Benha Veterinary Medical Journal 47 (2024) 52-57

Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

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

Asmaa S. Ghaniaa[1]*, Alaa E. Abdel-Ghaffara[1,2], Gamal A Sosaa[1], Magdy R Badrc[3], Mohsen A. Agaga[1], Mohamed M. M. Kandiel[1]

[1]*Theriogenology Department, Faculty of Veterinary Medicine, Benha University, Benha, Egypt.*
[2]*Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine, Delta University for Science and Technology, Gamasa, Dakahlia, Egypt.*
[3]*Department of Artificial Insemination and Embryo Transfer, Animal Reproduction Research Institute (ARRI), Agriculture Research Center (ARC), 12556 Haram, Giza, Egypt.*

ARTICLE INFO

Keywords

Antioxidant markers

Buffalo bull

Frozen semen

Green tea extract

Soy lecithin nanoparticles

Received 20/11/2024
Accepted 03/12/2024
Available On-Line 31/12/2024

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 3 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 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 the extenders 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 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, the use of extenders with suitable components is encouraged to improve the survival and functional status of spermatozoa after the thawing process (Bucak et al., 2008).

Soybeans, an ancient grain from China known as the "golden bean," contain over 40% protein and about 20% oil involving 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 the phospholipid of soy lecithin stabilizes cell membranes and improves the cryopreservation process (Moussa et al., 2002). Using soy lecithin as nanoparticles has been related to the enhancement of sperm motility and viability in bulls (Mousavi et al., 2019). Additionally, 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.

* Correspondence to: asmaasalah854@gmail.com

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

2. MATERAL 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 minutes (5 seconds On, 10 seconds 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., 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 distilled water, glycerol 7% (v/v) (Sigma-Aldrich), 500 IU/ml penicillin and 250 µg/ml gentamicin (Shokry et al., 2024). 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).

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 per each 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 hours, 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 the liquid nitrogen vapors for 10 min after which the straws were plugged in liquid nitrogen at -196 °C until the assessment (Khalifa, 2001). Frozen semen straws were thawed (n= 6 straws/ replicate) at 37 °C for 30 sec for the 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 setup specific 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 minutes 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 100× magnification.

2.5.3. Assessment of sperm plasma membrane integrity

According to Chan et al. (1991), the plasma membrane integrity was assessed using the hypo-osmotic swelling test (HOST). Briefly, 100 µL of the frozen-thawed semen was mixed with 500 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 minutes, and examined under a phase contrast microscope (Olympus Bx40, Japan) at 400× magnification. The spermatozoa displayed a swollen head and/or curled tail indicated 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 minutes. The slides stained with 1% acridine orange were examined under 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), reduced glutathione (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 test 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 integrities.

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).

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

Treatments	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

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

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 also 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).

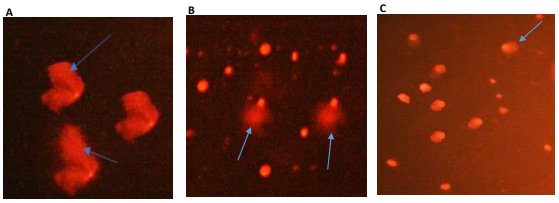


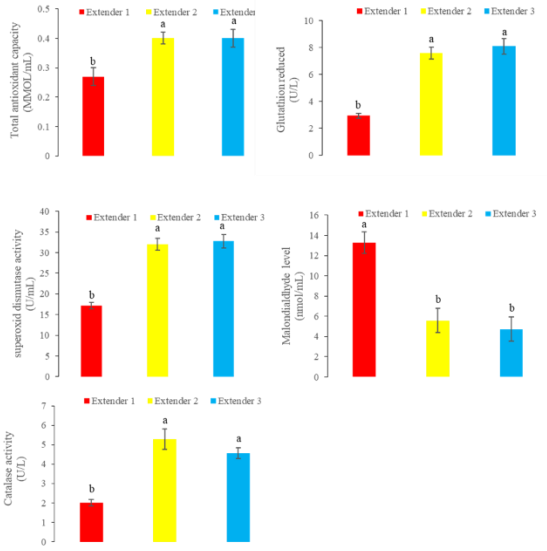
Table 2. Effect of soy lecithin and nano-soy lecithin with green tea extract in Tris-based extender on DNA integrity.

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.

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. DISCUSSION

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.

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. 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 potentiate lecithin's beneficial effects 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, which 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 a cryoprotectant. 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 is a critical factor in determining fertilization potential and embryo viability (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 the 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.

6. REFERENCES

1. Adami, L. N. G., Lima, B. T. de, Andretta, R. R., Bertolla, R. P. and Nichi, M., 2020. Carnosine treatment during human semen processing by discontinuous density gradient. Andrologia 52 (2), e13497.
2. Aguirre, A. and Borneo, R., 2019. Improving bioavailability of polyphenols using nanodelivery systems based on food polymers, In, Ronald, R. W (eds.), Polyphenols in plants. Elsevier, Pp. 59–65.
3. Ahmed, H., Andrabi, S. M. H. and Jahan, S., 2016. Semen quality parameters as fertility predictors of water buffalo bull spermatozoa during low breeding season. Theriogenology 86 (6), 1516- 1522.
4. Ahmed, H., Jahan, S., Khan, A., Khan, L., Khan, B. T., Ullah, H., Riaz, M. and Ullah, K., 2020a. Supplementation of green tea extract (GTE) in extender improves structural and functional characteristics, total antioxidant capacity and in vivo fertility of buffalo (*Bubalus Bubalis*) Bull Spermatozoa. Theriogenology 145, 190–197.

5. Ahmed, H., Jahan, S., Riaz, M., Khan, B. T. and Ijaz, M. U., 2020b. Epigallocatechin-3-Gallate (EGCG) addition as an antioxidant in a cryo-diluent media improves microscopic parameters, and fertility potential, and alleviates oxidative stress parameters of buffalo spermatozoa. Cryobiology 97, 101–109.
6. Akhter, S., Ansari, M. S., Andrabi, S. M. H., Rakha, B. A., Ullah, N. and Khalid, M., 2012. Soya-lecithin in extender improves the freezability and fertility of buffalo (*Bubalus Bubalis*) Bull spermatozoa. Reproduction in Domestic Animals 47 (5), 815–819.
7. Almadaly, E.A., Tawfik, F.S., El-Kon, I.I., Heleil, B.A. and M Fattouh, E.S., 2019. Effect of different cryoprotectants on the post-thaw sperm characteristics and in vivo fertility of buffalo (*Bubalus bubalis*) bull semen. Slovenian Veterinary Research/Slovenski Veterinarski Zbornik, 56 (22-Suppl)., 541-551.https://doi.org/10.26873/SVR-792-2019
8. Al-Mutary, M. G., 2021. Use of antioxidants to augment semen efficiency during liquid storage and cryopreservation in livestock animals: a review. Journal of King Saud University-Science 33 (1), 101226.https://doi.org/10.1016/j.jksus.2020.10.023
9. Alhelal, A. M. and Abdulkareem, T. A., 2023. Effect of Adding Resveratrol to Soybean-Lecithin Extender on Some Semen Attributes of Buffalo Bulls. Iraqi Journal of Agricultural Sciences 54 (4), 1074–1083.
10. Badr, M.A., Mary, G., Abd EL-Malak, Mohammed, K.M. and Ebtihal A. Ibrahim., 2012. Effect of soybean lecithin on freezability and fertilizing potentials of bovine spermatozoa. Assiut Veterinary Medical Journal, 58(133), 1-10.
11. Bendou, O., Gutiérrez-Fernández, I., Marcos-Barbero, E. L., Bueno-Ramos, N., González-Hernández, A. I., Morcuende, R. and Arellano, J. B., 2021. Theoretical and experimental considerations for a rapid and high throughput measurement of catalase in vitro. Antioxidants 11 (1), 21. https://doi.org/10.3390/antiox11010021
12. Boe-Hansen, G. B., Morris, I. D., Ersbøll, A. K., Greve, T. and Christensen, P., 2005. DNA integrity in sexed bull sperm assessed by neutral comet assay and sperm chromatin structure assay. Theriogenology 63 (6), 1789–1802.
13. Bucak, M. N., Ateşşahin, A. and Yüce, A., 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. Small ruminant research 75 (2–3), 128–134.
14. Campos-Shimada, L. B., Hideo Gilglioni, E., Fernandes Garcia, R., Rizato Martins-Maciel, E., Luiza Ishii-Iwamoto, E. and Luzia Salgueiro-Pagadigorria, C., 2020. Superoxide dismutase: a review and a modified protocol for activities measurements in rat livers. Archives of Physiology and Biochemistry 126 (4), 292–299.
15. Chan, P. J., Tredway, D. R., Corselli, J., Pang, S. and Su, B.C., 1991. Combined supravital staining and hypoosmotic swelling. Human Reproduction 6 (8), 1115-1118.
16. Chan, E.W.C., Lim, Y.Y. and Chew, Y.L., 2007. Antioxidant activity of camellia sinensis leaves and tea from a lowland plantation in Malaysia. Food Chemistry 102, 1214–1222
17. Chinoy, N. J., Ranga, G. M., Highland, H. N., D., Souza, K. J. and Sequeira, E., 1992. A modified method for the differential staining of spermatozoa using alcoholic acidic silver nitrate. International Journal of Fertility 37 (4), 232–236.
18. Fraser, L., 2004. Structural damage to nuclear DNA in mammalian spermatozoa: its evaluation techniques and relationship with male infertility. Polish Journal of Veterinary Sciences 7 (4), 311–321.
19. Gale, I., Gil, L., Malo, C., González, N. and Martínez, F., 2015. Effect of *Camellia sinensis* supplementation and increasing holding time on quality of cryopreserved boar semen. Andrologia 47, 505–512.
20. Gunawan, M., Kaiin, E.M., Mudita, G.S. and Chaidir, R.R.A., 2020. Soybean phospholipids-based extender as an alternative for bull sperm cryopreservation. Earth and Environmental Science 478, 012014. 10.1088/1755-1315/478/1/012014
21. Hosen, M. B., Islam, M. R., Begum, F., Kabir, Y. and Howlader, M. Z. H., 2015. Oxidative stress induced sperm DNA damage, a possible reason for male infertility. Iranian Journal of Reproductive Medicine 13 (9). 525-532.
22. Khalifa, T. A. A., 2001. Effect of some antioxidants on viability of preserved buffalo and ram semen. Ph. D. Thesis, Theriogenology Dept., Faculty of Veterinary Medicine. Cairo University, Egypt.
23. Koracevic, D., Koracevic, G., Djordjevic, V., Andrejevic, S. and Cosic, V., 2001. method for the measurement of antioxidant activity in human fluids. Journal of Clinical Pathology 54 (5), 356–361.
24. Le, N. T. T., Cao, V. Du, Nguyen, T. N. Q., Le, T. T. H., Tran, T. T. and Hoang Thi, T. T., 2019. Soy Lecithin-derived liposomal delivery systems: surface modification and current applications. International Journal of Molecular Sciences 20 (19), 4706. https://doi.org/10.3390/ijms20194706
25. Mata-Campuzano, M., Soleilhavoup, C., Tsikis, G., Martinez-Pastor, F., Graaf, S. P. De and Druart, X., 2015. Motility of liquid stored ram spermatozoa is altered by dilution rate independent of seminal plasma concentration. Animal Reproduction Science 162, 31–36.
26. Mehdipour, M., Kia, H. D., Najafi, A., Dodaran, H. V. and García-Álvarez, O., 2016. Effect of green tea (*camellia sinensis*) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. Cryobiology 73 (3), 297–303.
27. Mittal, P.K., Madan, A.K., Sharma, V., Gottam, G.S. and Gupta, B., 2019. Cryopreservation of buffalo bull semen-restriction and expectation: A Review. International Journal of Current Microbiology and Applied Science 8(1), 1351-1368.
28. Monakhova, Y. B. and Diehl, B. W. K., 2018. Automated multicomponent phospholipid analysis using 31p nmr spectroscopy: example of vegetable lecithin and krill oil. Analytical and Bioanalytical Chemistry 410 (30), 7891–7900.
29. Motlagh, M. K., Sharafi, M., Zhandi, M., Mohammadi-Sangcheshmeh, A., Shakeri, M., Soleimani, M. and Zeinoaldini, S., 2014. Antioxidant effect of rosemary (*Rosmarinus Officinalis L.*) extract in soybean lecithin-based semen extender following freeze–thawing process of ram sperm. Cryobiology 69 (2), 217–222.
30. Mousavi, S. M., Towhidi, A., Zhandi, M., Amoabediny, G., Mohammadi-Sangcheshmeh, A., Sharafi, M. and Hussaini, S. M. H., 2019. Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. Animal Reproduction Science 209. 106171. https://doi.org/10.1016/j.anireprosci.2019.106171
31. Moussa, M., Martinet, V., Trimeche, A., Tainturier, D. and Anton, M., 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen–thawed bull semen. Theriogenology 57 (6), 1695–1706.
32. Nadri, T., Towhidi, A., Zeinoaldini, S., Martínez-Pastor, F., Mousavi, M., Noei, R., Tar, M. and Sangcheshmeh, A. M., 2019. Lecithin nanoparticles enhance the cryosurvival of caprine sperm. Theriogenology 133, 38–44.
33. Naz, S., Umair, M. and Iqbal, S., 2018. Comparison of tris egg yolk-based, triladyl® and optixell® extender on post-thaw quality, kinematics and in vivo fertility of nili ravi buffalo (*Bubalus Bubalis*) Bull Spermatozoa. Andrologia 50 (8), e13063.
34. Ognjanović, B. I., Marković, S. D., Pavlović, S. Z., Žikić, R. V and Štajn, A. Š., 2008. Effect of chronic cadmium exposure on antioxidant defense system in some tissues of rats: protective effect of selenium. Physiological Research 57 (3), 403-411.
35. Pachuau, L., Laldinchhana, Roy, P. K., Zothantluanga, J. H., Ray, S. and Das, S., 2021. 'Encapsulation of bioactive compound and its therapeutic potential. Bioactive Natural Products for Pharmaceutical Applications 140, 687–714.
36. Partyka, A., Łukaszewicz, E. and Niżański, W., 2012. Lipid peroxidation and antioxidant enzymes activity in avian semen. Animal Reproduction Science 134 (3–4), 184–190.
37. Prastiya, R. A., Suprayogi, T. W., Debora, A. E., Wijayanti, A., Amalia, A., Sulistyowati, D. and Nugroho, A. P., 2023. Green tea extract addition into a tris-based egg yolk extender improves bali bull sperm quality. Animal Bioscience 36 (2). 209-217.
38. Rahman, M. J., Ambigaipalan, P. and Shahidi, F., 2018. Biological activities of camelina and sophia seeds phenolics: inhibition of LDL oxidation, DNA damage, and pancreatic lipase and α-glucosidase activities. Journal of Food Science 83

(1), 237–245.
39. Richens, J. L., Tyler, A. I. I., Barriga, H. M. G., Bramble, J. P., Law, R. V, Brooks, N. J., Seddon, J. M., Ces, O. and O'Shea, P., 2017. Spontaneous charged lipid transfer between lipid vesicles. Scientific Reports 7 (1), 12606-12611.
40. Röpke, T., Oldenhof, H., Leiding, C., Sieme, H., Bollwein, H. and Wolkers, W. F., 2011. liposomes for cryopreservation of bovine sperm. Theriogenology 76 (8), 1465–1472.
41. Roychoudhury, S., Agarwal, A., Virk, G. and Cho, C.-L., 2017. Potential role of green tea catechins in the management of oxidative stress-associated infertility. Reproductive Biomedicine Online 34 (5), 487–498.
42. Salmani, H., Towhidi, A., Zhandi, M., Bahreini, M. and Sharafi, M., 2014. In vitro assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. Cryobiology 68 (2), 276–280.
43. Sharafi, M., Zhandi, M. and Akbari Sharif, A., 2015. Supplementation of soybean lecithin-based semen extender by antioxidants: complementary flowcytometric study on post-thawed ram spermatozoa'. Cell and Tissue Banking 16. 261–269.
44. Sharma, R., Verma, S., and Kumar, D., 2021. Polyphenolics and therapeutic insights in different tissues extract and fractions of Camellia sinensis (L.) Kuntze (Kangra Tea). Food Bioscience. 42, 101164.https://doi.org/10.1016/j.fbio.2021.101164
45. Shokry, D. M., Badr, M. R., Sakr, A.-A. M., Elmesiry, A. M., Assy, M. M., Rawash, Z. and Abd Eldaim, M. A., 2024. Enhancement potential of *Moringa Oleifera* leaves extract on buffalo bull cryopreserved semen quality and fertilization capacity. Animal Reproduction Science 262. 107414.https://doi.org/10.1016/j.anireprosci.2024.107414.
46. Simpson, A. M., Swan, M. A. and White, I. G., 1986. Action of phosphatidylcholine in protecting ram sperm from cold shock. Gamete Research 15 (1), 43–56.
47. Tewari, S., Arora, N.K., and Miransari, M., 2016. Plant growth promoting rhizobacteria to alleviate soybean growth under abiotic and biotic stresses, in: Abiotic and Biotic Stresses in Soybean Production. Elsevier, pp. 131–155. https://doi.org/10.1016/B978-0-12-801536-0.00006-2
48. Toker, M. B., Alcay, S., Gokce, E. and Ustuner, B., 2016. Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. Cryobiology 72 (3), 205–209.
49. Üstüner, B., Alçay, S., Nur, Z., Sağırkaya, H. and Soylu, M. K. 2014. Effect of egg yolk and soybean lecithin on tris-based extender in post-thaw ram semen quality and in vitro fertility. Kafkas Üniversitesi Veteriner Fakültesi Dergisi 20 (3), 393–398.
50. Zandiyeh, S., Kalantari, H., Fakhri, A., Nikkhah, M., Janani, B. J. and Sabbaghian, M., 2024. A review of recent developments in the application of nanostructures for sperm cryopreservation. Cryobiology 115, 104890. https://doi.org/10.1016/j.cryobiol.2024.104890