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Comparative effects of melatonin and melatonin nanoparticle for cryopreservation of Barbari Buck semen in the summer season

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ABSTRACT

The study aimed to evaluate potential of melatonin and melatonin loaded nanoparticles to mitigate cryopreservation-induced cryo-damage using sperm related parameters of Barbari Buck semen. The pooled ejaculates were diluted using Tris-Egg-Yolk-Citric-Acid Fructose-Glycerol (TEYCAFG) extender and subsequently partitioned into three aliquots i.e. CON (without additives), MT (fortified with 1.0mM melatonin), and MTN (fortified with 5.0µM melatonin nanoparticles. Various seminal attribute viz. motility, viability, HOS reactive sperm, and the levels of GOT, and GPT was evaluated at pre-freeze and post-thaw stage. Significantly (P<0.05) higher motility and liveabilty in MTN (58.56±2.17 and 53.80±8.73%) and MT (55.95±2.6 and 51.84±8.72%) groups were recorded as compared those of CON (46.14±0.25 and 45.56±7.49%). Similarly, significantly (P<0.05) higher HOS reactive sperms and sperm acrosomal integrity was noted in MTN (57.99±2.11 and 67.24±1.22%) as compared to MT (54.18±2.12 and 64.92±2.05%) and CON (50.14±0.41and 61.28±0.26%). In addition, abnormality, and enzyme leakage (GPT and GOT) was significantly (P<0.05) declined in MTN (6.29±0.48%, 25.46±0.74 and 127.30±1.86 µM/L) as compared to MT (7.14±0.72%, 27.94±1.04 and 149.30±2.11 µM/L) and CON 8.50±0.11%, 30.14±0.00 and 157.70±0.00 µM/L). Overall, the incorporation of 5 µM melatonin nanoparticles in the semen dilutor significantly improved post-thaw seminal attributes of Barbari buck semen.

Keywords: Antioxidants, Buck, Cryopreservation, Fertility Assessment, Melatonin, Nanoparticles, Semen Quality

Climatic changes in India have spurred increased interest in goat breeding, as goats are adept at enduring heat stress, drought, and other severe circumstances in tropical climates (Danso *et al.* 2024). The cryopreservation is an assisted reproductive technology (ART) that can be utilized alongside artificial insemination (AI) to enhance goat population and productivity (Zou *et al*. 2022). Sperm death is mainly attributed to production of intracellular ice-crystals, osmotic and oxidative stress, as well as various other mechanisms collectively termed as sperm freezing damage (Hai *et al*. 2024). The buck spermatozoa exhibit greater susceptibility to oxidative stress than bull spermatozoa, attributable to variations in lipid composition and the interaction between seminal plasma and egg yolk diluents (Peris-Frau *et al*. 2020).

To retain semen for prolonged periods and cryopreserve spermatozoa, they must be diluted with a protective solution to sustain their fertilizing capability during *in-vitro* storage at subzero temperatures. Notwithstanding the constituents of extenders, the vitality of spermatozoa diminishes following storage at low temperatures (Paulenz *et al*. 2002). Indeed, unsaturated fatty acids of sperm membranes are vulnerable to ROS-induced lipid peroxidation, which in turn compromises sperm attributes (Wang *et al*. 2025). The antioxidant-defense-system of mammalian sperm cells, comprised of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), and reduced glutathione (GSH), mitigates lipid peroxidation-induced damage of mammalian sperm (Sarıkaya and Doğan 2020; Jomova *et al*. 2024). Antioxidants may mitigate apoptosis during spermatogenesis, sperm storage, and transit via the genital canal, as overproduction of reactive oxygen radicals is a principal contributor to sperm DNA damage (Sikka

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2001). Nonetheless, the antioxidant activity in sperm cells may be insufficient to prevent lipid peroxidation during the freeze-thaw process.

The pineal gland synthesizes N-acetyl-5-methoxytryptamine (Melatonin), a neurohormone exhibiting significant antioxidant characteristics, and can serve as an adjunct in cryopreservation of bull spermatozoa (Alam *et al*. 2024[a]). Melatonin can influence spermatozoan function and reproduction in animals (Makris *et al*. 2023; Casao *et al*. 2025). Melatonin and its bioactive compounds can neutralize free radicals, including reactive oxygen species, hence lipid peroxidation inhibition, as well as damage to proteins and DNA (Galano *et al.* 2018). It effectively enhances the capacity of antioxidant enzymes and diminishes peroxidase. Indeed, diminutive-sized nanoparticles, ranging from 10 to 100 nanometres, result in a substantial surface area of the biomaterial interacting with targeting moieties to ensure precise distribution of the drug payload to the target cells (Abdolvahab *et al.* 2024). The extensive variety of biomaterials accessible for nanoparticle formulation presents new opportunities for enhancing interaction with mucosal surfaces through the incorporation of mucoadhesive polymers in the formulation. The encapsulation of medications within the oil phase of nanoemulsions protects them from hydrolysis and oxidation, hence enhancing pharmaceutical stability (Liu *et al*. 2024). Their optimal design facilitates the incorporation of various oils with antioxidant properties or molecules such as melatonin, which possesses significant antioxidant potential.

MATERIALS AND METHODS

The experiment was conducted following ethical approval of the Institutional Animal Ethics Committee (vide reference no. P-3/2025) on six sexually mature, 1.5 to 3.0 year old Barbari bucks at Deep Frozen Semen Laboratory, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj-224229, Ayodhya (UP), India. The experimental area is situated at an elevation of 113 meters above mean sea level at 26°47'N latitude and 82°12'E longitude. The semen was collected using artificial vagina during the hot-dry months of May to June 2025, known as the "hot summer season", with maximum and ambient temperatures averaging 43 °C (109°F) and relative humidity averaging 40.5 %. Throughout the study period, the Temperature Humidity Index (THI) was recorded at 83.6 (Kumar et al. 2018; Tucker et al. 2008) signifying heat stress (West 2003; Upadhyay et al. 2009).

*Preparation of melatonin nanoparticles***:** Aqueous solution was prepared by dissolving 200 mg melatonin (Sigma-Alderich M5250) in a solution containing 50 ml triple distilled water and 50 ml ethyl alcohol (99.99%) in a glass beaker. Polyvinyl alcohol (500 mg) was incorporated into the aqueous melatonin mixture as a surfactant using a magnetic stirrer (stirring at 500 rpm for one hour) to achieve a stable, homogeneous aqueous 100 ml solution. The oil phase was generated utilizing a necessary volume of 10 ml maize oil (Sigma-Alderich C8267). To create a coarse emulsion, melatonin-polyvinyl alcohol and maize oil were combined under magnetic stirring until optimal conditions were achieved (Fayez, *et al*. 2024).

An ultrasonic probe (set to a 1-second on-off pulse interval at 60% amplitude) was used to sonicate the coarse emulsion and optimize nanoemulsion droplet dimensions reduction (Ghosh, *et al.* 2015). Melatonin nanoparticles were isolated by centrifuging the nanoemulsion at 15,000 rpm. Melatonin nanoparticles were subsequently washed to eliminate remaining oil and surfactants. Ultimately, the nanoparticle powder was recovered using lyophilisation.

*Characterisation of melatonin nanoparticles:*The physical and chemical evaluation of melatonin nanoparticles was conducted to assess their biological potential. Nanoparticles were analysed microscopically for characterisation, indexing, and identification using transmission electron microscopy (Thermo Scientific™ Model-Talos L120C G2 TEM)

*Preparation of dilutor:*The glycerolated egg yolk tris dilutor was made according to the procedure outlined by Gangwar et al. (2015) on the day of semen collection. The dilutor was formulated in two components: Part A consists of 3.604 g of Tris buffer (Sigma-Aldrich), 1.902 g of citric acid monohydrate (Merck, USA), 1.00 g of anhydrous fructose (Merck, USA), 6 ml of glycerol, 100,000 units of penicillin G sodium, 100 mg of dihydrostreptomycin sulphate, and glass-distilled water to a final volume of 100 ml. The resulting dilutor exhibited a pH of 6.8-6.9, and the ultimate osmolarity was 410 mOsm/litre. The chosen ejaculates (exhibiting ≥ 70% ocular motility) were diluted in a Tris-Egg-Yolk-Citric acid-Fructose-Glycerol extender. The diluted ejaculates were classified into three groups: one control group (CON) and two treatment groups (MT and MTN).

Group 1 (Control; CON): Semen, neat; tris, citric acid, fructose, yolk (15%), glycerol (6%).

Group 2 (MT): Neat semen + tris - citric acid - fructose - yolk (15%) - glycerol (6%) + 1.0 mM melatonin.

Group 3 (MTN): Neat semen combined with tris, citric acid, fructose, yolk (15%), glycerol (6%), and 5.0 µM melatonin nanoparticles.

A minimum of 12 French mini straws (0.25mL) were filled with PVA powder at room temperature using automatic filling and sealing (ISEVO, IMV, France) for each group. Subsequent to filling and sealing, the straws were promptly positioned on a pre-cooled (50°C) water tray for a four-hour equilibration process in a cold cabinet.

Freezing and thawing of semen straws: The equilibrated semen straws were pre-frozen in a Styrofoam box above 4 cm of liquid nitrogen (LN2) vapor to achieve a temperature of -170°C for 7-8 minutes. Subsequently, these pre-frozen semen straws were promptly transferred to a pre-cooled canister and immersed in an LN2 container for one week prior to further analysis (Evans and Maxwell 1987).

The activities of glutamic oxaloacetic transaminase

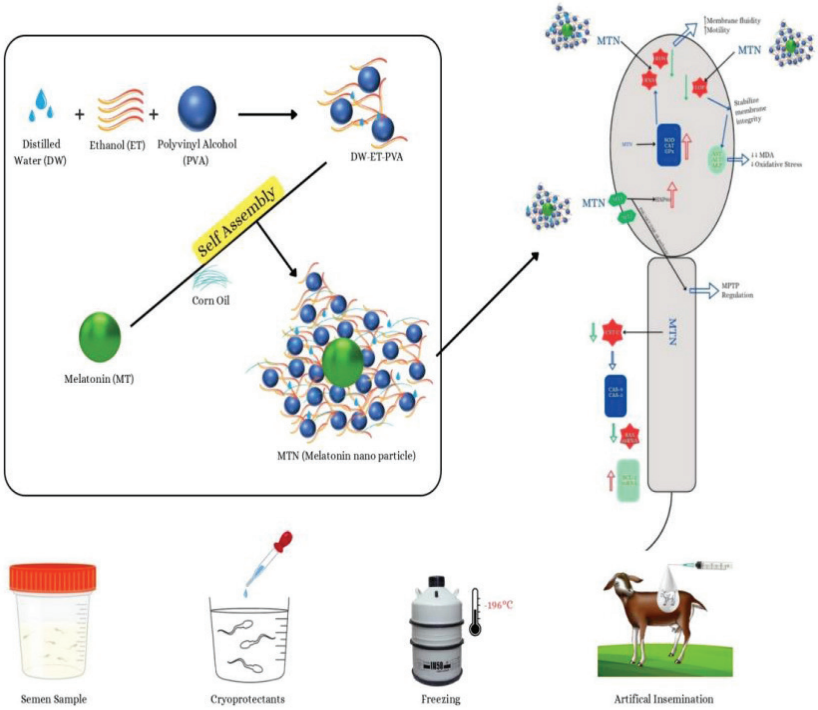

Fig. 1 Schematic representation of development of Melatonin nanoparticles

(GOT) and pyruvic transaminase (GPT) were assessed using BEACONB AUTO 200 serum analyzer and standard commercial kits. To achieve this purpose, seminal plasma was isolated from semen samples using centrifugation at 3000 rpm for 20 minutes. Post-thaw assessment of semen was done following thawing of straw (37°C for 30 s) in a water bath (Sari *et al.* 2024; Deka and Rao 1986).

Evaluation of post-thaw sperm quality: Following 7 days of cryopreservation in liquid nitrogen, frozen semen samples were assessed for physico-morphological seminal characteristics, including mass motility, total concentration, viability, abnormal morphology, structural and functional membrane integrity, and acrosomal integrity immediately after collection, before pooling. The post-thaw motility of semen samples was assessed according to the usual methodology (Galián*et al.* 2023; Hafez and Hafez 2000). The viability of sperm was evaluated using established procedures (Tanga *et al.* 2021). The semen samples were assessed for morphological abnormalities (Milewska 2024). The membrane function was evaluated using HOST (Jeyendran *et al.* 1984; Alam *et al.* 2024[b]). The Trypan Blue-Giemsa staining approach was employed to assess the acrosome integrity of spermatozoa (Sun *et al.* 2021).

*Statistical analysis:*Data were analysed using Graph Pad Prism version-5 data analysis software. The factorial model included the effect of MT and MTN as independent variables and percent post-thawed motility, liveability, abnormalities, acrosomal integrity, structural and functional plasma membrane integrity, and extracellular enzyme leakage as dependent variables. An ANOVA was used to assess differences among the treatment groups. Post hoc Tukey's test was conducted to know the significant difference between MT and MTN at $P < 0.05$.

RESULTS AND DISCUSSION

Based on the Transmission Electron Microscope images (Fig. 2), MTN exhibited a maximum size of 40–82 nm and were spherical in shape. The zeta size analysis indicated that the synthesised melatonin nanoemulsion was 18.2±0.1 nm in size with a zeta potential value of -3.87±0.05 mV (Fig. 3). The synthesized nanoemulsion with elevated zeta potential directly influenced the stability of the colloid in water, stemming from the significant melatonin nanoemulsion bioactivity.

In the present study, the alterations in various seminal attributes, including initial progressive motility, viability, sperm abnormalities, HOS reactivity, and GPT and GOT levels pre-freez and post-thawed in buck semen were noted (Table 1) and exhibited a statistically significant difference ($P<0.05$).

Pre-freeze Assessment: The pre-freeze (PF) values of glutamic-oxaloacetic transaminase (GOT) or aspartate

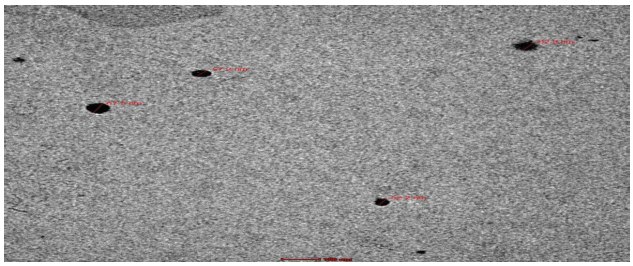

Fig. 2 Characterization of MTN with the help of TEM (40-82nm spherical shape)

Table 1. Effect of MT (1.0mM) and MTN (5.0µM) on seminal attributes of Barbary buck spermatozoa

Stage	Additive Concentration	Sperm motility (%)	Liveability (%)	Morphological Abnormalities	HOS (%)	Sperm Acrosomal Integrity (%)	GPT (µM/L)	GOT (µM/L)
Post diluted	C (T_0)	83.03±0.26^a	88.90±0.29^a	3.82±0.03^a	72.49±0.56^a	85.69±0.2^a	24.41±0.24^a	104.30±0.36^a
	MT 1.0 mM (T_1)	83.29±0.29^a	84.37±0.47^b	3.84±0.04^a	74.79±0.80^{ab}	86.05±0.92^a	22.30±0.32^b	94.15±0.55^b
	MTN 5 µM (T_2)	83.69±0.49^a	85.35±0.47^b	3.88±0.23^a	76.16±1.50^b	87.25±1.27^a	20.66±0.27^c	86.63±0.77^c
Post thaw	C (T_0)	46.14±0.25^a	45.56±7.49^a	8.50±0.11^a	50.14±0.41^a	61.28±0.26^a	30.14±0.00^a	157.70±0.00^a
	MT 1.0 mM (T_1)	55.95±2.6^b	51.84±8.72^b	7.14±0.72^{ab}	54.18±2.12^{ab}	64.92±2.05^{ab}	27.94±1.04^{ab}	149.30±2.11^a
	MTN 5 µM (T_2)	58.56±2.17^b	53.80±8.73^b	6.29±0.48^b	57.99±2.11^b	67.24±1.22^b	25.46±0.74^b	127.30±1.86^b

Means bearing different superscripts (a, b, c) within a column differ significantly (P<0.05) for the respective stage.

aminotransferase (AST) in semen were significantly reduced (P<0.05) in both the MT and MTN than those of CON. Both GPT and ALT were reduced in the MT and MTN groups; however, the changes were not statistically significant in the PF stage (Table 1). The initial motility, abnormality, acrosomal integrity, and GPT did not differ significantly (P<0.05) among CON, MT and MTN (Table 1). The sperm liveability of CON was significantly (P<0.05) higher than that of MT and MTN (Table 1). The HOS reactive sperm were significantly (P<0.05) higher in MTN compared to CON**.** The GOT values differed significantly (P<0.05) among CON, MT, and MTN.

Post-thaw assessment: The post-thaw (PT) motility and liveability did not differ significantly between MT and MTN; however, both values were significantly higher compared to CON (Table 1). The abnormality, acrosomal integrity, and GPT values of MTN was significantly higher than MT and CON moreover, the corresponding value between MT and MTN did not differ significantly (Table 1). The PT MTN group HOS reactivity differed significantly (P<0.05) compared to the CON group, but not to the MT group (Table 1). The PT GOT values were significantly (P<0.05) lower in MTN than those of CON and MT (Table 1). Sperm acrosomal integrity in the PT stage of bucks was 61.28±0.26%, 64.92±2.05%, and 67.24±1.22% in the CON, MT, and MTN groups, respectively, with the MTN group exhibiting a significantly higher (P<0.05) as compared to the CON group but not the MT group.

Semen cryopreservation enables prolonged storage of spermatozoa for artificial insemination in animals. The generation of reactive oxygen radicals like nitric oxide and superoxide radicals is a prevalent occurrence during cryopreservation, which compromises the spermatozoon membrane, resulting in diminished viability and reduced fertilisation potential (Xue *et al.* 2025). To preserve the integrity of sperm cell membranes and optimise extracellular fluid osmolarity during freezing and thawing, cryoprotectants and appropriate freezing methods safeguard sperm from dehydration, high salt concentrations, and thermal shock. Nevertheless, semen extenders and cryoprotectants are insufficient to completely safeguard sperm from the detrimental effects of ultra-rapid freezing, which generally leads to oxidative stress damage due to elevated cytoplasmic ROS levels (Bustani and Baiee 2021). Numerous researchers have previously found that the administration of various antioxidants can mitigate ROS-induced damage during cryopreservation techniques (Cao *et al.* 2022). This study demonstrated that the incorporation of MTN at a concentration of 5µM in sperm cryoprotectants significantly enhanced motility and viability, while also reducing sperm abnormalities, preserving sperm membrane and acrosomal integrity through the scavenging of excess reactive oxygen radicals. In the pre-freeze stage, sperm viability declined compared to fresh semen when treated with 5µM nano melatonin and 1.0 mM melatonin, likely to be due to melatonin's antioxidant properties influencing sperm capacitation and its potential to bind to calmodulin, an enzyme involved in sperm functions (Makris *et al.* 2023). Currently, numerous herbal and physiological antioxidants are receiving increased attention due to their safety profiles (Chen*et al.* 2022; Peng *et al.* 2022; Lu *et al.* 2023; Wang *et al.* 2023; Zhou, D., *et al.* 2023; Alam *et al.* 2024[a]; Alam *et al.* 2024[b]; Xi *et al.* 2025). Melatonin is a recognised and validated potent antioxidant and anti-inflammatory agent that neutralises reactive oxygen radicals and indirectly enhances the production of antioxidant enzymes, namely glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) (Joseph *et al.* 2024). The present observations evidenced that melatonin nanoparticles treated sperm cells exhibited considerably (P<0.05) enhanced motility metrics post-cryopreservation compared to both the melatonin and CON groups. Melatonin exhibits antioxidant properties that are tenfold more effective than traditional antioxidants in neutralising reactive oxygen radicals (Monteiro *et al.* 2024; Tan *et al.* 2015). One of the antioxidant mechanisms of melatonin may mitigate liver fibrosis by reducing ROS-mediated liver inflammation and mitopathy through the activation of Nrf2. (Zhu *et al*. 2023) also indicated that melatonin is correlated with a decline in malondialdehyde (MDA), a persistent byproduct of lipid peroxidation and an indirect indicator of elevated intracellular reactive oxygen species (ROS) formation. Earlier findings also report that melatonin administration leads to a reduction in lipid and peroxidation levels, as well as a decrease in cellular mortality (Aranarochana *et al.* 2021). Its anti-apoptotic

actions, safeguarding the testes from oxidative stress-induced damage, a significant contributor to male infertility and reproductive system dysfunction (Li *et al.* 2022). The lipophilic nature of melatonin facilitates its passage across cellular barriers; yet, its restricted solubility considerably constrains further application. The efficacy of melatonin is constrained due to its low bioavailability. Melatonin nanoparticles (MTN) have been engineered to address this constraint and enhance localised drug delivery (Franco *et al.* 2025). This study utilises melatonin loaded naoparticles as an antioxidant as a semen additive for the cryopreservation of buck semen. The MTN had a uniform distribution and a neutral zeta potential, which may have functioned as a protective layer adhering to the surfaces of sperm membranes during cryopreservation (Xi *et al.* 2025). Post-optimisation, the MTN size values are 60.6±3.5, presumably reflecting the dimensions of the nanoparticles. A negative zeta potential value signifies that the nanoparticles possess a negative charge. Which contributes to the stabilisation of the nanoparticle dispersion by inhibiting aggregation. The zeta potential's magnitude (Fig 3), though not exceedingly high, is adequate to ensure reasonable stability. The tight distribution indicated that the nanoparticles were relatively monodispersed, implying uniform size and consistent surface charge, which underpined the protective function. The prolonged medication release enabled the nanoparticles to maintain their protective properties

Fig. 3 The zeta size analysis indicated that the synthesised melatonin nanoemulsion is 18.2±0.1 nm in size with a zeta potential value of -3.87±0.05 mV

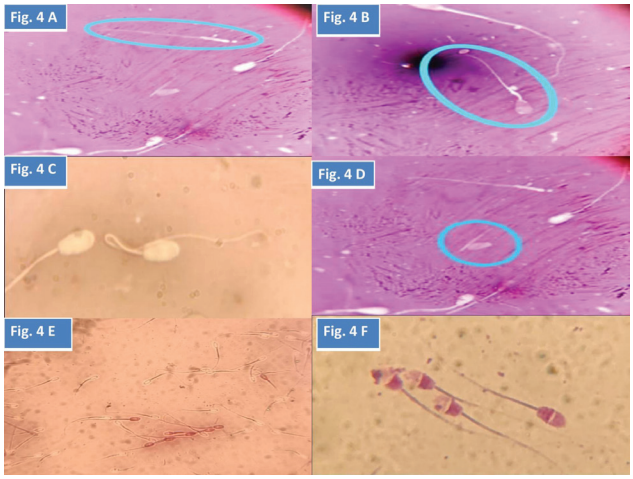

Fig. 4 Morphological assessments of the sperms in relation with abnormalities Small head (Fig 4A), Swollen mid piece (Fig 4B), Coiled tail (Fig 4C), Detached head (Fig 4D), sperm liveability (Fig 4E), and sperm acrosomal integrity (Fig 4F) .

surrounding the sperm. The current investigation employed a concentration of 5 µM MTN to assess its impact on sperm quality measures (Xi *et al.* 2025). MTN at 5 µM significantly protected sperm motility and viability in comparison to melatonin. The sperm DNA fragmentation index functions as a criterion for evaluating sperm quality, fertility, and embryonic development (Yang *et al.* 2025). The 5 µM MTN exhibited the highest protective effect on sperm morphological abnormalities (Fig 4), HOST positive and sperm acrosomal integrity as compared to the CON (Table 1). Conversely, the 1.0 mM melatonin dose did not yield a significant enhancement relative to the CON when compared to MTN for buck spermatozoa. While bull semen cryopreservation MT showed a protective effect against sperm morphological abnormalities, HOS positive, and sperm acrosomal integrity compared to CON (Alam *et al.* 2024[a]). The lipid bilayer of goat buck and bull spermatozoa differs, and these differences might affect their response to treatments like MTN. Melatonin, particularly when delivered through nanoparticles, can enhance sperm quality in both goats and bulls by protecting the membrane of sperm against cryodamage (Akhtarshenas *et al.* 2024). At the pathological level, elevated reactive oxygen species (ROS) can negatively affect the motility of sperm cells andcause DNA damage. Our findings indicated that MTN possesses anti-oxidative stress properties in post-thaw spermatozoa. Consequently, the protective benefits of sperm function were likely associated with the robust ROS scavenging capacity of MTN. The MTN therapy exhibited the largest proportion of viable sperm cells compared to the other groups. Moreover, MTN supplementation markedly enhances motility and fertility. In comparison to the CON and MT groups, the inclusion of MTN in the cryopreservation solution resulted in a markedly enhanced active oxygen scavenging capacity.

In conclusion, the identified cryoprotective effects of MTN on spermatozoa can be ascribed to its capacity to

mitigate ROS in freeze-thaw medium, hence improving viability and motility while reducing oxidative damage. This project involved the development of melatonin nanoparticles via self-assembly to alleviate oxidative stress and improve the quality of cryopreserved buck semen. These nanoparticles demonstrated PVA facilitated homologous targeted adhesion to spermatid cell surfaces, offering rapid protection. The MTN significantly improved sperm motility and viability, and inhibited the formation of reactive oxygen species (ROS). Moreover, MTN significantly enhanced the progressive mobility and motility of thawed spermatozoa. Overall, MTN demonstrates potential as an efficacious method for preserving cryopreserved sperm in buck semen.

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