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Eco-friendly Synthesized Zinc Oxide Nanoparticles Improved Frozen-thawed Semen Quality and Antioxidant Capacity of Rams

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Abstract

Zinc oxide nanoparticles (ZnO-NPs) have antimicrobial, antioxidants, and anticancer properties. This study aimed to improve the post-thawing semen characteristics of rams using three concentrations of ZnO-NPs. ZnO-NPs in 0.0, 0.5, 1.0, 1.5 mg/ml tris-based diluents were used to extend semen collected from five Awassi rams twice weekly for two months. Post-thaw semen evaluation and measurement of Malondialdehyde (MDA), nitric oxide (NO), ascorbic acid (AA), superoxide dismutase (SOD), glutathione peroxidase (GPx), total cholesterol (CHO), low density lipoproteins (LDL), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), zinc, and copper (Cu). With increasing ZnO-NPs concentrations, the post-thaw sperm total motility (STM %), progressive motility (SPM) %, viability (SV%), functional membrane integrity (FMI %), acrosome integrity (AI %), NO, LDH, ALP, Cu, and zinc ascended linearly ($P \leq 0.0001$) but the abnormal sperm (AS%) and SOD decreased ($P \leq 0.0001$). The addition of 1.0 mg/ml ZnO-NPs increased ($P \leq 0.0001$) GPx but decreased ($P \leq 0.0001$) CHO and LDL. All concentrations of ZnO-NPs preserved ($P \leq 0.0001$) high AA compared to 0.0mg/ml and did not influence MDA. The addition of 0.5 mg/ml ZnO-NPs reduced ($P \leq 0.059$) Cu concentrations. All semen characteristics had strong positive correlations with zinc, LDH, and ALP and negative correlations with SOD. AS % had negative correlations with zinc, ALP, and LDH and positive correlation with SOD. All the three added concentrations of ZnO-NPs improved the post-thaw semen motility, viability, fertilizing capacity, and antioxidant capacity but 1.5mg/ml semen diluents proved the best concentration for semen preservation.

KEYWORDS
Oxidants-Antioxidants, Rams, Post-Thaw Semen Characteristics, Tris-Based Semen Diluents, Zinc Oxide Nanoparticles

INTRODUCTION

Cryopreservation decreases post-thaw sperm motility and fertilizing capacity compared to the fresh one. The advent of semen extenders for the cryopreservation of semen of any species aims to obtain high post-thaw semen characteristics, viability, and fertilizing capacity. Because of ram sperm are extremely vulnerable to temperature changes during freeze-thawing procedures, several additives are added to freezing extenders seeking for improved post-thaw motility, livability, and fertility potential. The high amount of polyunsaturated fatty acids in the plasma membrane and insufficient level of cytoplasmic antioxidants in ram semen render sperm cells sensitive to lipid peroxidation in presence of reactive oxygen species (ROS) (Al-badry *et al*., 2018). The excessive production of ROS impairs kinematic parameters, viability, and fertilizing capacity of spermatozoa due to the induction of biochemical and structural alterations, ATP depletion, and DNA fragmentation (Topraggaleh *et al*., 2014). After cryopreservation, the activities of total LDH, SOD, CAT and GSH-Px increased in seminal plasma and decreased in spermatozoa (Huang *et al*., 2014). Antioxidants (Banday *et al*., 2017), Butylated hydroxytoluene (BHT), vitamins, minerals are usually added to semen extenders prior to semen storage to decrease the oxidative stress and lipid peroxidation (LPO) induced by semen processing and the low temperature. When BHT was added to egg yolk-deficient (2.5%) extenders, it significantly improved viability of chilled-stored semen together with the motility (48.5%) and the fertility (62.5%) of frozen-thawed spermatozoa (Khalifa *et al*., 2008). Supplementation of Tris-egg yolk extender with 3% lyophilized royal jelly (RJ) had a protective effect on chilled and cryo-preserved ram spermatozoa (Amini *et al*., 2019).

To combat bacterial growth or contamination during semen processing, antibiotics must be added to the semen extender during semen processing. During the last decade, the application of nanotechnology gained great interest in the veterinary medicine. Iron oxide and silver nanoparticles had been used in the extended ram semen to minimize the bacterial growth and overcome the drug resist venereal disease (Yousef *et al*., 2021). When selenium nanoparticles were supplemented to male Boer goat in the diet, it improved sperm motility and viability and reduced abnormalities (Shi *et al*., 2010). In vitro, silver nanoparticles at concentrations of 30, 60, 125, 250 and 500 µM incubated with human semen at 37°C for 60-120 min showed a slight insignificant toxicity (Moretti *et al*., 2013). However, the addition

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of 0.05 µg./ml selenium to Barki rams' semen extender improved all sperm post-thaw characteristics and reduced abnormalities and LPO (Hozyen *et al*., 2019). Zinc nanoparticles showed protective effects on the testicular tissue and semen in rats treated with nicotine (Mohamed and Abdelrahman, 2019), doxorubicin (El-Maddawy and Abd El Naby, 2019), and cisplatin (Erfani Majd *et al*., 2021). Zinc-nanoparticles added to semen diluents had reduced cryopreservation mediated sperm damage (Isaac *et al*., 2017; Jahanbin *et al*., 2021).

Both alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities decrease in semen extenders rich in antioxidants additives due to their action in scavenging ROS produced by live and motile spermatozoa but their increasing activities in fresh semen were directly related to high dead and abnormal sperm percent. The addition of ascorbic acid to semen extender of stallions decreased the activities of these enzymes (Alamaary *et al*., 2020). The correlation between LDH and progressive motility and viability could be a sign that extracellular LDH ensures metabolism of spermatozoa. The ALP enzyme is also isolated from the sperm plasma membrane (Bucci *et al*., 2014). Furthermore, LDH enzyme is found in the cytosol, mitochondria and on the sperm plasma membrane (O'Flaherty *et al*., 2005). Reduced LDH indicates poor semen quality and high dead sperm percent (Aksoy *et al*., 2002). So, the objective of the current study was to add zinc oxide nanoparticles (ZnO-NPs) prepared by the green method using plant extract to the semen extender of rams in three different concentrations and explore their effects on post-thaw sperm motility and viability and on seminal plasma oxidants, antioxidants, trace minerals, and enzymes.

MATERIALS AND METHODS

Animals

Before conducting the current study, Animal care and use approval certificate was obtain from The Faculty of Veterinary Medicine (Cairo University) Animal Care and Use Committee. (Vet CU 8/3/2022/399). Five fertile and clinically healthy Awassi rams belonged to the faculty of Veterinary Medicine Cairo University (weighing 50-65 kg and aging 3-5 years old) were kept under natural environment (ambient temperature and daylight) and supplied with same quantity of feed in accordance with NRC recommendations (each ram fed daily 1.25 kg ration composed of 850 g wheat straw, green forage and 400 g pelleted concentrated ration with free access to fresh water).

Preparation and characterization of ZnO-NPs

Zinc sulphate (20 g) was added to 200ml of the filtrated *Cymbopogon schoenanthus* (20g/200 ml distilled water kept in a shaker incubator at 37°C for 72 h) aqueous extract and kept in a shaker incubator at 37°C until the complete evaporation of the water content. The powder was put for 1 h in the muffle furnace at 400°C then was ready to use. A scanning and transmission electron microscope (Figure 1) and XRD were performed (Dey and Somaiah, 2022).

Semen collection and processing

Semen was collected using lubricated and pre-warmed (40-42ºC) short artificial vagina twice weekly for two months. Immediately after collection, freshly ejaculated semen samples were transformed to the laboratory at 37ºC for examination and processing. In order to eliminate the individual variations among rams, collected ejaculates from each ram were pooled. Pooled semen samples with a final volume of about 5.00 mL were evaluated for basic semen characteristics including motility, percentage of live spermatozoa and sperm cell concentration. Semen volume was estimated using a graduated tube and sperm concentration was assessed with a hemocytometer. Samples showing more than 75% motility, 75% live sperm and $3x10^9$ sperm cell/mL were processed for experiments.

Pooled semen samples were divided into 12 equal aliquots and diluted 1:5 (V/V) with Tris citrate glucose (Tris (hydroxyl methyl amino methane; 3.634 g), glucose (0.5 g), citric acid monohydrate (1.99 g), fresh chicken egg yolk (15 ml), glycerol (7 ml), penicillin G sodium (100.000 i.u.), streptomycin sulphate (100 mg) and glass distilled water to 100 ml (Evans and Maxwell, 1987). ZnO-NPs in 0, 0.50, 1.00, 1.50 mg/ml concentrations were added to semen extender. Diluted semen samples were kept in the refrigerator at 5°C (in graduated falcon tubes) for 90 min. For cryopreservation, diluted semen samples were equilibrated at 5°C for 15 min and then loaded in 0.25 ml straws and sealed with polyvinyl powder. After that, the straws were placed horizontally at 6.5 cm over liquid nitrogen for 10 min and then plunged into liquid nitrogen tank for storage. Frozen ram semen was thawed by removing two straws from liquid nitrogen container and dropping them in a water bath at 40°C for 30 seconds (Kumar *et al*., 2003).

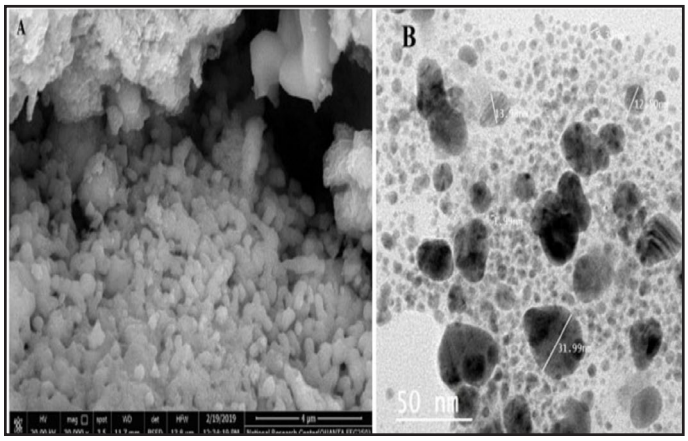

Fig. 1. The scanning (A) and transmission (B) electron microscope of zinc oxide nanoparticles (ZnO-NPs) showing aggregates (A) and spherical shape of 12-32nm in diameter (B).

Evaluation of frozen semen

Post-thaw motility

Frozen-thawed semen samples were subjectively assessed immediately after thawing and expressed in percentages by using heated stage (37ºC) optical microscope (Olympus BH-2, Olympus Optical Co. Ltd., Japan) (400×).

Determination of sperm viability and sperm abnormalities

Alive sperm percentage was evaluated using eosin-nigrosine stain (Evans and Maxwell, 1987). Each sample was stained by mixing a drop of frozen-thawed semen sample with 2 drops of the stain on a warm glass slide. A total of 200 sperm cells were examined randomly using a bright field microscope (1000×). Normal alive sperm exclude the eosin stain and appear white in color, whereas dead sperm (those with loss of membrane integrity) take up the eosin stain and appear pinkish in color. On the same slides, spermatozoa with morphologic defects in head, tail, or neck-mid piece were classified as abnormal. The percentages

of live spermatozoa and abnormal sperm cells were calculated.

Functional membrane integrity

To evaluate the functional membranes integrity (FMI) of the spermatozoa, hypo osmotic swelling test was used (Revell and Mrode 1994). Briefly, 30 µl of frozen-thawed semen sample was mixed with a 300 µl of 100 mOsm/kg hypo-osmotic (9 g fructose plus 4.9 g sodium citrate per liter of distilled water) solution. This mixture was incubated at 37°C for one hour, 0.2 ml of the mixture was placed on a microscope slide and mounted with a cover slip and immediately evaluated under the bright field microscope (400×). Sperm cells with resistant membranes exhibited a swelling around the tail, such that the flagella became curled and the membrane maintained a swollen 'bubble' around the curled flagellum. A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded.

Acrosome integrity

Acrosome integrity was evaluated using patent specific stain (Spermac) according to Ghallab *et al*. (2019). In brief, dried sperm smear prepared from each sample after cooling was fixed in a solution composed of 10% formalin for 10 min. Each slide was then processed through stain solutions A, B and C for 1 min at room temperature. After air drying, the slides were analyzed under oil immersion (×1000). Two hundred sperm cells were counted and the percentage of spermatozoa with undamaged acrosome was recorded.

Biochemical evaluation

Determination of lipid peroxide product (Malondialdehyde, MDA nmol/ml), nitric oxide (NO umol/L), Glutathione peroxidase (GPx mU/mL), superoxide dismutase (SOD (U/ml), and ascorbic acid (mg/L), zinc, copper, total cholesterol, low density lipoproteins (LDL) were assayed calorimetrically using commercial kits (Biodiagnostics, Egypt).

Lactate dehydrogenase and alkaline phosphatase (MG, Science and Technology Center"STC") of sensitivity 1U/100ml and the intra- and inter- test precisions are 0.56% and 1.1%.

Statistical analysis

The obtained data were expressed as Mean ± SEM. The effect of different concentrations of the ZnO-NPs added to semen diluent on the semen parameters were tested using one-way analysis of variance (ANOVA). Duncan's Multiple Range Test was used to differentiate between significant means at P<0.05. Pearson correlation coefficient was also processed using IBM-SPSS version 20.0 (2016).

RESULTS

The scanning electron microscope (SEM, Fig. 1A) showed aggregation of ZnO-NPs which were spherical using Transmission electron microscope (TEM) of diameter ranged from12-32 nm (Fig.1B). The addition of different concentrations of zinc oxide nanoparticles (ZnO-NPs) to semen extender (Table 1) had increased (P<0.001) STM, SPM, SV, FMI, and AI with increasing the concentrations of ZnO-NPs. The increase in ZnO-NPs concentration to the semen diluent resulted in a linear decrease (P<0.001) of AS%. The addition of 1.5 mg/ml diluents resulted in the highest semen parameters with the lowest AS% (Table 1).

All diluents supplemented with ZnO-NPs had insignificantly low lipid peroxide product (MDA; Table 2). Nitric oxide concentration ascended (P<0.001) with increasing ZnO-NPs concentration per ml diluent (Table 2). All diluents with ZnO-NPs concentrations from 0.5 to 1.5 contained high (P<0.01) ascorbic acid with no significant difference between them. Semen diluent with 1.5 mg had the lowest (P<0.001) GPx and SOD activities (Table 2). The concentrations of zinc increased (P<0.001) with increasing ZnO-NPs in the semen diluent (Table 2). The highest copper concentrations can be noticed in semen diluent supplemented with 1.5 mg ZnO-NPs. Total cholesterol and LDL decreased (P<0.001) in all semen extenders supplemented with ZnO-NPs with the lowest concentrations observed after adding 1.0 mg/ml ZnO-NPs.

LDH and ALP activities increased (P<0.001) in semen diluents enriched with 1.0 mg and 1.5 mg compared to 0.0 mg (control), and 0.5 mg ZnO-NPs with the lowest activities were noticed when 0.5 mg and highest ones were observed when 1.5 mg ZnO-NP was added (Table 2).

NO tended to correlate (Table 3) with SPM (r=0.52; P=0.085), FMI (r=0.50; P=0.096), and AI (r=0.50; P=0.097) but had a negative correlation with SA (r=-0.68; P=0.016). Zinc concentrations correlated with STM (r=0.73; P=0.008), SPM (r=0.85; P=0.001), SV (r=0.73; P=0.008), FMI (r=0.97; P<0.001), and AI (r=0.96; P<0.001) but showed a negative correlation with AS% (r=-0.90; P<0.001). SOD correlated negatively with STM (r=-0.62; P=0.033), SPM (r=-0.62; P=0.033), and live sperm% (SV; r=-0.61; P=0.035). SOD correlated positively with abnormal sperm (r=0.69; P=0.014) and

Table 1. Effect of different concentrations of ZnO-NPs concentrations on the percentages of TM, SPM, SV, SA and FMI, and AI of frozen-thawed ram semen

Semen parameters	ZnO-NPs concentrations				P-value
	0 mg/ml	0.5 mg/ml	1.0 mg/ml	1.5 mg/ml	
STM%	39.98±1.17^{a}	45.43±1.34^{b}	46.23±1.02^{b}	70.44±1.24^{c}	0.000
SPM %	30.33±1.08^{a}	42.52±1.65^{b}	44.22±1.52^{b}	67.42±1.35^{c}	0.000
SV %	43.25±1.32^{a}	48.85±1.66^{b}	49.53±1.42^{b}	73.85±1.62^{c}	0.000
SA %	30.42±1.55^{c}	25.34±1.08^{b}	23.45±1.72^{b}	18.04±1.55^{a}	0.000
FMI%	65.32±1.38^{a}	75.22±1.54^{b}	76.38±1.57^{b}	82.15±1.64^{c}	0.000
AI%	62.03±1.41^{a}	72.25±1.32^{b}	74.43±1.65^{b}	81.03±1.12^{c}	0.000

Data are expressed as Mean ±standard error of mean (SEM).
Means with different superscripts (a, b, c) in the same row are significantly different at P<0.05
n=16, STM= sperm total motility; SPM= sperm progressive motility; SV= sperm viability; SA= sperm abnormalities; FMI= functional membrane integrity; AI= acrosome integrity.

tended to have a positive correlation with AI (r=0.54; P=0.068). ALP correlated with STM (r=0.94; P<0.001), SPM (r=0.89; P<0.001), SV (r=0.94; P<0.001), FMI (r=0.68; P=0.015), and AI (r=0.71; P<0.01) but had a negative correlation with AS (r=-0.81; P=0.002). LDH correlated with STM (r=0.63; P<0.05), SPM (r=0.68; P=0.016), SV (r=0.63; P=0.029), FMI (r=0.67; P=0.017), and AI (r=0.72; P=0.009), with obtaining a negative correlation with AS (r=-0.69; P=0.013).

DISCUSSION

Zinc is an important trace mineral involved in many enzymes controlling fertility. The addition of 100 µM zinc prior to cryopreservation resulted in higher percentage of sperm with intact DNA mitochondrial function and progressive motility (Kotdawala *et al*., 2012). In contrast to bull semen supplemented with 10-6, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} ZnONPs did not influence total sperm motility, progressive sperm motility, and viability (Jahanbin *et al*., 2021). In the investigated rams, semen extenders containing 0.50, 1.00, and 1.50 mg/ml achieved higher post-thaw total motility (70.4%), progressive motility (67.4%), and viability (73.85%) especially with the highest concentration compared to control. Moreover, 40 and 80 ppm of ZnO-NPs supplemented orally in rams improved sperm motility and viability (Abaspour Aporvari *et al*., 2018). In contrast to bull semen supplemented with 10^{-6},10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} ZnO-NPs (Jahanbin *et al*., 2021), the abnormal sperm percentage of rams in this study declined linearly with the increase of ZnO-NPs in their semen extenders. Also, the abnormal sperm % declined when rams were supplemented 40 and 80 ppm ZnO-NPs orally (Abaspour Aporvari *et al*., 2018). The sperm plasma membrane functionality indicated by FMI% based on the membrane resistance to lose permeability barriers under stress condition (stretching in a hypo-osmotic medium) increased by 25.77% with the highest ZnO-NPs (P= 0.0001). In agreement with the obtained results, the FMI% of bull semen extender supplemented with 10^{-6} and 10^{-2} M ZnO-NPs which ranges from 81mg/ml to 814mg/ml was improved (69.3 and 62.4%) significantly (P < 0.05) compared to control (51.3%) group (Jahanbin *et al*., 2021). Only 80 ppm of ZnO-NPs improved the membrane resistance to lose permeability FMI% test (Abaspour Aporvari *et al*., 2018). Similar to human semen supplemented with ZnO-NPs (Isaac *et al*., 2017), cryopreserved ram semen of this study kept their fertilizing activity as indicated by increasing the percentages of acrosome integrity by 16.48%, 19.99%, and 30.63% from the lowest to the highest ZnONPs concentration compared to controls. This improvement in post-thaw semen characteristics was referred to the presence of ZnONPs around the spermatozoa (Isaac *et al*., 2017).

Although ZnO-NPs slightly decreased LPO in ram semen, but this decrease was not significant. The slight insignificant decrease in membrane LPO product (MDA) in extenders supplemented by 0.50, 1.00, and 1.50 mg/ml ZnONPs prove that nanoparticles efficiently scavenge the free radicals generated during freeze-thaw process as recorded in human (Isaac *et al*., 2017) and bull semen

Table 2. Effect of different concentrations of ZnO-NPs concentrations on seminal plasma (MDA, NO, Ascorbic acid, glutathione peroxidase (GPx), superoxide dismutase (SOD).

Oxidants/	ZnO-NPs concentrations				
antioxidants	0.0 mg/ml	0.5 mg/ml	1.0 mg/ml	1.5 mg/ml	P-value
MDA nmol/ml	6.67±1.03	5.89±0.42	5.50±0.20	6.03±0.26	NS
NO umol/L	$50.00±2.78^{a}$	$53.91±0.42^{ab}$	$56.50±0.31^{bc}$	$59.33±0.25^{c}$	0.000
AA g/L	$824.4±14.9^{a}$	$871.4±6.3^{b}$	$879.3±6.4^{b}$	$864.8±5.4^{b}$	0.001
GPx mU/mL	$82.14±11.21^{a}$	$73.49±10.86^{a}$	$170.75±35.99^{b}$	$32.42±6.95^{a}$	0.000
SOD U/ml	$2625±244^{c}$	$3000±92^{c}$	$1750^{b}±53.30^{c}$	$1250±141^{a}$	0.000
Zinc ug/dl	$94.41±0.31^{a}$	$237.27±1.33^{b}$	$265.84±3.97^{c}$	$305.59±4.50^{d}$	0.000
Cu µg/dl	$540.3±52.5^{ab}$	$491.87±34.29^{a}$	$580.57±34.38^{ab}$	$688.0±73.05^{b}$	0.059
CHO mg/dl	$160.7±10.0^{c}$	$144.27±2.21^{bc}$	$100.80±13.40^{a}$	$133.20±1.84^{b}$	0.000
LDL mg/dl	$117.29±9.35^{c}$	$82.47±5.64^{b}$	$48.54±13.23^{a}$	$83.18±11.33^{b}$	0.000
LDH U/L	$4857±104^{a}$	$4825±46^{a}$	$6938±211^{b}$	$7003±143^{b}$	0.000
ALP IU/L	$137.9±0.79^{b}$	$131.76±1.90^{a}$	$144.88±0.74^{c}$	$186.90±1.30^{d}$	0.000

Data are expressed as Mean ±standard error of mean (SEM).
Means with different superscripts (a,b,c,d) are significantly different at P<0.05, non-significant (NS). (n=16), lipid peroxide product (MDA, Malondialdehyde), Nitric oxide (NO), glutathione peroxidase (GPx), superoxide dismutase (SOD), Ascorbic acid (AA), Copper (Cu), Total cholesterol (CHO), Low density lipoproteins (LDL), Lactate dehydrogenase (LDL), Alkaline phosphatase (ALP)

Table 3. Pearson correlation coefficient between post-thaw semen characteristics and measured blood variables.

Semen parameters	NO	Zinc	SOD	GPx	ALP	LDH	Cu	CHO	LDL	AA
STM%	0.47	$.73^{**}$	$-.62^{*}$	-0.36	$.94^{**}$	$.63^{*}$	-0.23	-0.13	-0.12	0.2
SPM %	$.52^{\#}$	$.85^{**}$	$-.62^{*}$	-0.29	$.89^{**}$	$.68^{*}$	-0.21	-0.21	-0.22	0.3
SV %	0.46	$.73^{**}$	$-.61^{*}$	-0.36	$.94^{**}$	$.63^{*}$	-0.24	-0.12	-0.13	0.2
AS %	$-.68^{*}$	$-.90^{**}$	$.69^{*}$	0.08	$-.81^{**}$	$-.69^{*}$	-0.1	0.33	0.27	-0.31
FMI %	$.50^{\#}$	$.97^{**}$	-0.48	-0.09	$.68^{*}$	$.67^{*}$	-0.22	-0.34	-0.44	0.49
AI %	$.50^{\#}$	$.96^{**}$	$-.54^{\#}$	-0.16	$.71^{**}$	$.72^{**}$	-0.23	-0.35	-0.39	0.47

** Significant at P<0.01, * significant at P<0.05, # tended to be significant at P>0.05.
Sperm total motility (STM), Sperm progressive motility (SPM%), Sperm Viability (SV%), Abnormal sperm (AS%), functional membrane integrity (FMI)%, Acrosome integrity (AI%) Copper (Cu), Total cholesterol (CHO), Low density lipoproteins (LDL), nitric oxide (NO), Alkaline phosphatase (ALP), Ascorbic acid (AA), superoxide dismutase (SOD), glutathione peroxidase (GPx), Lactate dehydrogenase (LDH).

(Jahanbin *et al*., 2021). Similar to zinc nanoparticles, extenders containing 0.5 and 1 µg/ml selenium nanoparticles (SeNPs) improved post-thaw sperm motility, and viability index and reduced membrane integrity, acrosome defects, DNA deterioration and MDA concentrations in ram seminal plasma (Hozyen *et al*., 2019).

The role of nitric oxide (NO) in regulating spermatogenesis, sperm motility and maturation (Perera *et al*., 1996) has been confirmed in rams supplemented L-arginine which is nitric oxide precursor (Ozer Kaya *et al*., 2020). Though 0.50 mg of ZnO-NPs slightly increased NO either 1.00 or 1.50mg/ml ZnO-NPS supplemented diluents significantly elevated NO in the post-thaw ram semen of this work. In agreement with the negative correlation between abnormal sperm morphology and NO observed in rams supplemented ZnO-NPS of the present study, the rate of abnormal spermatozoa in the L-arginine group showed a significant decrease in the abnormal sperm morphology compared to the control rams (Ozer Kaya *et al*., 2020). The significant decrease in SOD activity with increasing ZnO-NPs to the ram semen and the marked negative correlations with good quality semen observed in the current study was also noted when 800 ppm of ZnO-NPs were supplemented orally to rams (Abaspour Aporvari *et al*., 2018). The increase in glutathione peroxidase values in post-thaw semen in rams of the current study is similar to the increase of antioxidant capacity in rams orally supplemented with 80 ppm of ZnO-NPs (Abaspour Aporvari *et al*., 2018). Contrary to the absence of any correlation between GPx and abnormal sperm in the current study, positive correlation was found between GPx activity in seminal plasma and abnormal tails were observed in buffaloes (Waheed *et al*., 2013). Similarly, significant correlation of GPx with both post-thaw viability indices and increased motility were noted (Waheed *et al*., 2013). The observation of the lowest GPx levels in the semen extended supplemented with 1.50 mg/ml ZnO-NPs and the highest levels semen extended supplemented with 1.00 mg/ml in the cryopreserved ram semen of this study, the addition of the three concentrations of ZnO-NPs to semen extenders preserved the values of ascorbic acid higher than controls and indicate that ascorbic acid was not consumed during scavenging reactive oxygen species and ZnO-NPs reduced OS evolution. The high insignificant correlations between ascorbic acid with FMI% and acrosome integrity agree with the addition of ascorbic acid to the bovine semen diluents during freezing and thawing to reduce cell damage due to its reactive oxygen species scavenging property (Beconi *et al*., 1993). In agreement with the association of ascorbic acid with ram FMI% and acrosome integrity, the addition of 4.5mg/ml ascorbic acid to bovine semen extender improved the motility and viability and achieved the highest membrane and acrosome integrities (Hu *et al*., 2010). In stallions, the addition of antioxidants such as ascorbic acid and cysteine to semen extender reduced the levels of ALP and LDH with no differences in sperm viability, sperm membrane integrity and morphology (Alamaary *et al*., 2020).

The higher positive correlations of zinc with the STM, SPM, SV, FMI and AI and its negative correlation with SA indicate that zinc acts as a powerful antioxidant, decreases the ROS production and reduces LPO so prevents cells from being destructed (Rogalska *et al*., 2009). The increase in zinc concentrations with the increasing supplemented dose from 0.50 to 1.50 mg/ml diluents indicates that ZnO-NPs did not penetrate the sperm plasma membrane (Isaac *et al*., 2017). Zinc is involved in sperm maturation (Henkel *et al*., 1999; Leonhard-Marek, 2001) and physiological zinc concentrations in seminal plasma are known to block the influx of ionized calcium to avoid early induction of acrosome reaction (Leonhard-Marek, 2001). In agreement with the obtained results, the increased release of zinc from ZnO-NPs correlated with an increased oxygen input and motility (Henkel *et al*., 2003)

Copper is necessary for many enzymes like the Cu– Zn–superoxide-dismutase (SOD), which is involved in cell protection against ROS. Copper is also needed for the cytochrome c oxidase that is responsible for energy supply and for cellular and humoral immunity (Leonhard-Marek, 2001) Elevated copper concentrations reduce oxidative processes and glycolysis which may cause sperm immobility and reduced viability. The increase of copper concentrations in semen diluent supplemented with 1.50 mg/ml semen and the negative non-significant low correlation of copper with all ram semen characteristics of this study indicate that the increased copper did not reach toxic levels that disrupts sperm motility and viability (Knazicka *et al*., 2012).

The significant increase ALP activity in seminal plasma of ram semen supplemented with 1.00 and 1.50 mg/ml diluents correlates with sperm quality parameters (Bucci *et al*., 2016). In normal stallions, seminal plasma alkaline phosphatase activity is considered a sperm-independent marker, (Turner and McDonnell, 2003). However, the increase of LDH activity indicated highest number of dead and abnormal sperm (Ezazi *et al*., 2019), Increase of LDH activity in post-thaw ram semen extenders supplemented with 1.00mg and 1.50mg/ml ZnO-NPs in semen diluents are associated with high sperm motility, viability, FMI%, and acrosome integrity and low abnormal sperm morphology. In agreement with the obtained findings, after cryopreservation, the activities of total LDH, increased in seminal plasma and decreased in spermatozoa (Huang *et al*., 2014).

Glycerol is routinely used as a membrane permeable cryoprotectant for freezing of ram spermatozoa (, Bucak *et al*., 2009; Başpınar *et al*., 2011; Çoyan *et al*., 2011; Bucak *et al*., 2013). LDL alone as a component in egg yolk protects sperm from cold shock during the cryopreservation processing by various mechanisms to maintain the integrity of sperm membrane (Snoeck *et al*., 2017). The lowest LDL and total cholesterol in semen extenders supplemented with 1.00mg/ml ZnO-NPs were not related to any post-thaw sperm motility, viability, FMI%, and acrosome integrity parameters. Similar to our results, LDL did not offer any additional benefits in terms of post-thaw motility in comparison with 20% egg yolk regardless of the presence/absence of permeable cryoprotectant or addition of cholesterol or phosphatidylcholine (Dong *et al*., 2011).

CONCLUSION

All zinc oxide nanoparticles concentrations added to the cryopreserved rams' semen (0.50, 1.00, 1.50 mg/ml) showed improvement in post-thaw semen motility and acrosome integrity, reduced the oxidative stress production, total cholesterol, LDL, alkaline phosphatase and lactate dehydrogenase with reduced the activity of SOD and abnormal sperm %. The recommended concentration of choice is 1.5mg/ml tris-based semen diluents.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

REFERENCES

Abaspour Aporvari, M.H., Mamoei, M., Tabatabaei Vakili, S., Zareei, M., 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. Arch. Razi Inst. 73, 121-129. doi: 10.22092/ari.2018.120225.1187.

Aboelmaaty, A.M., Omara, S.T., Aly, M.S., Kotpa, M.S., Ali, A.H., 2022. The antibacterial and anti-inflammatory effects of zinc oxide nanoparticles synthesized by Thymus vulgaris medicinal plant against Escherichia coli and Escherichia coli lipopolysaccharides. Egypt. Pharm. J. 21. DOI: 10.4103/epj.epj_98_21

Aksoy, M., Kaya, A., Vatansev, H., Tekeli T., 2002. Testosterone secretion and semen plasma enzyme activity in rams with genital pathology stimulated with GnRH. Theriogenology 57, 1907-1916. doi: 10.1016/s0093-691x02)00700-8.

Alamaary, M.S., Haron, A.W., Hiew, M.W.H., Ali, M., 2020. Effects of cysteine and ascorbic acid in freezing extender on sperm characteristics and level of enzymes in post-thawed stallion semen. Vet. Med. Sci. 6, 666-672, doi: 10.1002/vms3.315.

Al-badry, K.I., Aboud, Q.M., Zalzala, S.J., Ibrahim, F.F., Lateef, W.Y., 2018. Effect of trehalose and month of collection on DNA fragmentation in Holstein bull semen during dilution, cooling and thawing.

Online J. Vet. Res. 22, 237–246.
Amini, S., Masoumi, R., Rostami, B., Shahir, M.H., Taghilou, P., Arslan, H.O., 2019. Effects of supplementation of Tris-egg yolk extender with royal jelly on chilled and frozen-thawed ram semen characteristics. Cryobiology 88, 75-80. doi: 10.1016/j.cryobiol.2019.03.008.
Banday, M.N., Lone, F.A., Rasool, F., Rashid, M., Shikari, A., 2017. Use of antioxidants reduce lipid peroxidation and improve quality of crossbred ram sperm during its cryopreservation. Cryobiology 74, 25–30. doi: 10.1016/j.cryobiol.2016.12.008.
Başpınar, N., Coyan, K., Bucak, M.N., Tuncer, P.B., 2011. Effects of Dithioerythritol on ram semen after the freeze-thawing process. Cryobiology 63, 152–156. doi: 10.1016/j.cryobiol.2011.08.004.
Beconi, M.T., Francia, C.R., Mora, N.G., Affranchino, M.A., 1993. Effect of natural antioxidants on frozen bovine semen preservation. Theriogenology 40, 841–851.
Bucak, M.N., Keskin, N., Taşpınar, M., Çoyan, K., Başpınar, N., Cenariu, M.C., Bilgili, A., Öztürk, C., Kurşunlu, A.N., 2013. Raffinose and hypotaurine improve the post-thawed Merino ram sperm parameters. Cryobiology 67, 34–39. doi:10.1016/j.cryobiol.2013.04.007.
Bucak, M.N., Tuncer, P.B., Sarıözkan, S., Ulutaş, P.A., Coyan, K., Başpinar, N., Ozkalp, B., 2009. Effects of hypotaurine, cysteamine and aminoacids solution on post-thaw microscopic and oxidative stress parameters of Angora goat semen. Res. Vet. Sci. 87, 468-472. doi: 10.1016/j.rvsc.2009.04.014.
Bucci, D., Isani, G., Giaretta, E., Spinaci, M., Tamanini, C., Ferlizza, E., Galeati, G., 2014. Alkaline phosphatase in boar sperm function. Andrology 2, 100-106. doi: 10.1111/j.2047-2927.2013.00159.x.
Bucci, D., Giaretta, E., Spinaci, M., Rizzato, G., Isani, G., Mislei, B., Mari, G., Tamanini, C., Galeati, G., 2016. Characterization of alkaline phosphatase activity in seminal plasma and in fresh and frozen-thawed stallion spermatozoa. Theriogenology 85, 288-295. e2. doi: 10.1016/j.theriogenology.2015.09.007.
Çoyan, K., Başpinar, N., Bucak, M.N., Akalin, P.P., 2011. Effects of cysteine and ergothioneine on post-thawed Merino ram sperm and biochemical parameters. Cryobiology 63, 1–6. doi: 10.1016/j.cryobiol.2011.04.001.
Dey, A., Somaiah, S., 2022. Green synthesis and characterization of zinc oxide nanoparticles using leaf extract of *Thryallis glauca* (Cav.) Kuntze and their role as antioxidant and antibacterial. Microsc Res Tech. Apr 16. doi: 10.1002/jemt.241
Dong, Q-X., Rodenburg, S.E., Hill, D., Vandevoort, C.A., 2011. The role of low-density lipoprotein LDL) and high-density lipoprotein HDL) in comparison with whole egg yolk for sperm cryopreservation in rhesus monkeys. Asian J. Androl. 13, 459-464. doi: 10.1038/aja.2010.145.
El-Maddawy, Z.K., Abd El Naby, W.S.H., 2019 . Protective effects of zinc oxide nanoparticles against doxorubicin induced testicular toxicity and DNA damage in male rats. Toxicol. Res. Camb. 8, 654-662. doi: 10.1039/c9tx00052f.
Erfani Majd, N., Hajirahimi, A., Tabandeh, M.R., Molaei, R., 2021. Protective effects of green and chemical zinc oxide nanoparticles on testis histology, sperm parameters, oxidative stress markers and androgen production in rats treated with cisplatin. Cell Tissue Res. 384, 561-575. doi: 10.1007/s00441-020-03350-2.
Evans, G., Maxwell, W.M.C., 1987. Salamon's artificial insemination of sheep and goats 2nd Ed., Butterworths.
Ezazi, H., Abdi-Benemar, H., Taghizadeh, A., Khalili, B., Seifdavati, J., Jafaroghli, M., Elghandour, M.M., Salem, A.Z., 2019. The influence of dietary sunflower oil, rich in n-6 polyunsaturated fatty acids, in combination with vitamin C on ram semen parameters, sperm lipids and fertility. J. Sci. Food Agri. 99, 3803-3810. doi: 10.1002/jsfa.9602.
Ghallab A.M. Abou-Ahmed M.M., Fadl-Aya M., Shahat A.M., El- Badry D.A., Moawad, A.R., 2019. Optimization of the Protocol for Cryopreservation of Arabian Stallion Spermatozoa: Effects of Centrifugation, Semen Extenders and Cryoprotectants. Cryo Letters, 40: 129-138.
Henkel, R., Bittner, J., Weber, R., Hüther, F., Miska, W., 1999. Relevance of zinc in human sperm flagella and its relation to motility. Fertil. Steril. 6, 1138–1143. doi: 10.1016/s0015-028299)00141-7.
Henkel, R., Baldauf, C., Schill, W.B., 2003. Resorption of the element zinc from spermatozoa by the epididymal epithelium. Reprod. Dom. Anim. 38, 97-101. doi: 10.1046/j.1439-0531.2003.00417.x.
Hozyen, H.F., El Shamy, A.A., Farghali, A.A., 2019. In vitro supplementation of nano selenium minimizes freeze-thaw induced damage to ram spermatozoa. Inter. J. Vet. Sci. 84, 249-254.
Hu, J.H., Tian, W.Q., Zhao, X.L., Zan, L.S., Wang, H., Li, Q.W., Xin, Y.P., 2010. The cryoprotective effects of ascorbic acid supplementation on bovine semen quality. Anim. Reprod. Sci.211, 72-77.
Huang, X., Zhuang, P., Zhang, L., Zhao, F., Liu, J., Feng, G., Zhang, T., 2014. Effects of cryopreservation on motility characteristics and enzyme activity of sperm in a Chinese fish Nibea albiflora. Cryo Letters. 354, 267-276.
Isaac, A.V., Kumari, S., Nair, R., Urs, D.R., Salian, S.R., Kalthur, G., Adiga, S.K., Manikkath, J., Mutalik, S., Sachdev, D., Pasricha, R., 2017. Supplementing zinc oxide nanoparticles to cryopreservation medium minimizes the freeze-thaw-induced damage to spermatozoa. Biochem. Biophys. Res. Commun. 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. Biol. Trace Elem. Res. 199, 126-135.
Khalifa, T.A., Lymberopoulos, A.G., El-Saidy, B.E., 2008. Testing usability of butylated hydroxytoluene in conservation of goat semen. Reprod. Domest. Anim. 435, 525-530.
Knazicka, Z., Tvrda, E., Bardos, L., Lukac, N. 2012. Dose and time dependent effect of copper ions on the viability of bull spermatozoa in different media. J. Environ. Sci. Health 9, 1294-1300.
Kotdawala, A.P., Kumar, S., Salian, S.R., Thankachan, P., Govindraj, K., Kumar, P., Kalthur, G., Adiga, S.K., 2012. Addition of zinc to human ejaculate prior to cryopreservation prevents freeze-thaw-induced DNA damage and preserves sperm function. J. Assist. Reprod. Genet. 29,1447-1453.
Kumar, S., Millar, J.D., Watson, P.F., 2003. the effect of cooling rate on the survival on cryopreserved bull, ram and boar spermatozoa: a comparison of two controlled rate-cooling machine. Cryobiology 46, 246-253.
Leonhard-Marek, S., 2001. Influence of drugs, pollution and trace elements on male fertility. In: Busch W, Holzmann A, editors. Andrology in Veterinary Medicine. Schattauer: Stuttgart, pp. 474–481.
Mohamed, D.A., Abdelrahman, S.A., 2019. The possible protective role of zinc oxide nanoparticles ZnONPs) on testicular and epididymal structure and sperm parameters in nicotine-treated adult rats a histological and biochemical study. Cell Tissue Res. 375, 543-558.
Moretti, E., Terzuoli, G., Renieri, T., Iacoponi, F., Castellini, C., Giordano, C., Collodel, G., 2013. In vitro effect of gold and silver nanoparticles on human spermatozoa. Andrologia 456, 392-6.
O'Flaherty, C., Breininger, E., Beorlegui, N., Beconi, M.T., 2005. Acrosome reaction in bovine spermatozoa: Role of reactive oxygen species and lactate dehydrogenase C4. Biochim. Biophysic. Acta-General Subjects 17261, 96–101. https://doi.org/10.1016/j.bbagen.2005.07.012
Ozer Kaya, S., Kandemir, F.M., Gur, S., Erisir, M., Benzer, F., Kaya, E., Turk, G., Sonmez, M., 2020. Evaluation of the role of L-arginine on spermatological parameters, seminal plasma nitric oxide levels and arginase enzyme activities in rams. Andrologia 521, e13439.
Perera, D.M.D., Katz, M., Heenbanda, S.R., Marchant, S., 1996. Nitric oxide synthase inhibitor NG-monomethyl-l-arginine preserves sperm motility after swim-up. Fertil. Steril. 665, 830-833.
Revell, G., Mrode, R.A., 1994. An osmotic resistance test for bovine semen. Anim. Reprod. Sci. 36: 77-86.
Rogalska, J., Brzoska, M.M., Roszczenko, A., Moniuszko Jakoniuk, J., 2009. Enhanced zinc consumption prevents cadmium-induced alterations in lipid metabolism in male rats. Chem. Biol. Interact. 177, 142-152.
Shi, L.G., Yang, R.J., Yue, W.B., Xun, W.J., Zhang, C.X., Ren, Y.S., Shi, L., Lei, F.L., 2010. Effect of elemental nano-selenium on semen quality, glutathione peroxidase activity, and testis ultrastructure in male Boer goats. Anim. Reprod. Sci. 1182, 248-254.
Snoeck, P.P.N., Moura, L.C.O., Silva, M.C., Machado-Neves, M., Melo, M.I.V., Heneine, L.G.D., Henry, M., 2017. Effect of storage conditions on the LDL effectiveness in ovine sperm cryopreservation. Cryobiology 75, 88-90. doi: 10.1016/j.cryobiol.2017.01.007.
Topraggaleh, T.R., Shahverdi, A., Rastegarnia, A., Ebrahimi, B., Shafiepour, V., Sharbatoghli, M., Esmaeili, V., Janzamin, E., 2014. Effect of cysteine and glutamine added to extender on post-thaw sperm functional parameters of buffalo bull. Andrologia. 46, 777–783.
Turner, R.M., McDonnell, S.M., 2003. Alkaline phosphatase in stallion semen: characterization and clinical applications. Theriogenology 601, 1-10.
Waheed, M. M., Gouda, E.M., Khalifa, T.A.A., 2013. Impact of seminal plasma superoxide dismutase and glutathione peroxidase on cryopreserved buffalo spermatozoa. Anim. Reprod. Sci. 142, 126-130.
Yousef, M.S., Abdelhamid, H.N., Hidalgo, M., Fathy, R., Gómez-Gascón, L., Dorado, J., 2021. Antimicrobial activity of silver-carbon nanoparticles on the bacterial flora of bull semen. Theriogenology 161, 219-227.