو می تواند به عنوان یک افزودنی مناسب برای بهبود کیفیت اسپرم بز در هنگام انجام فرایند انجماد-یخگشایی باشد.

کلمات کلیدی: بز، ذخیره سرمایی، رقیق کننده، میتوتمپو، اسپرم.