Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.111

RESEARCH ARTICLE

Effect of Spirulina Nanoparticles or Selenium-Coated Spirulina Nanoparticles Supplemented to Freezing Extender on Bull Sperm Freezability

Mahmoud A. E. Hassan[1]†, Ahmed. M. Shehabeldin[1]†, Mohamed. E. A. Omar[1], Wael A. Khalil[2]*, Ayman A. Swelum[3]*, Yangqing Lu[4], and Sameh A. Abdelnour[5]

[1]Animal Production Research Institute, Agriculture Research Centre, Ministry of Agriculture, Dokki, Giza 12619, Egypt.
[2]Department of Animal Production, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt.
[3]Department of Animal Production, College of Food and Agriculture Science, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia.
[4]State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Animal Science and Technology, Guangxi University, Nanning, 530004, China.
[5]Department of Animal Production, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt.
*Corresponding author: W.A.K.: w-khalil@mans.edu.eg, A.A.S.; aswelum@ksu.edu.sa
†These authors contributed equally to this work

ARTICLE HISTORY (23-412)

Received: September 12, 2023
Revised: December 22, 2023
Accepted: December 24, 2023
Published online: December 28, 2023

Keywords:
Bull
Cryopreservation
Nanoparticles
Selenium
Semen
Sperm quality
Spirulina platensis

ABSTRACT

Cryopreservation has adverse effects on sperm function and structure, while nanotechnology can be used to optimize this process. Thereafter, this research aimed to investigate the effects of adding *spirulina platensis* nanoparticles (SPNP) or selenium-coated spirulina nanoparticles (SPSE) to a freezing extender on the quality of post-thawed bull sperm and anti-oxidant-related markers. Five healthy proven-fertile bulls were used for semen collection using an artificial vagina. The semen samples were divided into three groups: the first one was extended without supplementation (CON), the second group was supplemented with spirulina nanoparticles (SPNP; 10 µg/mL), and the third group was supplemented with selenium (0.5 µg/mL) coated spirulina nanoparticles (SPSE; 10 µg/mL). Compared to the CON group, the supplemented groups showed significant improvements in the percentages of progressive motility, livability, and plasma membrane integrity ($P<0.05$). Additionally, the percentages of sperm abnormalities and chromatin damage were significantly decreased in the supplemented groups. The SPSE group had the highest activity of total antioxidant capacity ($P<0.001$). All supplemented groups significantly improved superoxide dismutase ($P<0.001$), the viable sperm, but significantly reduced the malondialdehyde levels and the apoptotic sperm. The addition of SPNP or SPSE sustained the sperm bull ultrastructure (plasma membrane, acrosome integrity, and normal mitochondria). In conclusion, the results indicate that adding SPNP or SPSE to the freezing extender significantly improves the quality of post-thawed bull sperm by enhancing antioxidant biomarkers, reducing lipid peroxidation, and maintaining sperm ultrastructure.

To Cite This Article: Hassan MAE, Shehabeldin AM, Omar MEA, Khalil WA, Swelum AA, Lu Y and Abdelnour SA, 2023. Effect of spirulina nanoparticles or selenium coated spirulina nanoparticles supplemented to freezing extender on bull sperm freezability. Pak Vet J, 43(4): 739-747. http://dx.doi.org/10.29261/pakvetj/2023.111

INTRODUCTION

The use of frozen-thawed sperm for artificial insemination is a vital technology in the dairy industry (Tippenhauer *et al*., 2023), and can be used to preserve the genetic material of valuable animals or endangered species (Lafontaine *et al.,* 2023). The cryopreservation process has been found to negatively affect both the functionality and structure of sperm affecting fertilizing capacity (Abdelnour e*t al.,* 2020). The cryopreservation can stimulate the production of OS (oxidative stress) and decrease the antioxidant capacity in sperm (Abdelnour *et al*., 2022), resulting in mitochondrial dysfunction of spermatozoa (Hassan *et al.,* 2022), consequently decreasing sperm survival and fertilizing ability after thawing. For many decades, scientists have used various types of antioxidants, such as minerals, phytochemicals, herbal extracts, amino acids, and many others, to protect

the functional and structure of sperm from the negative impacts of the cryopreservation process (Hassan *et al.*, 2022; Kandeel *et al*., 2022; Maleki *et al.,* 2023).

In the last decade, the application of nanotechnology has been applied rapidly in various scopes including biomedical, biological, and pharmaceutical uses *(Khan et al*., 2022**;** Barroso *et al.*, 2023). Nano minerals are new types of nanotechnology that present many advantages like their superior solubility, more sustainability, and better bioavailability (Abdelnour *et al.,* 2020; Hassan *et al.*, 2022). according to many previous trials, (Mehdipour *et al.,* 2017; Abdelnour *et al.,* 2023), extenders enriching with nanoparticles can enhance the sperm freezability by enhancing sperm function and supporting the fertility capacity. Selenium (Se) is a crucial element in maintaining reproductive aspects and has a significant ability to improve sperm quality and reproductive features in various animals (Ali *et al.,* 2023) and humans (Pieczyńska and Grajeta, 2015). Selenium nanoparticles (SeNPs) have recently gained popularity as dietary supplements due to their antioxidant, antimicrobial, and anti-inflammatory impacts. Additionally, previous trials have shown that adding SeNP to the freezing extenders of various animals such as roosters (Safa *et al.,* 2016), bulls (Li *et al.,* 2023), goats (Abedin *et al.,* 2023), and fish (Zhu *et al.*, 2023) exhibited significant improvements in sperm quality, function, and fertility consequences.

The improvement of sperm quality and fertility by SeNPs may be associated with its ability to reduce OS synthesis and promote the antioxidant capacity (Safa *et al.,* 2016), through activating AMP-activated protein kinase (AMPK) for producing energy suppliers for sperm (Zhu *et al.,* 2023) and support the function of mitochondria (Abedin e*t al.,* 2023; Li *et al.*, 2023). *Spirulina platensis* (SP) is a filamentous cyanobacterium rich in numerous phyto-contents, including pigments, amino acids, fatty acids, minerals, and carotenoids. In buffalo, cryopreserved semen enriched with SP extract (10 µg/mL) in the freezing extender enhanced post-thawed sperm quality and decreased sperm cryodamage (Badr *et al.*, 2021).

Recently, SP has been synthesized in the nano-form (Shaman *et al.,* 2022; Guo *et al.,* 2023). Many reports have proved that SP nanoparticles (SPNP) exhibit robust antioxidant, anti-inflammatory, and anti-apoptotic actions (Shaman *et al.*, 2022).

Moreover, SPNP has been shown to act as a protective mediator against reproductive dysfunction caused by diabetes (Shaman *et al*., 2022), due to its anti-apoptotic effect and up-steroidogenesis genes. According to various studies (Bashar *et al.,* 2022; El-Ratel *et al.,* 2023), SP and SeNP can improve the reproductive capacity and health status of male rabbits exposed to summer conditions. Despite the large number of published works on the use of SeNP and/or SP in the livestock industry as dietary feed additives, antioxidants, and anti-heat stress agents, the addition of SPNP or SPSE in the freezing extender of post-thawed bull sperm remains unexplored. Thereafter, this study aims to explore the potential effects of adding SPNP or SPSE in the freezing extender on sperm quality, oxidative stress, sperm apoptosis, and ultrastructure changes of post-thawed bull sperm for the first time.

MATERIALS AND METHODS

Preparation of Spirulina nanoparticles (SPNP) and coated SPNP: The dried *spirulina platensis* (SP) was acquired from the SCAD Company, New Salhia, Egypt. For the green synthesis of Spirulina nanoparticles (SPNP), we followed the ball milling method (Elabd *et al.*, 2020). Selenium was purchased from Sigma Aldrich (Product Number: 919519). Following the protocol of (Shin *et al.,* 2007), selenium nanoparticles (SeNP) were synthesized. While SeNP-coated SPNP was prepared as the following, 0.5 µg mg of SeNP was added to 10 µg SPNP/mL. The blend was transferred into a Teflon-lined stainless-steel autoclave (50 mL) and kept stationary for 24 hours at 100-160°C.

Characteristics of spirulina nanoparticles (SPNP) and Selenium coated Spirulina nanoparticles (SPSE): The morphological characterization of synthesized *Spirulina platensis* nanoparticles (SPNP) and selenium-coated *Spirulina platensis* nanoparticles (SPSE) was evaluated using transmission electron microscopy (TEM; JEOL-JEM-2100) at 200 kV. Additionally, the Zetasizer Nano (Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK) was used to assess the mean size, Z potential, and polydispersity index (PDI) of SPSE.

Ethical approval: All experimental procedures used were approved by the Institutional Animal Use and Care Committee (IACUC) at Zagazig University (Approval No: ZU IACUC/2/F/173/2022), based on the ARRIVE guidelines.

Animal management and semen collection: The semen samples were obtained from five verified fertile bulls (30 ejaculates) using the artificial vagina method. The semen samples were immediately transferred to the laboratory for initial assessment and further processing. The healthy bulls were housed separately in pens and fed identical diets consisting of a blend of farm fodder: apple pomace, vitamin premix, maize silage, basic minerals, and haylage. The bulls had unrestricted access to fresh water. Semen samples were collected from each bull using the artificial vagina method. Over six consecutive weeks, a total of 30 ejaculates were collected for this experiment. Ejaculates with over 75% progressive motility, 80% livability, sperm cell concentration (500 $X10^6$/mL), and abnormality less than 15% were selected, pooled, and used for freezing assessment (Khalil *et al.,* 2019).

Experimental Design and Freezing Procedures: The freezing extender was prepared according to the technique of (Khalil *et al.,* 2019) following standard construction procedures at the International Livestock Management Training Center (ILMTC) with some adjustments. The freezing medium consisted of 7.0mL of glycerol, 1.25 g of fructose, 1.675 g of citric acid, 3.028 g of Tris, 20mL of egg yolk, and 100 µg/mL of streptomycin and 100 IU/mL of penicillin, in double distilled water and the volume was made up to 100mL. The fresh semen was then divided into three equal fractions and treated before cryopreservation. The first group; semen was cryopreserved with the basic extender without any

addition, which served as the control group (CON). The other 2nd and 3rd groups: extender was fortified with 10 µg/mL spirulina nanoparticles (SPNP) or selenium coated spirulina nanoparticles (SPSE; 10 µg/mL of SPNP and 0.5 µg/mL of selenium nanoparticles), respectively. Then, semen was automatically packaged into straws (0.25 mL) equilibrated for two hours at 4°C, and placed at 5 cm overhead the liquid nitrogen for 10 min, and then followed by dropping into liquid nitrogen (− 196°C).

Post-thawed Sperm Assessment

Progressive motility: After thawing the straws at 37 °C for 30s, semen samples were placed on a slide in a volume of 5–10µL, covered with a coverslip, and examined for motility using a phase contrast microscope (Leica DM 500, Schweiz) at a constant temperature of 37°C. The subjective assessment data were obtained from this examination.

Sperm viability and abnormality: Sperm viability was considered based on the (Moskovtsev and Librach, 2013) protocol by eosin-nigrosine staining. A small sample (10µL) of extended semen was mixed with the eosin-nigrosine solution on a sterilized slide and thoroughly mixed. The slides containing the mixture were left for 2-3 minutes and then smeared on a glass slide to dry in the air. Sperm were then counted using a light microscope at × 400 magnifications (Leica DM 500, Schweiz). The spermatozoa stained red were considered non-viable, while the viable ones remained colorless. Additionally, in the same field, any sperm cells with irregularities in head and tail morphology were also noted, as previously mentioned.

Plasma membrane integrity: For assessing the sperm plasma membrane integrity, the HOST (hypoosmotic swelling test; fructose and sodium citrate, 1.35 and 0.73 grams, respectively) was performed. Thawed semen (10 µL) was extended in a hypoosmotic solution (100 µL). The resulting mixture was then incubated at 37 °C (Lone *et al.,* 2019). A contrast-phase microscope (Leica DM 500, Schweiz) with 400x magnification was utilized to explore the plasma membrane of sperm cells.

Assessing sperm chromatin damage: Toluidine blue staining was done as formerly identified by (Sobeh *et al.,* 2017) with some adjustments. Sperm cells were fixed with ethanol-acetic acid (3:1, v/v), and 70% ethanol for 1-3 minutes, respectively. Then, the fixed smears were hydrolyzed in 4 mM hydrochloric acid for twenty minutes, rinsed with distilled water, and air-dried. Fixed sperm cells were washed with 0.025% toluidine blue in McIlvaine buffer (sodium citrate-phosphate, pH 4.0), and then covered with a coverslip. The slides were examined using light microscopy at a magnification of 1000x to determine the percentage of chromatin damage in each field. A total of 300 sperm cells were analyzed in each group. Sperm cells with a green to light stain were considered to have normal chromatin, while those stained dark blue to violet were classified as having injured chromatin, based on our previous research (Abdelnour *et al.,* 2020; Abdelnour *et al.*, 2022).

Oxidative-related biomarkers Assays: For assessing the oxidative-related biomarkers, post-thawed extended semen (150 µL) was centrifuged (1600 × *g*) for 5 min. The supernatant was detached and precipitated in 1% Triton (1/3) for 20 min for extraction according to the previous methodology of (Ramazani *et al.*, 2023). The sample was centrifuged at 4000 × g and 25°C for 30 minutes. This step was repeated, and the supernatant was collected for analysis. Both antioxidant enzymes including superoxide dismutase (SOD, U/mL) and total antioxidant activity (TAC, mmol/L) were tested by the spectrophotometric method using ELISA kits provided by (Jiancheng Bioengineering Institute) following the method of (Mehdipour *et al.*, 2017)**.** Malondialdehyde (MDA, nmol/mL) content was determined using the thiobarbituric acid (TBA) technique by biological reaction kits (Jiancheng Bioengineering Institute), which were accomplished following the manufacturer's directions as defined earlier (Kadirve *et al.,* 2014).

Flow cytometry for assessing apoptotic sperm: Flow cytometry was used for detecting the apoptotic sperm using the Annexin V staining technique as termed by (Sharaf *et al.,* 2022). Sperm cells were incubated with binding buffer, and suspended with Annexin V (fluorescein isothiocyanate [FITC] labeled) and 5µL of phycoerythrin BD (Pharmingen™) and incubated for fifteen min in the dark conditions at room temperature.

Following this step, the samples were suspended in a binding buffer (200 µL). Then, the flow cytometric analysis was applied by an Accuri C6 Cytometer (BD Biosciences, San Jose, CA, USA), as described by (Masters and Harrison, 2014). The percentages of PI-positive or negative (PI+ or PI−), annexin-positive or negative (A+ or A−), and double-positive cells were counted. Sperm cells were divided into four categories according to (Peña *et al.,* 2003).

Sperm Ultrastructure Evaluation: Transmission electron microscopy (TEM) was used for studying the changes in sperm ultra-morphological changes in sperm following the (Khalil *et al.,* 2020) method. The semen samples were centrifuged at 1600 *g* 25°C for 5 min, then the samples were re-suspended in a 4% PBS (phosphate buffer saline, fixative solution) at 4°C for 2 h.

The fixed samples were exposed to dehydrated ethanol, preserved with propylene oxide, and embedded in ultrathin Epon resin, then sectioned (60-70nm). The images can be directly taken by TEM.

Statistical analysis: The collected data was assessed by the SPSS program (Statistical Package for the Social Sciences; version 26, Chicago, USA) by the one-way ANOVA and Tukey's post hoc test. The statistical difference was considered when $P \leq 0.05$. The analyzed data was imaged by the GraphPad prism (version 8).

RESULTS

Characterization of SPNP and SPSE: The TEM (transmission electron microscope) images of synthesized *Spirulina platensis* nanoparticles (SPNP, Fig. 1A) and selenium-coated *Spirulina platensis* nanoparticles (SPSE, Fig. 1B). The values of (Fig. 1C) and zeta potential of SPSE (Fig. 1D) were 141-152nm, and -24m/V.

Effects on post-thawed bull sperm quality: The percentages of progressive motility (Fig. 2A; P<0.001), and livability (Fig. 2B; P<0.001) were significantly improved in both treated groups. The SPSE group had the highest progressive motility and livability values, followed by SPNP. Supplementation with SPSE resulted in higher (P<0.001) plasma membrane integrity compared to other groups (Fig. 2C). Additionally, the untreated group had the lowest value of plasma membrane integrity. In contrast, the SPNP group had intermediate values (P<0.001). The inclusion of SPNP or SPSE in bull freezing extenders significantly decreased the percentages of sperm abnormalities (Fig. 2D; P<0.001) and chromatin damages (Fig. 2E; P<0.01) in opposition to the CON group.

Effects on oxidative-related biomarkers: The activity of TAC (Fig. 3A; P<0.001) was the highest in the SPSE group, while no significant differences were noticed between CON and SPNP (P>0.05). All supplemented groups significantly improved the activity of SOD (Fig. 3D; P<0.001) and significantly reduced MDA levels (Fig. 3C; P<0.01). The activities of TAC and SOD were improved by 71.2% and 45.32% in the SPSE group in opposition to the CON group, while MDA was reduced by 53.35% and 47.37% in the SPSE and SPNP groups, respectively.

Effects on sperm apoptosis status: The SPSE group had the highest values of viable sperm (Fig. 4A; P<0.01) compared to the other groups. Viable sperm was improved by 155.63 and 62.03% in the SPSE and SPNP groups, respectively, compared to the control. Supplementation with SPNP or SPSE had no significant effects on the percentages of early apoptotic sperm (Fig. 4B; P>0.05); however, higher values of early apoptotic spermatozoa were noticed in both SPNP and SPSE groups. Apoptotic sperm was significantly decreased by the addition of SPNP or SPSE (Fig. 4C; P<0.01) with the SPSE group having the lowest values of apoptotic sperm (P<0.01). Similar data was recorded for necrotic sperm between the CON and SPNP groups (Fig. 4D; P>0.05).

Sperm Ultrastructure: Fig.5 (A-F) illustrates the protective effects of SPNP or SPSE in maintaining sperm ultrastructure after bull sperm cryopreservation. In the control group (Fig. 5-A, B), the sperm head had a nucleus with necrotic chromatin (N), surrounded by a thin layer of cytoplasm, and the damaged plasma membrane (PM) and disrupted acrosome (AC) are depicted in Fig. 5A. Additionally, Fig. 5B shows damage to the mitochondrial membranes and distorted cristae (MI) in the sperm midpiece, as well as a disarranged flagellum in the axon tail (AX). SPNP treatment reduced the rates of acrosomal and plasma membrane rupture (Fig. 5C), maintained the sperm axon tail, and improved mitochondria shape (Fig. 5D). The addition of SPSE to the freezing extender of buffalo sperm reduced plasma membrane damage, increased intact plasma membrane, maintained acrosome status (Fig. 5E), and revealed homogenous mitochondria (Fig. 5F).

DISCUSSION

The current research indicates that enriching the freezing extender of bull with SPNP (10µg) or SPSE (10µg and 0.5µg of Se) could improve post-thawed sperm quality. This improvement is realized by increasing sperm motility, livability, and plasma membrane integrity while reducing sperm abnormality, apoptotic sperm, and chromatin damage compared to the control group. These positive effects could be attributed to the ability of SPNP or SPSE to maintain sperm morphology and structure through MDA and enhance the levels of SOD and TAC. In this study, we clarified the safety and efficacy of using nano-minerals to improve sperm freezability in bulls.

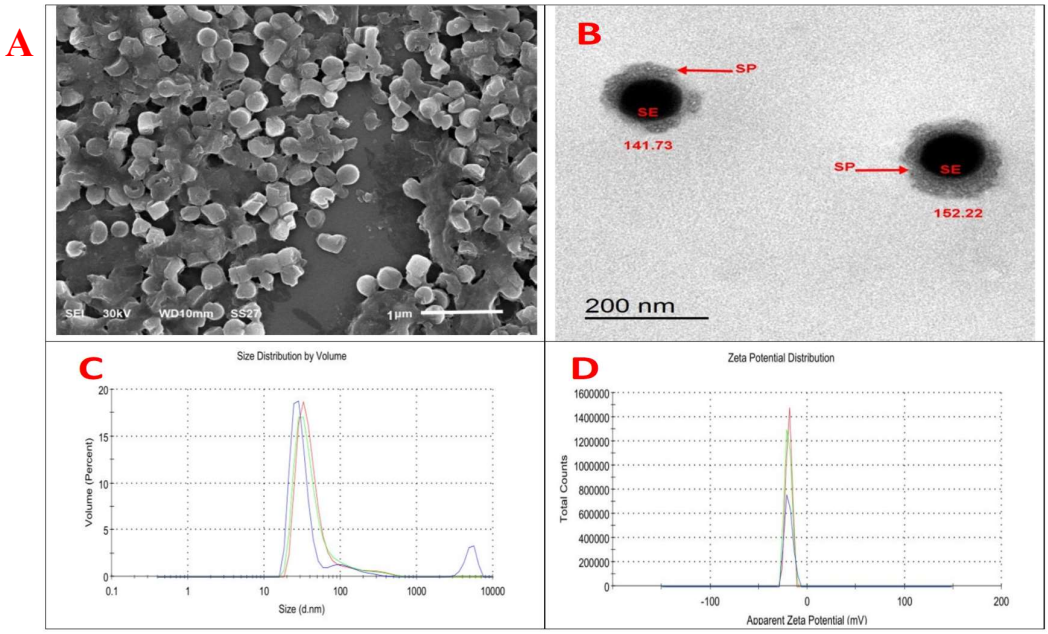
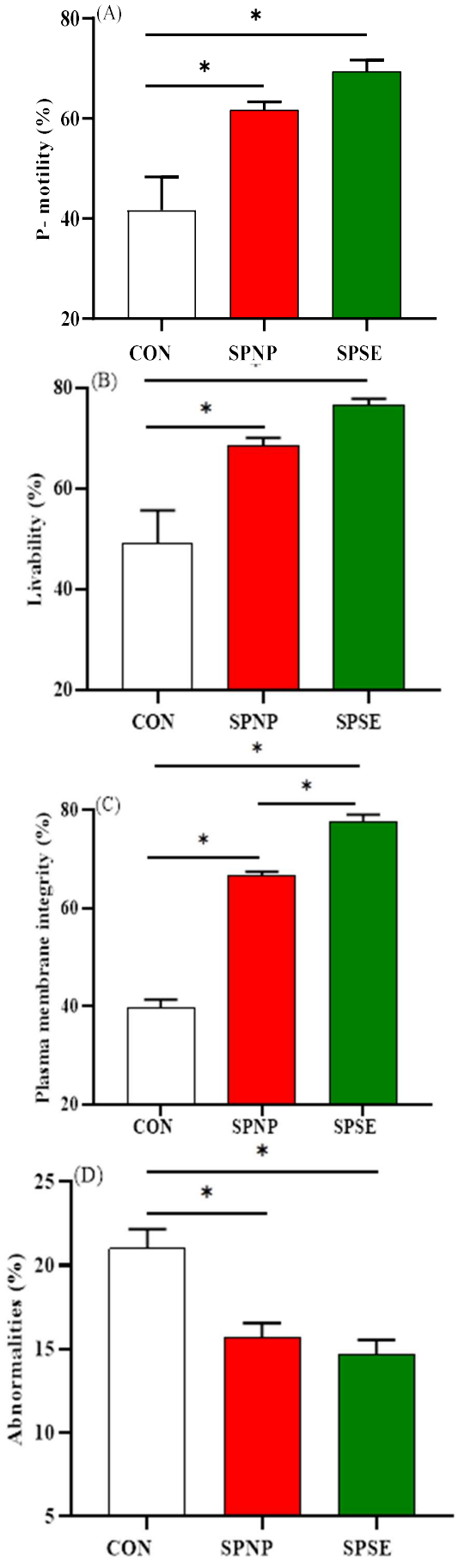


Fig. 1 (A-D): The TEM (transmission electron microscope) images of synthesized Spirulina platensis nanoparticles (SPNP, Fig. 1A) and selenium-coated Spirulina platensis nanoparticles (SPSE, Fig. 1B). The values of (Fig. 1C) and zeta potential of SPSE (Fig. 1D) were 141-152nm, and -24m/V.



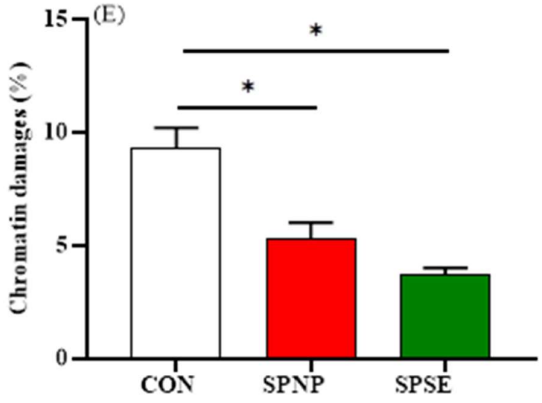


Fig. 2: Effects of *Spirulina platensis* nanoparticles (SPNP) or nano selenium coated with *Spirulina platensis* nanoparticles (SPSE) in freezing extender on motility (A), livability (B), membrane integrity (C), abnormalities (D) and chromatin damages (E) of post-thawed bull sperm. Data offered as mean ± SEM, *P < 0.05, compared to control.

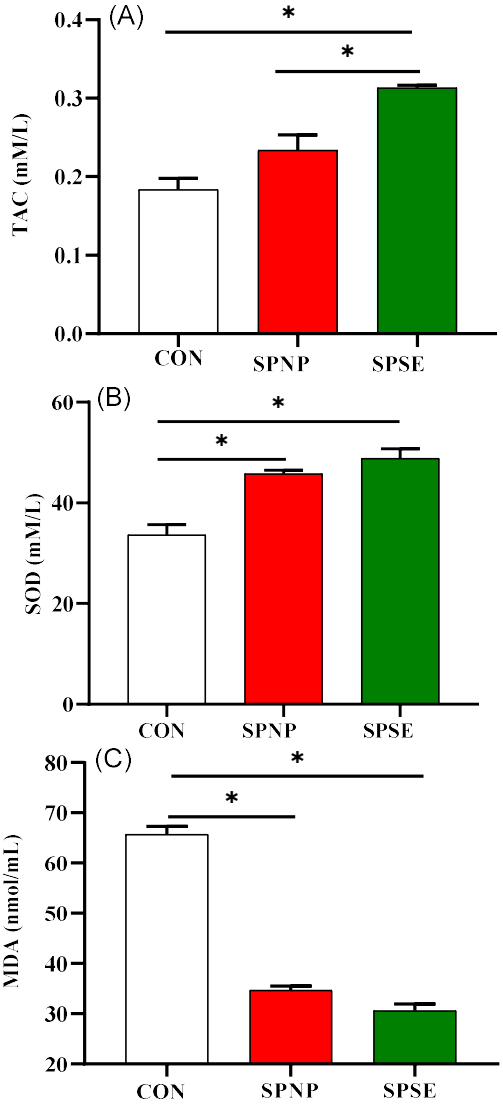


Fig. 3: Effects of *Spirulina platensis* nanoparticles (SPNP) or nano selenium coated with *Spirulina platensis* nanoparticles (SPSE) in freezing extender on total antioxidant capacity (A), SOD (B), and MDA (C) of post-thawed bull sperm. Data offered as mean ± SEM, *P < 0.05, compared to control.

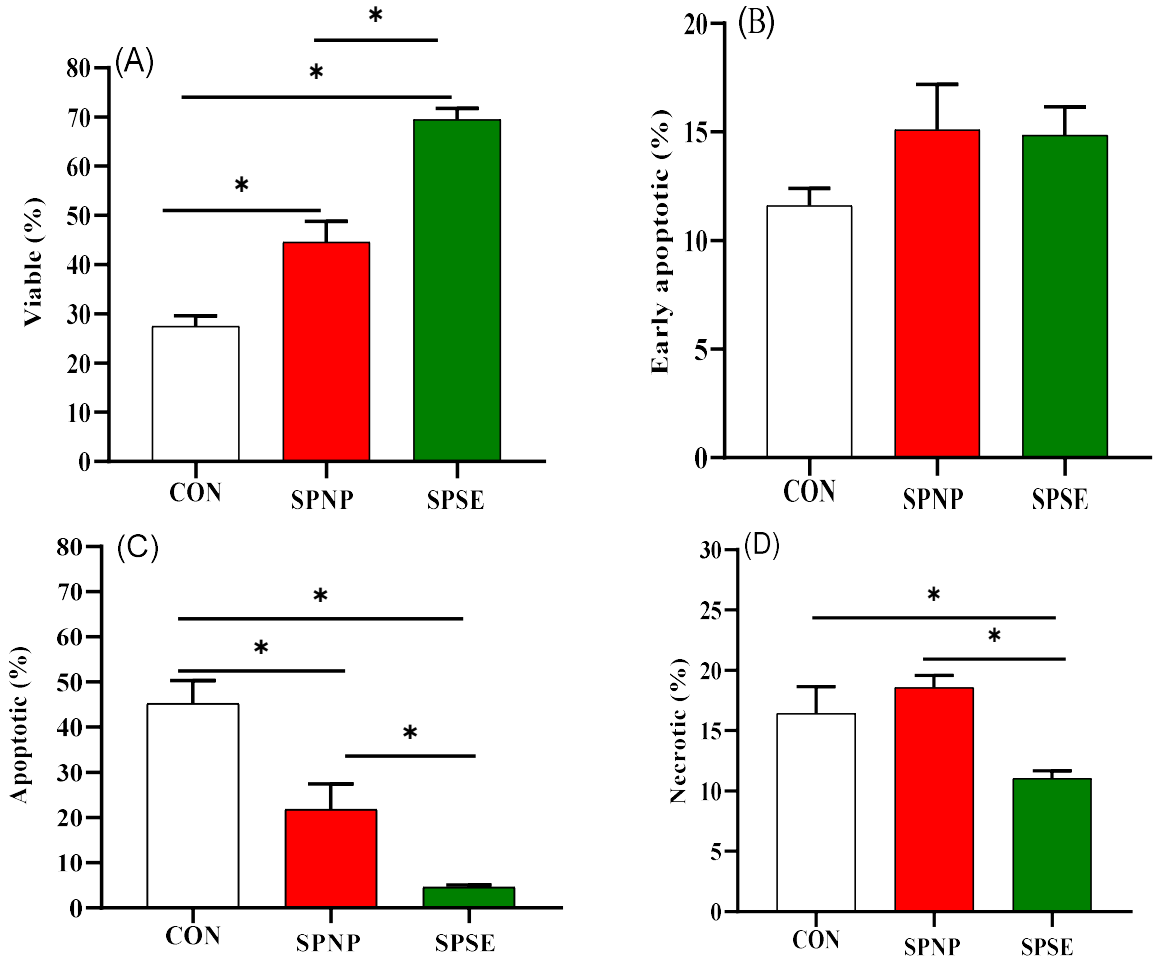


Fig. 4: Effects of Spirulina platensis nanoparticles (SPNP) or nano selenium coated with Spirulina platensis nanoparticles (SPSE) in freezing extender on apoptotic-like change (viable (A), early apoptotic (B), apoptotic (C) and necrotic spermatozoa (D) of post-thawed bull sperm. Data offered as mean ± SEM, *P < 0.05, compared to control.

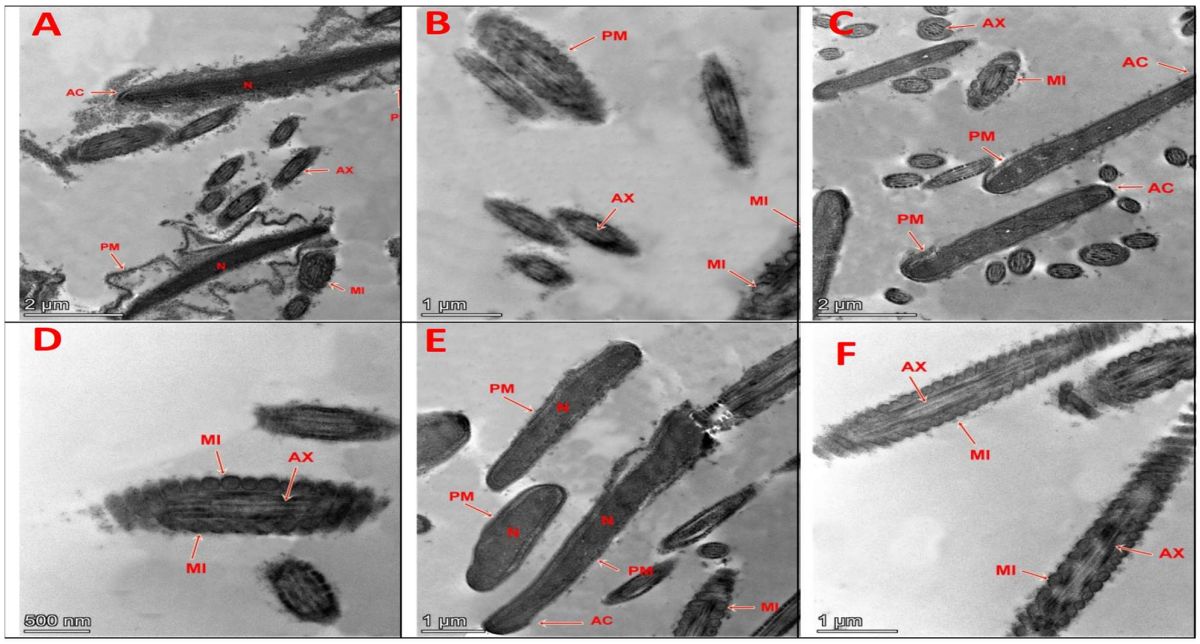


Fig. 5: Effects of *Spirulina platensis* nanoparticles (SPNP) or coated with nano selenium (SPSE) in freezing extender on sperm ultra-structure of post-thawed bull sperm.

It also opens up new possibilities for developing new types of extenders supplemented with nanotechnology.

Sperm motility is an important factor in fertility. It has been revealed that cryopreservation induces a significant decrease in sperm motility after thawing. This may be related to the excess OS generated during cryopreservation (Li e*t al.*, 2023). The addition of SPNP or SPSE to the freezing extender significantly enhanced post-thawed bull sperm, as evidenced in the present research. Similar to our work, (Li *et al.,* 2023) and (Khalil *et al.,* 2019) found that the supplementation of SeNP significantly improved the motility and viability of equilibrated bull spermatozoa. Moreover, several studies have also indicated that SeNP improved sperm quality after cryopreservation in goats (Abedin *et al.,* 2023), bull (Li *et al.,* 2023), and fish (Zhu *et al.,* 2023). Selenium nanoparticles have a protective effect against various damages, a previous study clarified that selenium nanoparticles maintained ATP content and sperm function during storage, possibly because they improved the glucose uptake capability of sperm by sustaining the level of phosphorylated AMP-activated protein kinase (p-AMPK) (Zhu e*t al.*, 2023). SP contains vital compounds such as nutraceutical pigments, protein, minerals, and essential fatty acids (Nakamoto *et al*., 2023). SP exhibits several biological activities including anti-oxidants (Badr *et al.*, 2021) and anti-apoptotic effects (Shaman e*t al.,* 2022). Furthermore, (Badr e*t al.,* 2021) found that adding SP (10 µg/mL) to the freezing extender improved significantly the post-thawing motility and reduced the proportion of acrosomal damage in buffalo ($P<0.05$). Furthermore, this research indicates that freeze-thawing has multi-injurious impacts on the plasma membrane and livability percentages. These findings are consistent with our previous data on rabbit bucks (Abdelnour *et al*., 2020; Abdelnour *et al.,* 2022), buffalo (Badr *et al.,* 2021; Hassan *et a*l., 2022; Abdelnour e*t al.,* 2023), and bull (Khalil *et al.,* 2019). Several studies have also confirmed the improvement of sperm parameters after the addition of various nanoparticles (Mehdipour *et al*., 2017; Abdelnour *et a*l., 2023; Li *et al.*, 2023; Zhu *et al.*, 2023).

We suggested that the synergistic effects of SPSE could be beneficial in minimizing the harmful effects of the cryopreservation procedure. The new form of nano SP coated with Se may cover the outer layer surface of the sperm membrane, stabilizing and protecting the cell membrane structure from cryo-damage. Few studies have explored the impacts of SP on sperm quality after cryopreservation in buffalos (Badr *et al.,* 2021) and rams preserved at 4°C (Ben Moula *et al.*, 2023). The addition of SeNP to the freezing extender has been found to positively affect sperm quality in various animal species, including bulls and boars (Khalil *et al.,* 2019; Zhu *et al*., 2023). Furthermore, to the best of our knowledge, no investigations have been conducted on the influence of SP-coated SE on sperm bull cryopreservation. Our research informs that the SPNP or SPSE improved post-thawed sperm parameters.

The integrity of sperm chromatin offers better prognostic and diagnostic tools (Hekmatdoost *et al*., 2009).

To preserve the genetic material of spermatozoa, the structures of chromatin and plasma membrane must be maintained during the freeze-thaw cycle. It's well accepted that cryopreservation can induce the generation of OS, resulting in DNA effects on male infertility. Consequently, counteracting the negative effects of OS and preserving the structures of chromatin and plasma membrane are essential for enhancing post-thawed sperm quality using nanoparticles-based extenders.

It has been indicated that the oral administration of SPNP caused a considerable decrease in MDA in sperm (Shaman *et al.,* 2022). Furthermore, similar to our data (Abedin *et al.*, 2023) found that the activities of antioxidant enzymes were significantly improved by SeNP incorporated into freezing extender in goats. The beneficial effects of SeNP may be attributed to the increase of GPX4, a critical element in maintaining oxidative homeostasis, as observed in fish (Zhu *et al*., 2023). Both SeNP and SP have been shown to mitigate the negative effects of HS in rabbits (Bashar *et al.,* 2022) and broilers (Abdel-Moneim e*t al.*, 2022) by supporting antioxidant indices and counteracting the impacts of OS. Additionally, SPNP enhanced SOD and catalase levels in fish (Mabrouk *et al.,* 2022), while SeNP improved sperm DNA integrity in rats (Mohammed *et al.*, 2023).

Despite the use of coated Se with SPNP being unexplored in cryopreserved semen, the beneficial effects of SeNP synthesized by SP were reported due to antioxidant and antimicrobial effects (Zhang *et al., 2*023), and cytotoxic impacts (Abbas *et al*., 2021). In this study, SPSE or SPNP showed significant effects on sperm quality by supporting TAC and SOD and reducing MDA in post-thawed sperm extenders. Nanoparticles can enhance semen quality by protecting sperm cells from damage and increasing the number of active, healthy sperm cells (Mohammed *et al*., 2023). Selenium has been identified as a promising trace element that can counteract OS and induce apoptosis in stressed cells. Numerous studies have elucidated the role of SeNP in reducing apoptotic sperm caused by the cryopreservation process in bulls (Khalil *et al.*, 2019; Li *et al.*, 2023). Several authors have also evidenced the anti-apoptotic activity of SeNP (Shahin *et al.*, 2020; Mohamed *et al.*, 2023). In our study, the addition of SPNP or SPSE can significantly reduce the necrosis and apoptotic sperms in cryopreserved bull semen. Additionally, the dietary addition of selenium significantly improved post-thaw sperm quality and fertility (Dou *et al.,* 2023).

Cryopreservation can induce damage to the structure and function of sperm cells (Abdelnour *et al.,* 2023; Ramazani *et al.,* 2023). Hence, adding natural protective compounds can effectively maintain normal plasma membrane, DNA integrity, and mitochondrial function. The current research indicates that the SP-coated SE stabilizes membranes by binding to spermatozoa membrane lipids (Dou *et al.,* 2023; Li *et al.,* 2023; Zhu *et al.*, 2023), thus improving sperm viability, sustaining membrane integrity, and reducing damage triggered by cryopreservation. Further, previous reports (Khalil *et al.,* 2019; Li *et al*., 2023) have demonstrated that 1-2 µg/mL of Se nanoparticles enhanced sperm quality in bulls via reducing OS, improving mitochondrial activity, and preserving sperm morphology. Adding nanoparticles such as nano-liposomes (propolis and alpha-lipoic acid), nano-curcumin, and Se nanoparticles to the freezing extender

maintained sperm membranes, nucleus, mitochondria and acrosome based on the sperm ultrastructure assessment (Khalil *et al.*, 2019; Abdelnour *et al.,* 2020; Hassan *et al.,* 2022; Abdelnour *et al.,* 2023). However, further investigations are needed to validate the beneficial uses of SPSE in the cryopreservation of bull semen based on the molecular pathways**.**

Conclusions: In light of the results, the addition of SPNP or SPSE to cryomedia boosted bull sperm quality, including livability, motility, and plasma membrane integrity. Furthermore, antioxidant-related biomarkers (TAC and SOD) were also enhanced. Additionally, the percentages of apoptotic sperm, chromatin damages, lipid peroxidation, and sperm ultrastructure maintenance were substantially decreased by SPNP or SPSE addition. Future investigations should utilize the findings of this research to explore the specific pathways of cryo-injury and the potential modulatory effects of other nanoparticles in improving the cryopreservation process.

Author contributions: Conceptualization, methodology, MAEH, AMS, MEAO and WAK; software, MAEH; validation, MAEH, AAS, YQL, AMS, MEAO, WAK, and SAA; formal analysis, AAS, YQL, AEH; investigation, resources, data curation, writing-original draft preparation, MAEH, AMS, MEAO, AAS, WAK and SAA writing-review and editing, MAEH, YQL, WAK AAS, and SAA. All authors have read and approved the published version of the manuscript.

Funding: The authors extend their appreciation to the Researchers Supporting Project number (RSPD 2023R971), King Saud University, Riyadh, Saudi Arabia for funding this research.

Data availability statement: Data are available with the request to the corresponding authors.

Acknowledgments: The authors extend their appreciation to the Researchers Supporting Project number (RSPD2023R971), King Saud University, Riyadh, Saudi Arabia for funding this research. Authors thank their universities and institutions.

Conflicts of interest: There are no declared conflicts of interest for the authors.

REFERENCES

Abbas HS, Abou Baker DH, Ahmed, EA, 2021. Cytotoxicity and antimicrobial efficiency of selenium nanoparticles biosynthesized by *Spirulina platensis.* Arch Microbiol 203:523-32.

Abdel-Moneim AME, Shehata AM, Selim DA, *et al.,* 2022. *Spirulina platensis* and biosynthesized selenium nanoparticles improve performance, antioxidant status, humoral immunity, and dietary and ileal microbial populations of heat-stressed broilers. J Therm Biol 104:103195.

Abdelnour SA, Hassan MAE, El-Ratel IT, *et al.,* 2022. Effect of addition of l-carnitine to cryopreservation extender on rabbit post-thaw semen parameters, antioxidant capacity, mitochondrial function, apoptosis and ultrastructure changes. Reprod Domest Anim 57:902-11.

Abdelnour SA, Hassan MAE, Mohammed AK, *et al.,* 2020. The effect of adding different levels of curcumin and its nanoparticles to extender on post-thaw quality of cryopreserved rabbit sperm. Animals 10:1508.

Abdelnour SA, Hassan MAE, Shehabeldin AM, *et al.,* 2023. Effects of propolis-loaded nanoliposomes fortification in extender on buffalo semen cryopreservation. Sci Rep 13:10621.

Abedin SN, Baruah A, Baruah KK, *et al.*, 2023. *In vitro and in vivo* studies on the efficacy of zinc-oxide and selenium nanoparticle in cryopreserved goat (*capra hircus*) spermatozoa. Biol Trace Elem Res 1-20.

Ali ME, Abd-Elkariem MFA, Fahmy S, *et al.,* 2023. Dietary supplementation with different nano and organic selenium doses improves ovarian activity, fertility rate, and progesterone level in Ossimi ewes. Ital J Anim Sci 22:651-9.

Badr M, Rawash Z, Azab A, *et al.,* 2021. *Spirulina platensis* extract addition to semen extender enhances cryotolerance and fertilizing potentials of buffalo bull spermatozoa. Anim Reprod 18.

Barroso PAA, Nascimento DR, Lima Neto MFD, *et al.,* 2023. Therapeutic potential of nanotechnology in reproduction disorders and possible limitations. Zygote 1-8.

Bashar A, Abdelnour S, El-Darawany A, Sheiha A, 2022. Effect of selenium nanoparticles and/or *spirulina platensis* on growth, hematobiochemical, antioxidant status, hormonal profile, immunity, and apoptosis of growing rabbits exposed to thermal stress. Egypt J Rabbit Sci 32:77-103.

Ben Moula A, Rahim A, Chentouf M, *et al.,* 2023. Inclusion of *Spirulina platensis* and *Salvia verbenaca* extracts to boost semen quality and fertilization ability in sheep. Reprod Domest Anim 58:637-45.

Dou X, Qiao L, Song X, *et al.,* 2023. Biogenic selenium nanoparticles alleviate intestinal epithelial barrier injury by regulating mitochondria–lysosome crosstalk. Food Function 14:4891-904.

El-Ratel IT, Elbasuny ME, El-Nagar HA, et al., 2023. The synergistic impact of Spirulina and selenium nanoparticles mitigates the adverse effects of heat stress on the physiology of rabbits bucks. Plos One 18:e0287644.

Elabd H, Wang HP, Shaheen A, *et al.* 2020. Nano spirulina dietary supplementation augments growth, antioxidative and immunological reactions, digestion, and protection of Nile tilapia, *Oreochromis niloticus,* against *Aeromonas veronii* and some physical stressors. Fish Physiol Biochem 46:2143-55.

Guo J, Gu X, Du, L, *et al*., 2023. Spirulina platensis protein nanoparticle-based bigels:Dual stabilization, phase inversion, and 3D printing. Food Hydrocoll 135:108160.

Hassan ME, Khalil WA, Abdelnour SA, *et al.,* 2022. Supplementation of Alpha-lipoic acid-loaded nanoliposomes in semen extender improves freezability of buffalo spermatozoa. Sci Rep 12:22464.

Hekmatdoost A, Lakpour N, Sadeghi MR, 2009. Sperm chromatin integrity:etiologies and mechanisms of abnormality, assays, clinical importance, preventing and repairing damage. Avicenna J Med Biotechnol 1:147-60.

Kadirve G, Kumar S, Ghosh SK, *et al.,* 2014. Activity of antioxidative enzymes in fresh and frozen thawed buffalo (*Bubalus bubalis*) spermatozoa in relation to lipid peroxidation and semen quality. Asian Pac J Reprod 3:210-7.

Kandeel M, Rehman TU, Akhtar T, *et al*., 2022. Anti parasitic applications of nanoparticles:a review. Pak Vet J 42:135-40.

Khalil WA, Abdel-Khalek AKE, Falchi, L et al., 2020. Effects of extender and packaging method on morphological and functional characteristics of cryopreserved Ossimi ram semen. Asian Pac J Reprod 9:148-55.

Khalil WA, El-Harairy MA, Zeidan AE, *et al.,* 2019. Impact of selenium nano-particles in semen extender on bull sperm quality after cryopreservation. Theriogenology 126:121-7.

Khan I, Zaneb H, Masood S, *et al*., 2022. Supplemental selenium nanoparticles-loaded to chitosan improves meat quality, pectoral muscle histology, tibia bone morphometry and tissue mineral retention in broilers. Pak Vet J 42:236-40.

Lafontaine S, Labrecque R, Blondin P, *et al.,* 2023. Comparison of cattle derived from in vitro fertilization, multiple ovulation embryo transfer, and artificial insemination for milk production and fertility traits. J Dairy Sci 106:4380-96.

Li S, Ren J, Zhang W, *et al*., 2023. Glutathione and selenium nanoparticles have a synergistic protective effect during cryopreservation of bull semen. Front Vet Sci 10:1093274.

Lone FA, Naikoo M, Khatun, A, *et al.,* 2019. Idebenone improves quality of ram sperm by mitigating oxidative stress during cryopreservation. Cryobiology 90:15-20.

Mabrouk MM, Ashour M, Labena *A, et al.,* 2022. Nanoparticles of Arthrospira platensis improves growth, antioxidative and

immunological responses of Nile tilapia (*Oreochromis niloticus*) and its resistance to Aeromonas hydrophila. Aquac Res 53:125-35.

Maleki K, Ayen E, Khaki A, *et al.,* 2023. Comparison of sperm characteristics and antioxidant and oxidant levels in bull semen frozen with four widely used extenders. Veterinary Research Forum. Faculty of Veterinary Medicine, Urmia University, pp:373-9.

Masters A and Harrison P, 2014. Platelet counting with the BD AccuriTM C6 flow cytometer. Platelets 25:175-80.

Mehdipour M, Daghigh KH, Nazari M, *et al.,* 2017. Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. Cryobiology 78, 34-40.

Mohamed KM, Abdelfattah MS, El-khadragy M, *et al.,* 2023. Rutin-loaded selenium nanoparticles modulated the redox status, inflammatory, and apoptotic pathways associated with pentylenetetrazole-induced epilepsy in mice. Green Process Synth 12:20230010.

Mohammed AIA, AL-Ahmed HI, Ben Ahmed A, 2023. Evaluation of green synthesis (*withania somnifera*) of selenium nanoparticles to reduce sperm DNA fragmentation diabetic mice induced with streptozotocin. Appl Sci 13:728.

Moskovtsev SI and Librach CL 2013. Methods of sperm vitality assessment. Spermatogenesis:methods and protocols 13-19.

Nakamoto MM, Assis M, de Oliveira Filho JG, *et al.,* 2023. Spirulina application in food packaging:Gaps of knowledge and future trends. Trends Food Sci Technol 133:138-47.

Peña FJ, Johannisson A, Wallgren M, *et al.,* 2003. Assessment of fresh and frozen–thawed boar semen using an Annexin-V assay:a new method of evaluating sperm membrane integrity. Theriogenology 60:677-89.

Pieczyńska J and Grajeta H, 2015. The role of selenium in human conception and pregnancy. J Trace Elem Med Biol 29:31-8.

Ramazani N, Mahd Gharebagh F, Soleimanzadeh A, e*t al.,* 2023. The influence of L-proline and fulvic acid on oxidative stress and semen quality of buffalo bull semen following cryopreservation. Vet Med Sci 9:1791-802.

Safa S, Moghaddam G, Jozani RJ, *et al.,* 2016. Effect of vitamin E and selenium nanoparticles on post-thaw variables and oxidative status of rooster semen. Anim Reprod Sci 174:100-6.

Shahin MA, Khalil WA, Saadeldin IM, *et al.,* 2020. Comparison between the effects of adding vitamins, trace elements, and nanoparticles to shotor extender on the cryopreservation of dromedary camel epididymal spermatozoa. Animals 10:78.

Shaman AA, Zidan NS, Atteia HH, *et al.,* 2022. *Arthrospira platensis* nanoparticles defeat against diabetes-induced testicular injury in rat targeting, oxidative, apoptotic, and steroidogenesis pathways. Andrologia 54:e14456.

Sharaf AE, Khalil WA, Khalifa EI, *et al.* 2022. The Supplementation of bee bread methanolic extract to egg yolk or soybean lecithin extenders can improve the quality of cryopreserved ram semen. Cells 11.

Shin Y, Blackwood JM, Bae IT, *et al.,* 2007. Synthesis and stabilization of selenium nanoparticles on cellulose nanocrystal. Mater Lett 61:4297-300.

Sobeh M, Hassan SA, El Raey MA, *et al.,* 2017. Polyphenolics from Albizia harveyi exhibit antioxidant activities and counteract oxidative damage and ultra-structural changes of cryopreserved bull semen. Molecules 22:1993.

Tippenhauer C, Plenio JL, Heuwieser W, *et al.,* 2023. Association of activity and subsequent fertility of dairy cows after spontaneous estrus or timed artificial insemination. J Dairy Sci 106:4291-4305.

Zhang T, Qi M, Wu, Q, *et al.,* 2023. Recent research progress on the synthesis and biological effects of selenium nanoparticles. Front Nutr 10.

Zhu C, Li L, Liu Q, *et al.*, 2023. Effect of selenium nanoparticles (SeNPs) supplementation on the sperm quality of fish after short-term storage. Aquaculture 562:738876.