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Supplementation of semen extender with zinc oxide nanoparticles improves the post-thaw quality of ram spermatozoa

Omid Maleki[1], Mahdi Khodaei-Motlagh[1], Reza Masoudi[2], Mehdi Kazemi-Bonchenari[1] and Mohammad Yahyaei[1]*

[1]Department of Animal science, Faculty of Agriculture and Natural Science, Arak University, Arak 38156-8-8349, Iran
[2]Animal Science Research Institute of Iran (ASRI), Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran

[*]Corresponding author,
E-mail address:
mmotlagh2002@gmail.com

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ORCID
Omid Maleki
0009-0005-4192-495X
Mahdi Khodaei-Motlagh
0000-0002-1281-7152
Reza Masoudi
0000-0002-1095-9392
Mehdi Kazemi-Bonchenari
0000-0002-4051-1097
Mohammad Yahyaei
0000-0002-8611-8469

Abstract Supplementation of the cooling medium with antioxidants has been suggested as an effective way to improve sperm quality during the chilling process. This study was designed to investigate the effect of supplementing sperm cooling medium with zinc oxide nanoparticles (NZnO) on ram semen quality. Semen samples were diluted in extenders supplemented with five different concentrations of NZnO (0, 50 µM/mL, 100 µM/mL and 200 µM/mL) or 100 µM/mL zinc oxide (ZnO) then frozen using a standard protocol. Various *in vitro* sperm qualities were assessed after thawing. No significant differences in average path velocity, straight-line velocity, curvilinear velocity, acrosome integrity and acrosome integrity were found among the treatments. Extender supplementation with 100 µM/ml NZnO presented higher (P<0.05) total motility, progressive motility, membrane integrity, mitochondria membrane potential, viability and lowfer malondialdehyde concentration compared to other groups. A dose of 200 µg/mL of NZnO had a toxic effect on these parameters (P≤0.05). In conclusion, NZnO as an antioxidant can enhance the quality of sperm in ram after of post- thawed process.

Keywords: antioxidant, cryopreservation, ram, sperm, zinc oxide nanoparticles

Introduction

Cryopreservation of farm animals' spermatozoa is gaining a lot of importance in recent years due to its application in preservation of genetic resources, a steady supply of semen, and improvement programs using artificial insemination technique (Hot, 2000; Bucak et al., 2007). However cryopreservation can cause significant damage to spermatozoa cells which contain DNA fragmentation, acrosomal defects (Ali et al., 2017), membrane lipid peroxidation (Najafi et al., 2019), loss of mitochondrial potential and apoptotic changes (Shah et al., 2016). Therefore it has been found fresh spermatozoa to have greater fertilizing capacity than frozen-thawed spermatozoa (Najafi et al., 2014). Cryopreservation injuries are mainly due to the formation of reactive oxygen species (ROS) and the occurrence of oxidative stress during cryopreservation (Len et al., 2019). In addition, ROS help to maintain fertilization ability through regulating capacitation and acrosome reaction of spermatozoa. However, excess levels of ROS can have a negative impact on fertilization rates by disrupting these pathways (Alvarez and Storey, 1982). Therefore, In order to maintain normal spermatozoa functions, a delicate balance between ROS generation and removal is needed.

Although semen does have an antioxidant system, which includes glutathione peroxidase(GPx), superoxide

dismutase (SOD), catalase (CAT), and other antioxidants, their activity is not only inefficient but is also reduced through cryopreservation process. Therefore, the addition of exogenous antioxidant compounds to the freezing extender could be mitigated cryopreservation's negative effects (Naijian et al., 2013; Salmani et al., 2013; Longobardi et al., 2017;).

Zinc (Zn), a prevalent mineral in seminal plasma, is directly related to the normal property of sperm cells such as motility, membrane strength, and tail morphology (Colagar et al., 2009). It is a cofactor in wide range of indigenous antioxidant enzymes that have ROS scavenging activity (Zago and Oteiza, 2001) and DNA-repairing roles (Ebisch et al., 2007). Various reports has been reported the potential of Zn for sperm motility and viability improvement through ROS scavenging in human (Gavella and Lipovac, 1998) and different animals (Abaspour Aporvari et al., 2018).

In Nano scale, inorganic nanoparticles could offer more antioxidant capabilities due to the high surface to volume ratio (Kim et al., 2017). From the reproduction perspective some biocompatible metal nanoparticles such cerium oxide (Falchi et al., 2018), selenium (Khalil et al., 2019), and manganese (Orzołek et al., 2021) have well-documented antioxidant properties during frozen-thawed process. NZnO is efficiently have been used to improve spermatozoa quality in animals. Supplementation of bull semen extender with NZnO decreased lipid peroxidation and improved the plasma membrane integrity of spermatozoa (Jahanbin et al., 2021). In goat (Abedin et al., 2023) and camel (Shahin et al., 2020), NZnO improved sperm quality parameters by enhanced the activity of antioxidant enzymes. The NZnO (100 µg/mL) were also found beneficial to preserve rooster semen quality during cooling storage period and transportation to other flocks (Khodaei-Motlagh et al., 2022). Moreover, Supplementations of dog epididymal spermatozoa extender with NZnO improved membrane integrity, acrosome integrity while decreasing the malondialdehyde (MDA) concentration (Fayez et al., 2023).

The literature review reveals a lack of information about the potential benefits of using NZnO for improving the post-thaw quality of ram semen. Therefore, this study was conducted to evaluate the effects of supplementing the freezing extender with different concentrations of NZnO on the quality of ram semen.

Materials and methods

Chemicals and ethics

All chemicals were provided from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA) companies. This study was approved by the Animal Science Research Institute of Iran's Research Ethics Committees.

Semen sample collection

Semen samples (n=30) were collected using an artificial vagina twice a week from five mature fat-tailed Zandi rams (3–4-years of age) in six replicates. Samples were evaluated and included in the experiment if the following criteria were met: volume of 1-2mL; sperm concentration of 3×10^9 spermatozoa/mL, total motility≥70% and normal morphology≥85%. To eliminate individual differences, semen samples were pooled and processed.

Extender preparation and cryopreservation

The extender medium was composed of 1.0% (w/v) soybean lecithin, 2.7g Tris, 1.4g citric acid, 1.0g fructose, 7.0% glycerol (v/v), 200IU/mL catalase, 1.0mg streptomycin and 100IU penicillin. Osmolality and pH were set at 320mosm/kg and 7.2, respectively (Motlagh et al., 2014). Semen samples were diluted with extender not supplemented or supplemented with various concentrations of NZnO and ZnO as follows: $NZnO_0$ (extender without NZnO), $NZnO_{50}$ (extender with 50µg NZnO), $NZnO_{100}$ (extender with 100µg NZnO), $NZnO_{200}$ (extender with 200µg NZnO) and ZnO_{100} (extender with 100µg ZnO). After dilution, semen samples were aspirated into 0.25 mL French straws (100×10^6 spermatozoa/mL) and sealed with polyvinyl alcohol powder and equilibrated at 5^0C for 2h. Then the straws were subsequently placed in static nitrogen vapor (-70^0C) for 10min, plunged into liquid nitrogen (LN2) and stored in LN2 until evaluation (Asadzadeh et al., 2021).

In vitro quality evaluation of post-thawed semen samples

Sperm motility variables

Sperm class analysis software (Version 5.1; Microptic, Barcelona, Spain) was used to analyze sperm motility and velocity parameters. The following motility values were recorded: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, m/s), straight-line velocity (VSL, m/s) and curvilinear velocity (VCL, m/s).

Abnormal sperm morphology

Hancock solution was used to evaluate post-thawed sperm abnormal morphology (Schäfer and Holzmann, 2000). Twenty-two µL of chilled semen samples were mixed with two mL of Hancock solution. Then, 10µL of sample were placed on a warm slide under a phase contrast microscope. Three-hundred spermatozoa were analyzed to detect abnormal morphology (acrosome and caput abnormalities, detached heads, the presence of cytoplasmic droplets, abnormal mid-pieces and tail defects) using a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan; ×1000 magnification; oil immersion).

Acrosome integrity

Pisum sativum agglutinin (PSA) was used to determine sperm acrosome integrity (Thys et al., 2009). A semen sample (5µL) was added to 100 µL ethanol and after 20min, 10µL of the suspended sample were mixed with 30µL of PSA on a glass slide. There were 200 spermatozoa evaluated on a slide using a fluorescent

microscope (BX51, Olympus) that had fluorescence illumination and a FITC filter (×400 magnification). The numbers of spermatozoa with and without green heads were recorded as having intact acrosomes and disrupted/damaged acrosomes, respectively (Figure 1).

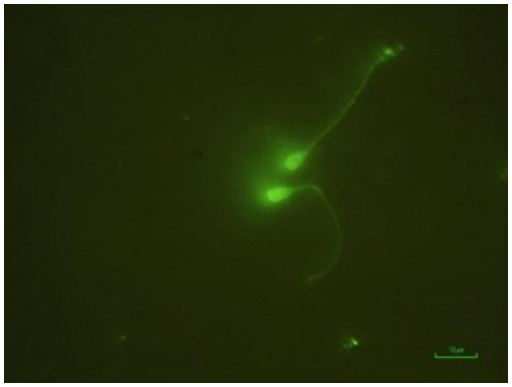

Figure 1. Assessment of acrosome integrity via PSA

Sperm membrane integrity

The hypo-osmotic swelling test was conducted to evaluate sperm membrane integrity (Revell and Mrode, 1994). With this procedure, 5µL of semen sample were mixed with 50µL hypo osmotic solution (57.6mM fructose and 19.2mM sodium citrate, 100 mOsm/L) and incubated for 20min. Thirty hundred spermatozoa were analyzed using a phase-contrast microscope (×400 magnifications) and samples with swollen and non-swollen tails were recorded.

Sperm mitochondrial activity

Rodamine 123 (R123:Invitrogen TM, Eugen, OR, USA) and propidium iodide (PI) was used to determine sperm mitochondrial activity. Samples were prepared based on our previous researches (Zarei et al., 2021; Khodaei-Motlagh et al., 2022). Briefly, 10µL of R-123 was added to 300µL of chilled semen sample and the suspension was incubated (20min) in a dark room. Then, samples were centrifuged (3min at 500g) and re-suspended again in Tris buffer. Next, 10µL of PI was added to the sample and 10,000 events were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, USA). Positive R123 and negative PI samples were recorded as active mitochondria. An argon-ion 488 nm laser excited fluorescent probes (R123 and PI). Probes of R123 and PI were measured in the channels of FL1 and FL2, respectively. Finally, the collected data were analyzed using FlowJo software (Treestar, Inc., USA) (Figure 2).

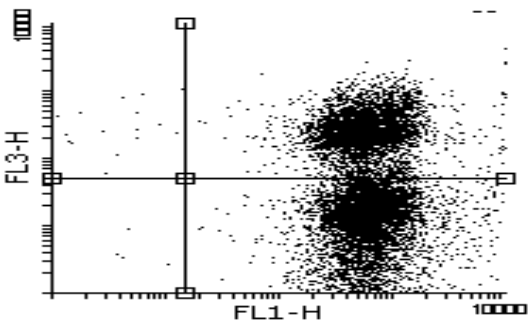

Figure 2. Evaluation of mitochondrial activity via Rh123

Sperm viability

The eosin–nigrosin staining technique was used to analyze sperm viability rate (Salmani et al., 2014). On a warm slide, semen samples were combined with two drops of eosin-nigrosin stain and evenly spread using a second slide. The viable and non-viable spermatozoa were determined under a phase contrast microscope (×400 magnification). Three-hundred sperm cells were counted and sperm cells with unstained or stained/partial stained heads were recorded as live cells.

MDA concentration

The concentration of MDA (as an indicator of lipid peroxidation) in semen samples was measured by using the thiobarbituric-acid reactive substances (TBARS) method (Esteribauer and Cheeseman, 1990). One mL of the diluted semen sample (400×10^6 sperm cells/mL) was mixed with 1mL of cold 20% (w/v) trichloro-acetic acid to precipitate the proteins. The precipitate was pelleted by centrifuging, and 1 mL of the supernatant was incubated with 1mL of 0.67 % (w/v) thiobarbituric in a water bath (100^0C) for 10 min. After cooling, a spectrophotometer (UV-1200, Shimadzu, Japan) recorded the absorbance at 532 nm.

Statistical analysis

Normal distribution was assessed by Shapiro–Wilk test. Data were analyzed using the Proc GLM in SAS (version 9.4; SAS Institute Inc., Cary, NC). Duncan's test determined statistical differences among the groups. P values ≤0.05 were considered to be statistically significant. Results were presented as mean±SE. Fertility data were assessed by using the Chi-Squared test in GENMOD procedure.

Results

Sperm motility variables

Table 1 shows the effect of NZnO and ZnO on sperm motility variables. The TM and PM of sperm were greater (P≤0.05) in the $NZnO_{100}$ treatment compared to the other treatments. The PM of sperm in $NZnO_0$ and $NZnO_{200}$ treatments was lower than in the others (P ≤0.05). The VAP, VSL and VCL of sperm were not affected by the treatments.

Abnormal morphology (AM), acrosome integrity (AI), membrane integrity (MI) and mitochondria membrane potential (MMP)

The effect of treatments on sperm AM, AI, MI and MMP is presented in Table 2. The MI and MMP were greater (P≤0.05) in semen samples supplemented with $NZnO_{100}$. The MI and MMP of sperm in $NZnO_0$ and $NZnO_{200}$ treatments were lower than in the others (P≤0.05). No significant differences in AM and AI were found among the treatments. No significant differences were found between other groups.

Viability and malondialdehyde (MDA) concentration

The NZnO and ZnO effects on sperm viability and malondialdehyde (MDA) concentration are shown in Table 3. Sperm viability was greater (P≤0.05) in $NZnO_{100}$ semen sample. Also, $NZnO_0$ and $NZnO_{200}$ semen samples had the lowest sperm viability (P≤0.05). The sperm MDA concentration in the $NZnO_0$ and $NZnO_{200}$ semen samples was greatest. There was no significant difference in the sperm MDA concentration among $NZnO_{50}$, $NZnO_{100}$ and ZnO_{100} semen samples.

Table 1. Effect of zinc oxide nanoparticles (NZnO) and zinc oxide (ZnO) on ram sperm motility parameters

Treatments	$NZnO_0$	$NZnO_{50}$	$NZnO_{100}$	$NZnO_{200}$	ZnO_{100}	SEM
Total motility (%)	40.5^b	41^b	43.5^a	39^b	41.4^b	1
Progressive motility (%)	17.2^c	20.1^b	22.4^a	16^c	19.5^b	1
Average path velocity (µm/s)	78.2	80	79.6	78	78.5	1.5
Straight-line velocity (µm/s)	70.6	72	71.5	69.6	71.3	1.7
Curvilinear velocity (µm/s)	150	152.6	152	148.9	150.5	2

Different concentrations of NZnO (0, 50,100 and 200 µM/ML) and ZnO (100 µM/ML); a,b,c: Within rows, mean values with common superscript (s) are not different (P>0.05; Duncan's test); SEM: standard error of the mean

Table 2. Effect of zinc oxide nanoparticles (NZnO) and zinc oxide (ZnO) on ram sperm characteristics

Treatments	$NZnO_0$	$NZnO_{50}$	$NZnO_{100}$	$NZnO_{200}$	ZnO_{100}	SEM
Abnormal morphology (%)	15.5	18.2	16.9	18.8	18	1.5
Acrosome integrity (%)	60	60	61.6	59.2	61.5	2
Membrane integrity (%)	44.1^c	48^b	53.2^a	45.4^c	46.3^b	1
Mitochondria membrane potential (%)	44.5^b	50^a	50^a	43.1^b	48.7^a	1.6

Different concentrations of NZnO (0, 50,100 and 200 µM/ML) and ZnO (100 µM/ML); a,b,c: Within rows, mean values with common superscript (s) are not different (P>0.05; Duncan's test); SEM: standard error of the mean

Table 3. Effect of zinc oxide nanoparticles (NZnO) and zinc oxide (ZnO) on ram sperm viability and lipid peroxidation

Treatments	$NZnO_0$	$NZnO_{50}$	$NZnO_{100}$	$NZnO_{200}$	ZnO_{100}	SEM
Viability (%)	48^c	51^b	54^a	48.4^c	51.8^b	1.2
Malondialdehyde (nmol/mL)	5.2^b	4.4^a	4.3^a	5.4^b	4.5^a	0.3

Different concentrations of NZnO (0, 50,100 and 200 µM/ML) and ZnO (100 µM/ML); a,b,c: Within rows, mean values with common superscript (s) are not different (P>0.05; Duncan's test); SEM: standard error of the mean

Discussion

Spermatozoa require a specific amount of ROS to function properly, but excess production of ROS, especially during cryopreservation, can damage their post-thaw quality and reduce their ability to fertilize (Susilowati et al., 2020). Studies have shown a correlation between elevated levels of reactive oxygen species (ROS) in spermatozoa and decreased motility, increased membrane lipid peroxidation and DNA damage (Liu et al., 2021). The addition of antioxidants to cryopreservation media is a suitable way to improve the success of cryopreservation by neutralizing the negative effect of ROS (Bucak et al., 2009). This study was performed to investigate the effect of NZnO as protective component on ram's semen quality parameters after frozen-thawed process.

In the present study, the supplementation of ram's semen extender with NZnO and ZnO showed positive effects on TM, PM, MI, MMP and viability. Additionally, it led to reduced concentrations of MDA. This result suggests that the nature (nano or micro) of ZnO significantly impact its performance. NZnO had a positive effect on measured parameters when their concentration was increased from 0 to 100, but beyond 100 the effect was reversed and became negative. So that, $NZnO_{100}$ group had the greatest performance in measured parameters, while $NZnO_{200}$ group had toxic effects. This suggests that there is a concentration-dependent effect of NZnO. In a comparative analysis, our study's results are consistent with findings from other studies and some existing differences can be attributed to method of nanoparticle synthesis, the composition of extender and the type of species.

Motility, velocity and kinematic parameters are key factors influencing spermatozoa fertility. We observed that the group treated with $NZnO_{100}$ showed higher levels of TM and PM compared to other groups. Adding zinc oxide nanoparticles (ZnO-NPs) to the extender is believed to directly affect spermatozoa motility by controlling ATP production, which is essential for sperm motion (Orzołek et al., 2021). Additionally, zinc enhances the antioxidant capacity of the sperm, protecting the plasma membrane from oxidative damage caused ROS (Afifi et al., 2015). By reducing oxidative stress, zinc preserves the integrity of the plasma membrane and promotes sperm motility. These combined effects contribute to the observed increase in motility in the group treated with ZnO-NPs. In line with our study Khalique et al. (2023) showed improved PM in cryopreserved buck spermatozoa supplemented with zinc oxide nanoparticle. Other studies also indicated that ZnO nanoparticles significantly improved post-thaw

motion kinetics of spermatozoa in humans and camels (Shahin et al. 2021).

The integrity of the plasma membrane plays a crucial role in sperm survival and fertilizing capacity. Our result showed that the use of NZnO at concentrations of 100 significantly improved MI compared to other groups. Similar findings have been reported in previous studies, where the addition of NZnO to bovine (Jahanbin et al., 2021), dog (Fayez et al., 2023) and camel (Shahin et al., 2020) spermatozoa during the freeze-thaw process resulted in enhanced plasma membrane integrity and improved mitochondrial activity. The positive effect NZnO on membrane integrity could be attributed to the antioxidant properties of NZnO. These nanoparticles likely acted as antioxidants, protecting the spermatozoa's plasma membrane from the harmful effects of ROS during the cryopreservation process.

Mitochondria play a vital role in maintaining normal sperm function and energy metabolism through ATP synthesis and oxidative phosphorylation. Cooling procedures can have a detrimental impact on sperm mitochondria, resulting in impaired ATP transport and decreased motility (Chen et al., 2005). In our study the addition of 50 and 100 µg NZnO in the extender effectively minimized mitochondrial damage during frozen-thawed process. In line with our results, Khodaei-Motlagh et al. (2022) indicated that supplementing rooster spermatozoa with 100 µg/mL ZnO nanoparticles significantly conserved sperm mitochondrial activity.

MDA is a natural byproduct of lipid peroxidation and oxidative stress induced at the membrane surface can be correlated with its level. Our result found that MDA concentrations were significantly lower in the groups which received NZnO or ZnO, which suggests that ZnO effectively scatters free radicals produced during the freeze-thaw process. Isaac et al stated ZnONP nanoparticles create a protective layer around sperms to scavenging excess free radicals, thereby maintaining membrane integrity and providing the normal activity of intracellular antioxidant enzymes (Isaac et al., 2017). In line with our results, adding ZnO-NPs to the freezing medium was reduced lipid peroxidation in bucks (Abedin et al., 2023), human (Isaac et al., 2017), rooster (Khodaei-Motlagh et al., 2022) and dog (Fayez et al., 2023).

Supplementing sperm samples with 100µg of NZnO improved sperm viability, likely due to the anti-apoptotic and plasma membrane protective effects of zinc which preserve and improve overall sperm quality. Zn can reduce sperm apoptosis by increasing the anti-apoptotic BCL2 protein and decreasing the pro-apoptotic Bax protein (Gualtieri et al., 2014).

Conclusion

The results of this study showed that NZnOs can be employed effectively as an antioxidant agent to protect ram spermatozoa from ROS-induced damage during cryopreservation. Adding ZnONPs (100 µg/mL) to semen extender had the best effect on total motility, progressive motility, membrane integrity, mitochondria membrane potential, viability and malondialdehyde concentration. However, a dose of 200 µg/mL ZnONPs appeared to be toxic, negatively affecting these parameters.

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Conflict of interests

The authors declare no conflicts of interest.

References

Abaspour, A., Mamoei, M., Tabatabaei Vakili, M., Zareei, S., Dadashpour Davachi, N., 2018. The effect of oral administration of zinc oxide nanoparticles on quantitative and qualitative properties of arabic ram sperm and some antioxidant parameters of seminal plasma in the non-breeding season. *Archives of Razi Institute* 73**,** 121-129.

Abedin, S. N., Baruah, A., Baruah, K.K., Kadirvel, G., Katiyar, R., Khargharia, G., Bora, A., Dutta, D.J., Sinha, S., Tamuly, S., 2023. In Vitro and In Vivo Studies on the Efficacy of Zinc-Oxide and Selenium Nanoparticle in Cryopreserved Goat (Capra hircus) Spermatozoa. *Biological Trace Element Research* 201, 4726-4745.

Afifi, M., Almaghrabi, O.A., Kadasa, N.M., 2015. Ameliorative effect of zinc oxide nanoparticles on antioxidants and sperm characteristics in streptozotocin-induced diabetic rat testes. *BioMed Research International* 2015, 1-6.

Ali, L., Andrabi, S., Ahmed, H., Hussain Shah, S.A., 2017. Freezability of water buffalo spermatozoa is improved with the addition of catalase in cryodiluent. *Cryo Letters* 38**,** 145-154.

Alvarez, J.G., Storey, B.T., 1982. Spontaneous lipid peroxidation in rabbit epididymal spermatozoa: its effect on sperm motility. *Biology of Reproduction* 27**,** 1102-1108.

Asadzadeh, N., Abdollahi, Z., Esmaeilkhanian, S., Masoudi, R., 2021. Fertility and flow cytometry evaluations of ram frozen semen in plant-based extender supplemented with Mito-TEMPO. *Animal Reproduction Science* 233**,** 233-240.

Bucak, M. N., Ateşşahin, A., Varışlı, Ö., Yüce, A., Tekin, N., Akçay, A., 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: microscopic and oxidative stress parameters after freeze–thawing process. *Theriogenology* 67**,** 1060-1067.

Bucak, M. N., Sarıözkan, S., Tuncer, P.B., Ulutaş, P.A., Akçadağ, H. İ., 2009. Effect of antioxidants on microscopic semen parameters, lipid peroxidation and

antioxidant activities in Angora goat semen following cryopreservation. *Small Ruminant Research* 81**,** 90-95.

Chen, H., Chomyn, A., Chan, D.C., 2005. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *Journal of Biological Chemistry* 280**,** 26185-26192.

Colagar, A.H., Marzony, E.T.,Chaichi, M.J., 2009. Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. *Nutrition Research* 29**,** 82-88.

Ebisch, I., Thomas, C., Peters, W., Braat, D., Steegers-Theunissen, R., 2007. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. *Human Reproduction Update* 13**,** 163-174.

Esteribauer, H., Cheeseman, K., 1990. Determination of aldehydic lipid peroxidation products: Malonaldehyde on related aldehyde. *Methods in Enzymology* 186**,** 407-421.

Falchi, L., Khalil, W. A., Hassan, M., Marei, W. F., 2018. Perspectives of nanotechnology in male fertility and sperm function. *International Journal of Veterinary Science and Medicine* 6**,** 265-269.

Fayez, E., El Sayed, M., Rawash, Z., Salama, A., 2023. Influence of the addition of zinc oxide nanoparticles to cryopreservation medium for dog epididymal spermatozoa. *Topics in Companion Animal Medicine* 52**,** 1-8.

Gavella, M., Lipovac, V., 1998. In vitro effect of zinc on oxidative changes in human semen. *Andrologia* 30**,** 317-323.

Gualtieri, R., Barbato, V., Fiorentino, I., Braun, S., Rizos, D., Longobardi, S., Talevi, R., 2014. Treatment with zinc, d-aspartate, and coenzyme Q10 protects bull sperm against damage and improves their ability to support embryo development. *Theriogenology* 82**,** 592-598.

Holt, W., 2000. Basic aspects of frozen storage of semen. *Animal Reproduction Science* 62**,** 3-22.

Isaac, A. V., Kumari, S., Nair, R., Urs, D. R., Salian, S. R., Kalthur, G., Adiga, S. K., Manikkath, J., Mutalik, S., Sachdev, D., 2017. Supplementing zinc oxide nanoparticles to cryopreservation medium minimizes the freeze-thaw-induced damage to spermatozoa. *Biochemical and Biophysical Research Communications* 494**,** 656-662.

Jahanbin, R., Yazdanshenas, P., Rahimi, M., Hajarizadeh, A., Tvrda, E., Nazari, S. A., Mohammadi-Sangcheshmeh, A., Ghanem, N., 2021. In vivo and in vitro evaluation of bull semen processed with zinc (Zn) nanoparticles. *Biological Trace Element Research* 199**,** 126-135.

Khalil, W.A., El-Harairy, M.A., Zeidan, A. E., Hassan, M.A., 2019., Impact of selenium nano-particles in

semen extender on bull sperm quality after cryopreservation. *Theriogenology* 126**,** 121-127.

Khalique, M. A., Rehman, H., Andrabi, S. M. H., Majeed, K. A., Ahmad, N., Fayyaz, M. H., Haider, M. S., Naz, S. S., Qureshi, I. Z. & Sulaiman, S., 2023. Antioxidant effects of zinc-oxide nanoparticles on post-thaw quality and in vivo fertility of Beetal buck spermatozoa. *Small Ruminant Research* 225**,** 1-9.

Khodaei-Motlagh, M., Masoudi, R., Karimi-Sabet, M., Hatefi, A., 2022. Supplementation of sperm cooling medium with Zinc and Zinc oxide nanoparticles preserves rooster sperm quality and fertility potential. *Theriogenology* 183**,** 36-40.

Kim, A., Ha, J.H., Park, S.N., 2017. Selective release system for antioxidative and anti-inflammatory activities using H2O2-responsive therapeutic nanoparticles. *Biomacromolecules* 18**,** 3197-3206.

Len, J.S., Koh, W.S.D., Tan, S.X., 2019. The roles of reactive oxygen species and antioxidants in cryopreservation. *Bioscience Reports* 39**,** 1-25.

Liu, X., Xu, Y., Liu, F., Pan, Y., Miao, L., Zhu, Q., Tan, S., 2021. The feasibility of antioxidants avoiding oxidative damages from reactive oxygen species in cryopreservation. *Frontiers in Chemistry* 9**,** 1-7.

Longobardi, V., Zullo, G., Salzano, A., De Canditiis, C., Cammarano, A., De Luise, L., Puzio, M. V., Neglia, G., Gasparrini, B., 2017. Resveratrol prevents capacitation-like changes and improves in vitro fertilizing capability of buffalo frozen-thawed sperm. *Theriogenology* 88**,** 1-8.

Motlagh, M., Sharafi, M., Zhandi, M., Mohammadi-Sangcheshmeh, A., Shakeri, M., Soleimani, M., 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**,** 217-222.

Naijian, H.R., Kohram, H., Shahneh, A. Z., Sharafi, M., Bucak, M. N., 2013. Effects of different concentrations of BHT on microscopic and oxidative parameters of Mahabadi goat semen following the freeze–thaw process. *Cryobiology* 66**,** 151-155.

Najafi, A., Kia, H.D., Mohammadi, H., Najafi, M. H., Zanganeh, Z., Sharafi, M., Martinez-Pastor, F., Adeldust, H., 2014. Different concentrations of cysteamine and ergothioneine improve microscopic and oxidative parameters in ram semen frozen with a soybean lecithin extender. *Cryobiology* 69**,** 68-73.

Najafi, A., Taheri, R.A., Mehdipour, M., Martínez-Pastor, F., Rouhollahi, A.A., Nourani, M.R., 2019. Improvement of post-thawed sperm quality in broiler breeder roosters by ellagic acid-loaded liposomes. *Poultry Science* 98**,** 440-446.

Orzołek, A., Rafalska, K. T., Otowska, W.A., Kordan, W., Korzekwa, A.J., Kozłowski, K., 2021. Influence of Zinc and Manganese nanoparticles on selected parameters of turkey spermatozoa stored in a liquid state at 4° C. *Animals* 11**,** 1-16.

Revell, S., Mrode, R., 1994. An osmotic resistance test for bovine semen. *Animal Reproduction Science* 36**,** 77-86.

Salmani, H., Nabi, M. M., Vaseghi-Dodaran, H., Rahman, M.B., Mohammadi-Sangcheshmeh, A., Shakeri, M., Towhidi, A., Shahneh, A.Z., Zhandi, M., 2013. Effect of glutathione in soybean lecithin-based semen extender on goat semen quality after freeze-thawing. *Small Ruminant Research* 112**,** 123-127.

Salmani, H., Towhidi, A., Zhandi, M., Bahreini, M., Sharafi, M., 2014. In vitro assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. *Cryobiology* 68**,** 276-280.

Schäfer, S., Holzmann, A., 2000. The use of transmigration and Spermac™ stain to evaluate epididymal cat spermatozoa. *Animal Reproduction Science* 59**,** 201-211.

Shah, S., Andrabi, S., Qureshi, I., 2016. Effect of equilibration times, freezing, and thawing rates on postthaw quality of buffalo (Bubalus bubalis) bull spermatozoa. *Andrology* 4**,** 972-976.

Shahin, M. A., Khalil, W. A., Saadeldin, I. M., Swelum, A.A., El-Harairy, M. A., 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**,** 1-16.

Shahin, M.A., Khalil, W.A., Saadeldin, I.M., Swelum, A. A., El-Harairy, M.A., 2021. Effects of vitamin C, vitamin E, selenium, zinc, or their nanoparticles on camel epididymal spermatozoa stored at 4 C. *Tropical Animal Health and Production* 53**,** 1-9.

Susilowati, S., Triana, I. N., Sardjito, T., Suprayogi, T. W., Wurlina, W., Mustofa, I., 2020. Effect of Simmental bull seminal plasma protein in egg yolk-citrate extender on Kacang buck semen fertility. *Cryobiology* 97**,** 20-27.

Thys, M., Nauwynck, H., Maes, D., Hoogewijs, M., Vercauteren, D., Rijsselaere, T., Favoreel, H., Van Soom, A., 2009. Expression and putative function of fibronectin and its receptor (integrin α5β1) in male and female gametes during bovine fertilization in vitro. *Reproduction* 138**,** 471-482.

Zago, M.P., Oteiza, P. I., 2001. The antioxidant properties of zinc: interactions with iron and antioxidants. *Free Radical Biology and Medicine* 31**,** 266-274.

Zarei, F., Kia, H. D., Masoudi, R., Moghaddam, G., Ebrahimi, M., 2021. Supplementation of ram's semen extender with Mito-TEMPO I: Improvement in quality parameters and reproductive performance of cooled-stored semen. *Cryobiology* 98**,** 215-218.