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Effect of soy lecithin and green tea extract supplementation in Tris-based extender on cryopreserved buffalo bull semen quality

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ABSTRACT

This study aimed to investigate the effect of soy lecithin (SL) or soy lecithin nanoparticles (nano-SL) together with green tea extract (GTE) on the frozen-thawed buffalo bull semen quality through its incorporation in the extended semen before cryopreservation. Semen was collected once weekly for six consecutive weeks from twelve Egyptian buffalo bulls of proven fertility (n= 72 samples), using a bovine artificial vagina. Semen was divided into three aliquots, each was extended with one of three extenders: Extender 1 (20% egg yolk-Tris), Extender 2 (4% SL+ 0.75% GTE-Tris), and Extender 3 (2% nano-SL+0.75% GTE-Tris), before semen processing (cooling, equilibration, and freezing) procedures. The frozen-thawed semen was assessed for motility and kinematics, acrosomal intactness, plasma membrane and DNA integrities, and antioxidant properties. The results showed that extender 2 and 3 considerably (P< 0.0001) improved sperm motility (total and progressive motilities), DNA intactness, and improved the semen antioxidant properties (increase the total antioxidant capacity, superoxide dismutase, catalase, reduced glutathione activities, and decreased malondialdehyde levels). In conclusion, the incorporation of soy lecithin or soy lecithin nanoparticles together with green tea extract in semen extenders improved the frozen-thawed buffalo bull semen quality.

1. INTRODUCTION

Semen cryopreservation is a pre-request for effective management and genetic enhancement of buffaloes (Mittal et al., 2019). However, freezing and thawing of semen are commonly faced with an increased reactive oxygen species (ROS), decreased semen quality, lowered sperm functions, and, as a result, reduced fertility rates (Mittal et al., 2019). As a solution, using extenders with suitable components is encouraged to improve spermatozoa survival and functional statuses after the thawing process (Bucak et al., 2008).

Soybean, an ancient grain from China, known as the "golden bean," contains over 40% protein and about 20% oil, and various phytochemicals e.g., lecithin. Lecithin can be derived from both plant and animal sources and is composed of mixtures of glycerophospholipids (Tewari et al., 2016). Soy lecithin plays a crucial role in semen cryopreservation due to its ability to encapsulate and protect sperm cells during the freezing and thawing processes (Almadaly et al., 2019). It has been recognized that soy lecithin as a phospholipid stabilizes cell membranes and improves the cryopreservation process (Moussa et al., 2002). It is assumed that the conversion of soy lecithin to nano-particles is more beneficial than the raw material. The smaller droplets of nano-soy lecithin have a greater surface area, which enhances the contact between antioxidants, semen-soluble free radicals, and diffusing oxygen (Mousavi et al., 2019).

Green tea, derived from the Camellia sinensis plant, is popular throughout Asia. It is characterized by its leaves, which are rich in bioactive components such as phenolic compounds, alkaloids, and other nutrients (Sharma et al., 2021). Green tea extract (GTE) is well-known for its strong antioxidant qualities owing to its content of polyphenols like epigallocatechin gallate, which can alleviate oxidative stress by lowering protein carbonylation and lipid peroxidation (Hosen et al., 2015). Green tea extract supplementation as a natural antioxidant to cryo-diluent media has promising outcomes in improving the overall quality of the cryopreserved semen (Prastiya et al., 2023).

A combination of lipid-based cryoprotectants with antioxidants can produce superior results than the use of each component alone (Mousavi et al., 2019). According to Mata-Campuzano et al. (2015), it would be advantageous to incorporate several enzymatic and non-enzymatic antioxidants into lecithin-based extenders. Previous studies showed that different cryoprotectants work well on their own (Naz et al., 2018; Almadaly et al., 2019), but little is known about the synergistic effect of soy lecithin nanoparticles and green tea extract (GTE) in improving buffalo freeze semen.

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This study aimed to evaluate the effect of soy lecithin (SL), or soy lecithin nanoparticles (nano-SL), in combination with Green tea extract (GTE) in improving the cryopreservation process as indicated by evaluating buffalo frozen-thawed semen characteristics and antioxidant properties.

2. MATERIAL AND METHODS

Research Ethics

The animal experiments and manipulations conducted in this study were approved by the ethics committee of the Faculty of Veterinary Medicine, Benha University, Egypt (Approval No. BUFVTM 04–02-23).

2.1. Nanoparticles preparation

A thin layer of soybean lecithin (Product No. KJ512547, Karlsruhe, Germany) was prepared and hydrated with 10 mL of sterile phosphate-buffered solution (PBS, pH 7). The solution was vortexed (XH-D Vortex mixer, Wincom Company, Ltd., China) for one hour at 2000 rpm to create a milky suspension. The coarse emulsion was sonicated (Misonix S 4000, QSONICA, USA) at 60% amplitude in an ice bath for 30 min (5 sec On and 10 sec Off) (Mousavi et al., 2019). A zeta analyzer (Microtrac MRB's NANOTRAC Wave II/Q/Zeta, Verder Scientific, UK) based on dynamic light scattering (DLS) was used to evaluate the size and potential of the nanoparticles. The mean size of nanoparticles was 28.09 nm, and their zeta potential was 19.6 mV and negatively charged.

2.2. Green tea extract preparation

The methanol extract of green tea was prepared according to Chan et al. (2007) and Gale et al. (2015) as follows: eight grams of dry, commercial green tea leaves (Product No. 9001, from Ragab El Attar Company, Cairo, Egypt) were ground into a fine powder and soaked in 400 mL of methanol at room temperature (25 °C) for 24 hrs. The mixture was centrifuged at 1500 rpm for 30 min., then the supernatant was filtered through 8~10 µm filter paper and stored at -20 °C until use.

2.3. Semen extenders preparation

The tris buffer (pH 6.90) was prepared from Tris, citric acid, and fructose at concentrations of 2.42 g, 1.48 g, and 1.00 g, respectively, in 100 mL of deionized bi-glass distilled water. Semen extenders were prepared as follows: Extender 1 was prepared from 20% egg yolk (EY) in Tris buffer. Extender 2 was prepared from 4% soy lecithin in Tris buffer and supplemented with 0.75% green tea extract (4% SL+0.75% GTE). Extender 3 was prepared from 2% nano-soy lecithin in Tris buffer and supplemented with 0.75% green tea extract (2% nano-SL+0.75% GTE). Glycerol 7% (v/v) (Sigma-Aldrich), and an antibiotic mixture (500 IU/ml penicillin and 250 µg/ml gentamicin) were added thereafter (Shokry et al., 2024).

2.4. Semen collection and processing

A pilot study was conducted before the experiment, testing various doses of soy lecithin and nano-soy lecithin (1.0-8.0%), alongside green tea extract (0.5-3.0%), to select the most suitable concentration adopted here. Twelve fertile Egyptian buffalo bulls, aged 5 -6 years, kept at the Animal Reproduction Research Institute's farm were used in this study. Semen was collected once weekly for six consecutive weeks (n= 72 samples) during the period between January to March 2024 with a bovine artificial vagina (Neustadt/Aisch Müller, Germany). Semen samples with motility and viability > 75%, and a concentration > 800 × 10^6 sperm/mL were selected for processing. The pooled semen was equally divided into 3 aliquots each was extended with one of the three prepared extenders as follows: Extender 1 (20% egg yolk-Tris), Extender 2 (4% soy lecithin + 0.75% green tea extract-Tris), and Extender 3 (2% nano-soy lecithin + 0.75% green tea extract-Tris). Extended semen was slowly cooled to 5 °C within 2 hrs, packed into 0.25 mL polyvinyl straws (IMV Co., France) (20 × 10^6 sperm/straw), and equilibrated at 5 °C for 4 hrs. After equilibration, semen was manually frozen by its exposure to liquid nitrogen vapor for 10 min after which the straws were plugged in liquid nitrogen at -196 °C until the assessment (Khalifa, 2001). Frozen semen straws (n= 6 straws/ replicate) were thawed at 37 °C for 30 sec prior to different evaluations.

2.5. Sperm quality evaluation

2.5.1. Assessment of sperm motility and kinematics

The sperm motility and kinetics were analyzed using computer-assisted sperm analysis (CASA; CEROS II, version 1.10; Hamilton Thorn Beverly) with a specific setup for buffalo bull semen (Naz et al., 2018). Briefly, 7 µL of frozen-thawed semen was mounted on a CASA slide and evaluated for total and progressive motility, velocity average path (VAP), distance path average (DAP), distance curved line (DCL), straight liner velocity (SLV), straightness (STR), beat cross frequency (BCF), wobble (WOB), linearity (LIN), and curved liner velocity (CLV).

2.5.2. Assessment of acrosomal integrity

The acrosome integrity was assessed using silver nitrate staining according to Chinoy et al. (1992). Briefly, a thin semen film was fixed with 70% then 95% ethyl alcohol for 2 min each, stained with silver nitrate for 2 hrs at 65 °C with 100% humidity, and examined with a phase contrast microscope (Olympus Bx40, Japan) at 100r× magnification.

2.5.3. Assessment of sperm plasma membrane integrity

According to Akhter et al. (2008), the plasma membrane integrity was assessed using the hypo-osmotic swelling test (HOST). Briefly, 100 µL of the frozen-thawed semen was mixed with 400 µL of hypo-osmotic swelling solution (0.735 g of sodium citrate and 1.35 g of fructose dissolved in 100 mL of distilled water, osmolality of 190 mOsmol/L), incubated at 37 °C for 30 min, and examined with a phase contrast microscope (Olympus Bx40, Japan) at 400× magnification. The spermatozoa displayed a swollen head and/or curled tail indicating plasma membrane intactness.

2.5.4. Assessment of sperm DNA integrity

The sperm DNA integrity was assessed by COMET assay according to Fraser (2004) and Boe-Hansen et al. (2005). Frozen-thawed semen was centrifuged at 1500 rpm for 15 min and the sperm pellets were diluted in PBS (1:10). To prepare the slides, 500 µL of 1% normal melting point agarose, heated to 45 °C, were applied to frosted microscopic slides. After the agarose dried, 1 mL of a mixture containing 6 µL of diluted semen and 2.5 mL of low-gelling temperature agarose was spread over each slide. After electrophoresis, the slides were placed on a pre-cooled tray, dried overnight at 5 °C, and rehydrated with distilled water for 10 min. The slides stained with 1% acridine orange were examined with a fluorescence microscope (Olympus, Japan) at 400× magnification. DNA integrity was assessed using an image analysis program (TriTek Comet-Score, software version 1.5) based on DNA integrity (%), tail length (µm), DNA in tail (%), and tail moment.

2.5.5. Assessment of semen antioxidant properties

Commercial diagnostic kits purchased from Bio Diagnostic (Cairo, Egypt) were used for evaluation of oxidative stress and lipid peroxidation markers as follows: total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), glutathione reduced (GSH), and malondialdehyde (MDA) levels (TA 25 13, SD 25 21, CA 25 17, GR 25 11, and MDA 25 29, respectively). Thawed semen was centrifuged, and the harvested seminal plasma was analyzed spectrophotometrically for TAC, SOD, CAT, GSH, and MDA at 505 nm, 560 nm, 405 nm, 510 nm, and 534 nm wavelength, following methods of Koracevic et al. (2001), Campos-Shimada et al. (2020), Bendou et al. (2021), Ognjanović et al. (2008), and Partyka et al. (2012), respectively.

2.6. Statistical analysis

Data were analyzed and presented as mean ± SEM using SPSS software (IBM® SPSS® Statistics Version 25) (Ho, 2013). The Values were tested for normality using the Shapiro-Wilk test. One-way ANOVA and Tukey's HSD post-hoc tests were used to define statistical differences between groups. The statistical significance was set at P< 0.05.

3. RESULTS

3.1. Effect of soy lecithin and nano-soy lecithin with green tea extract in Tris-based extender on sperm motility characteristics, acrosomal intactness, and membrane integrity.

As shown in Table (1), extender 2 (SL+ GTE) and extender 3 (nano-SL+ GTE) significantly (P< 0.0001) improved the sperm total and progressive motility compared to extender 1 (EY). Furthermore, extender 2 markedly (P< 0.01) increased the distance path average (DAP) while the sperm straightness and linearity significantly (P< 0.001) decreased in extender 2 (SL+ GTE) and extender 3 (nano-SL+ GTE) compared to extender 1 (EY). The sperm acrosomal and plasma membrane integrities significantly (P< 0.001) increased in extender 2 (SL+ GTE) and extender 3 (nano-SL+ GTE) compared to extender 1 (EY).

3.2. Effect of soy lecithin and nano-soy lecithin with green tea extract in Tris-based extender on sperm DNA integrity.

The results presented in Table (2) and Figure (1) revealed that both extender 2 (SL+ GTE) and extender 3 (nano-SL+ GTE) significantly (P< 0.0001) improved the sperm DNA integrity compared to extender 1 (EY). Additionally, extender 2 (SL+ GTE) and extender 3 (nano-SL+ GTE) showed a marked (P< 0.0001) reduction in tail length and tail moment compared to extender 1 (EY).

3.3. Effect of soy lecithin and nano-soy lecithin with green tea extract in Tris-based extender on antioxidant and lipid peroxidation indices of frozen-thawed buffalo semen.

As shown in Figure 2, extender 2 (SL+ GTE) and extender 3 (nano-SL+ GTE) significantly (P< 0.0001) increased the levels of TAC, SOD, CAT, and GSH compared to extender 1 (EY). Meanwhile, the level of MDA markedly (P< 0.0001) reduced in extender 2 (SL+ GTE) and extender 3 (nano-SL+ GTE) compared to extender 1 (EY).

4. DISUCCUSION

Former studies highlighted the potential of soy lecithin (SL) as an effective cryoprotectant and green tea (*Camellia sinensis*) extract as a strong antioxidant for the protection of spermatozoa against cryo-damage (Mehdipour et al., 2016). The present study provided valuable insights into the effectiveness of soy lecithin or soy lecithin nanoparticles (nano-SL) with green tea extract (GTE) in enhancing the post-thaw quality of Egyptian buffalo bull semen.

Table 1 Effect of soy lecithin and nano-soy lecithin with green tea extract in Tris-based extender on sperm motility characteristics, acrosomal intactness, and membrane integrity.

Treatments	Abbreviation	Unit	Extender 1 (Egg yolk-Tris)	Extender 2 (4% SL+ 0.75% GTE)	Extender 3 (2% nano-SL+ 0.75% GTE)	P value
Total motility	-	%	46.22 ± 2.07^{b}	73.33 ± 4.00^{a}	80.53 ± 4.60^{a}	0.0001
Progressive motility	-	%	27.63 ± 2.69^{b}	59.82 ± 3.06^{a}	63.39 ± 1.94^{a}	0.0001
Average path velocity	VAP	µm/s	35.10 ± 1.61	40.20 ± 2.74	35.51 ± 1.24	0.13
Distance path average	DAP	µm	15.16 ± 0.33^{b}	17.12 ± 0.53^{a}	15.84 ± 0.14^{ab}	0.01
Distance curved line	DCL	µm	24.87 ± 1.43	28.75 ± 1.40	26.39 ± 1.09	0.11
Straight-line velocity	VSL	µm/s	25.93± 1.38	25.40 ± 1.38	22.87 ± 0.10	0.14
Straightness ratio	STR	%	0.77 ± 0.35^{a}	0.63 ± 0.11^{b}	0.64 ± 0.01^{b}	0.001
Beat cross frequency	BCF	Hz	20.56 ± 1.80	21.53 ± 1.00	23.51 ± 0.48	0.53
Wobble	WOB	%	0.62 ± 0.04	0.65 ± 0.03	0.72 ± 0.03	0.07
Linearity	LIN	%	0.47 ± 0.01^{a}	0.37 ± 0.02^{b}	0.38 ± 0.01^{b}	0.001
Curvilinear velocity	VCL	µm/s	57.15 ± 3.60	67.17 ± 5.74	59.07 ± 2.72	0.20
Distance straight Line	DSL	µm	11.56 ± 0.46	10.78 ± 0.16	10.78 ± 0.25	0.24
Acrosomal integrity	-	%	78.33± 1.50^{b}	85.00 ± 2.01^{a}	86.67 ± 1.50^{a}	0.001
Plasma membrane integrity	-	%	37.33 ± 2.02^{b}	59.33 ± 2.33^{a}	56.5 ± 2.64^{a}	0.0001

SL = soy lecithin, GTE = green tea extract. Values (Mean ± SEM) with different superscript letters within the same row were statistically different.

Table 2 Effect of soy lecithin and nano-soy lecithin with green tea extract in Tris-based extender on DNA integrity.

Treatments	Extender 1 (Egg yolk-Tris)	Extender 2 (4% SL+ 0.75% GTE)	Extender 3 (2% Nano-SL+ 0.75% GTE)	P value
DNA integrity (%)	80.51± 0.75^{a}	90.89 ± 0.78^{b}	91.98 ± 0.99^{b}	0.0001
Tail length (µm)	15.69 ± 0.40^{a}	7.50 ± 1.04^{b}	6.95 ± 1.10^{b}	0.0001
DNA in tail (%)	12.73 ± 1.34	9.50± 0.44	10.51 ± 0.69	0.07
Tail moment	2.24 ± 0.15^{a}	0.63 ± 0.08^{b}	0.50 ± 0.07^{b}	0.0001

SL = soy lecithin, GTE = green tea extract. Values (Mean ± SEM) with different superscript letters within the same row were statistically different.

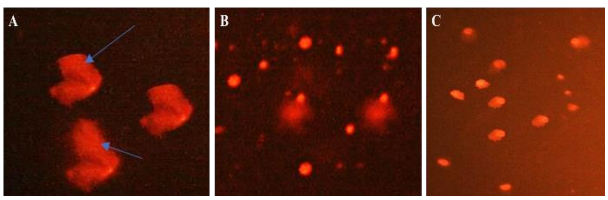

Figure 1 Comet assay of buffalo spermatozoa extended using extender 1 (egg yolk-Tris (A)), extender 2 (4% soy lecithin + 0.75% green tea extract (B)), and extender 3 (2% nano-soy lecithin + 0.75% green tea extract (C)). The comet's tail is indicated by blue arrows.

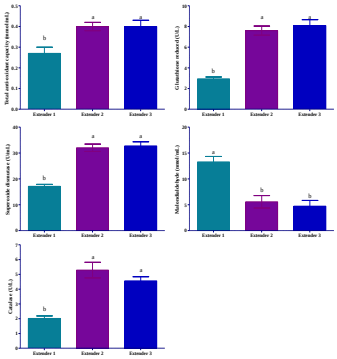

Figure 2 Changes in antioxidant and lipid peroxidation indices in frozen-thawed buffalo semen extended with egg yolk-Tris (extender 1), 4% soy lecithin plus 0.75% green tea extract (extender 2), and 2% nano-soy lecithin plus 0.75% green tea extract (extender 3). Data were presented as (mean ± SEM). columns with different letters indicated significant differences (P< 0.05).

The present results revealed that the combination of SL or nano-SL with GTE resulted in superior sperm motility, acrosomal, plasma membrane, and DNA integrities, and improved antioxidant defense mechanisms. Soybean lecithin has been used instead of egg yolk for bulls (Akhter et al., 2012; Bader et al., 2012), ram (Üstüner et al., 2014), and goat (Salmani et al., 2014) in the cryo-diluent media. Besides, using soy lecithin as nanoparticles has enhanced sperm motility and viability in Holstein bulls (Mousavi et al., 2019). It has been recorded that the combination of nano-lecithin extender with antioxidants resulted in improved post-thaw sperm quality in bull semen (Mousavi et al. (2019). Soy lecithin contains phospholipids; hence, it creates a protective barrier surrounding sperm cell membranes to stabilize them during the freeze-thaw process (Gunawan et al., 2020). Additionally, SL affects membrane permeability and allows the entrance of glycerol to protect against osmotic shock and sperm cell rupture by ice crystals (Röpke et al., 2011). The reduction in particle size may amplify these benefits because of the larger surface area to volume ratio, which may potentially affect lecithin's beneficial impacts on spermatozoa (Nadri et al., 2019). Additionally, the negatively charged lipid is transferred when charged donor particles are included; therefore, the negative charge of nano-lecithin may also augment these effects (Richens et al., 2017). Furthermore, the lecithin impact may be amplified by the characteristics of nanoparticles, which include high absorption, reactivity, and active surface area (Zandiyeh et al., 2024).

Green tea extract is well-known for its potent antioxidant properties, mainly due to its high content of polyphenols, particularly epigallocatechin gallate (Hosen et al., 2015). We assumed that this protective effect is further enhanced by the nano-SL which could encapsulate and transport natural bioactive chemicals, hence enhancing GTE stability and bioavailability (Pachuau et al., 2021).

One of the most noteworthy outcomes was the superior performance of extender 2 (SL+ GTE) and extender 3 (nano-SL+ GTE), which yielded the highest post-thaw total and progressive motilities and DAP than extender 1 (EY). These findings matched those of Mehdipour et al. (2016), who showed that a lecithin-based extender with GTE significantly enhanced the cryopreservation of ram spermatozoa with improvements in motility. This may be because the SL increases ATP synthesis, that is essential for the energy-dependent processes involved in sperm movement, including flagellar motion and membrane function (Adami et al., 2020), and reduces oxidative stress alongside the antioxidant effect of GTE, which neutralizes free radicals (Motlagh et al., 2014; Ahmed et al., 2020b).

Acrosome intactness is crucial for effective fertilization (Ahmed et al., 2016). The high sperm acrosomal intactness shown in our study indicated the protective advantages of supplementing extender 2 (SL+GTE) and extender 3 (nano-SL+GTE) in the semen. Our observation is in line with those of Toker et al. (2016) and Alhelal and Abdulkareem (2023). The successful integration mechanism of SL or nano-SL with GTE could be attributed to the phosphatidylcholine of SL, which has a protective effect on acrosomes (Simpson et al., 1986). Furthermore, the antioxidant effects of GTE are excreted by flavonoids and polyphenol contents, which decrease oxidative stress and maintain the structure and function of sperm cells (Roychoudhury et al., 2017).

The present study demonstrated the ability of extender 2 (SL+ GTE) and extender 3 (nano SL+ GTE) to improve sperm cell membrane intactness, which aligns with the findings of Mousavi et al. (2019) in bull semen and Sharafi et al. (2015) and Mehdipour et al. (2016) in ram semen. Due to SL biocompatibility and capacity to mimic the structure of biological membranes, forming a protective layer minimizes membrane rupture and leakage (Le et al., 2019). Hence, SL and its nanoparticles have gained attention for usage as cryoprotectants. Additionally, epigallocatechin gallate of GTE lowers oxidative stress and scavenges free radicals, both of which are critical in avoiding damage to sperm membranes during cryopreservation (Rahman et al., 2018; Al-Mutary, 2021).

DNA integrity controls spermatozoa fertilization potential and embryo development (Mousavi et al., 2019). The hypothesis that these two extenders provide better protection against cryo-induced damage is supported by the COMET assay results, which showed that the DNA integrity, tail length, and tail moment were empowered with extenders 2 (SL+ GTE) and 3 (nano- SL+ GTE). Similarly, fortification of soybean-lecithin extenders with antioxidant amino acids in rams (Toker et al., 2016) and with resveratrol antioxidants in buffalo bulls (Alhelal and Abdulkareem, 2023) significantly decreased DNA damage. This is consistent with lecithin's protective role in maintaining sperm membranes and the antioxidative properties of GTE, which reduce oxidative damage and prevent sperm DNA fragmentation during cryopreservation (Al-Mutary, 2021).

Recent studies highlighted the effectiveness of SL (Monakhova and Diehl, 2018) and GTE (Ahmed et al., 2020 a,b) in increasing the antioxidant power of frozen semen. The observed increases in TAC, the activity of SOD, CAT, and GSH, in addition to reduced MDA levels, indicated an improved enzymatic defense against oxidative stress. These findings are in agreement with results performed on bull semen extended with nano-SL and enzymatic antioxidants (Mousavi et al., 2019), and on ram semen supplemented with SL and GTE (Mehdipour et al., 2016). According to Aguirre and Borneo (2019), SL enhances the stability and transport of GTE, boosting its antioxidant activity. Moreover, the phosphatidylcholine antioxidant properties of SL (Monakhova and Diehl, 2018) were predicted to be increased by nano preparation of SL in addition to the GTE's polyphenols protective effect against lipid peroxidation (Hosen et al., 2015).

5. CONCLUSIONS

The substitution of egg yolk with SL (4%) or nano-SL (2%) fortified with GTE (0.75%) not only improved post-thawing semen characteristics (sperm motility, acrosomal, plasma membrane, and DNA integrities) but also enhanced the antioxidant defenses.

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