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Ameliorative Nexus of Nano Zinc on the Redox State of Chilled and Cryopreserved Barki Ram Semen

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Abstract

The current study aimed to modify rams' semen redox hemostasis by different concentrations of Nano zinc. Using artificial vagina (15) semen ejaculates were collected, evaluated, and pooled then subjected to processing using different concentrations of nano zinc (10, 50, 100, and 150µg/ml). The concentration of alanine and aspartate transaminases enzyme (ALT-AST), alkaline phosphatase Enzyme (ALP), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), superoxide dismutase (SOD), and total antioxidant capacity (TAC) of chilled and freeze-thawed Barki ram semen were assessed. Nano zinc significantly enhances semen quality in a dose-dependent manner. Where, 150µg/ml of nano zinc potentially reduced the extracellular concentrations of ALT (14.00±1.01 and 28.58±0.58 U/L), AST (39.67±1.54 and 54.83±2.33 U/L), ALP (174.90±1.90and 316.80±1.90 U/L), and MDA (5.45±0.02and 6.21±0.01 nmol/ml) after both chilling and freeze-thawing process. Moreover, 150µg/ml treating dose induced an efficient production of SOD (7417±16.67 and 8600±76.38U/ml) after both chilling and freeze-thawing process, respectively. SOD is considered the most important antioxidant enzyme in the sperm cell that scavenges generated free radicles like H_2O_2. In this respect, 150µg/ml treating dose reported the lowest H_2O_2 level (0.042±0.002 and0.065±0.005 mM/L), and highest TAC concentration (1.62±0.006 and 1.69±0.005 mM/L) after both chilling and freeze-thawing process, respectively. In conclusion, ZnO-NPs in semen extender can modify the sperm redox hemostasis in a dose-dependent manner. It reduces the extracellular transaminases and phosphatases leakage, augmenting SOD activity, and modifies TAC potential. ZnO-NPs supplementation to Barki sperm extender enhances its sperm quality and reduces cryo-injury.

KEYWORDS
Zn-ONPs, Barki, Ram, Semen, Redox, Hemostasis

INTRODUCTION

The sperm oxidative stress defined as a critical imbalance in the sperm redox homeostasis characterized by overproduction of the reactive oxygen species (ROS) that leads to potential sperm entity damage (Agarwal *et al*., 2003; Chetsawang *et al*., 2006; Partyka *et al*., 2012). The intensity and extent of any oxidative stress usually influences the degree of cell responses ranging adaptation, modification to apoptosis and necrosis (Agarwal *et al*., 2006). During semen processing, the antioxidant defense systems as well as their concentration regularly subjected to a potential reduction that subsequently impairing the sperm antioxidative capacity (Surai *et al*., 1998; Zhandi *et al*., 2020). In this scenario, semen cryopreservation steps entries on exposing the sperm cells to different types of stressors (as the low temperature, light, diluent effects) which negatively affecting to various degree the sperm structural and functional competences (Topraggaleh *et al*., 2014; Vichas *et al*., 2018). The excessive polyunsaturated fatty acids content (PUFA-Arachidonic and Docosatetraenoic) in the sperm plasma membrane is the key factor that accelerates these negative effects (Buhr *et al*.,1994; Samadian *et al*., 2010; Al-badry *et al*., 2018; Elokil *et al*., 2019). These greater PUFA content prone the sperm cells to intense levels of lipid peroxidation especially in the sperm flagellum more than in the head (Griveau *et al*., 1994), these changes consequently affecting semen quality as well as its fertilizing potential (Vishwanath and Shannon, 2000; Medeiros *et al*., 2002; Partyka *et al*., 2012). For this reason, an efficient sperms' antioxidant defense system should build to protect them during all cryopreservation process. This hopeful antioxidant system should operate on many levels: preventing ROS generation, hindering ROS reactivity with the cellular components, breaking down ROS chain reactions, and finally scavenging the oxidative reaction products (Fraczek and Kurpisz, 2005). This trend was achieved by enriching the semen extenders with appropriate antioxidants that reduce ROS drastic effects on the sperm quality (Forouzanfar *et al*., 2010; Seifi-Jamadi *et al*., 2016). In between these powerfully nominated antioxidants is Zinc, which has excellent antioxidant properties by acting on most of the levels discussed above (Zago and Oteiza, 2001). For example, Zinc promotes metallothionein synthesizing which inhibits ROS propagation (Maret, 2000). It acts as a structural and catalytic co-factor for superoxide dismutase (SOD) which reduces ROS toxicity (Fer-

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reira and Matsubara, 1997), also acting as a cofactor of the zinc/copper iso-enzyme of superoxide dismutase (CuZnSOD) (Orr and Sohal, 1994; Atig *et al*., 2012). Finally, the presence of zinc prevents oxidation by binding to sulfhydryl (SH) groups in cell membrane proteins (Zago and Oteiza, 2001). Depending on the previous mechanisms, Zinc supplementation to semen extender was found to have a powerful antioxidant capability that reduces the cryopreservation mediated sperm damage (Hammadeh *et al*., 2009; Harris *et al*., 2011; Swain *et al*., 2016; Isaac *et al*., 2017; Mousavi *et al*., 2019; Jahanbin *et al*., 2021). Via regulating the rate of lipid peroxidation, which in turn resulted in low level of the toxic malondialdehyde (Rogalska *et al*., 2009; Dawei *et al*., 2009; Fayyad and Mahmood., 2019; Zhandi *et al*., 2019). Furthermore, zinc decreases DNase activity (Pizzino *et al*., 2017), maintains the sperm plasma membrane integrity (Aitken, 2018). Recently zinc oxide nanoparticles (ZnONPs, 1-100 nm) gained much attention in animal reproduction: rat and mouse (Afifi *et al*., 2015; Talebi *et al*., 2013), and human (Barkhordari *et al*., 2013), but little known about its effects on Barki rams' sperm cryopreservation. To the best of the authors' knowledge, the current study is the first trial that assessed *in vitro* the effect of different ZnONPs concentrations on the redox state hemostasis of cooled and cryopreserved Barki ram semen.

MATERIALS AND METHODS

Animals

Five mature trained Barki rams with an average of 2-3 years of age, weighted 50-70 Kg, were kept at Animal Reproduction Research Institute (A.R.R.I) Al-Haram, Giza farm were used for semen collection. Rams are clinically healthy and free from external parasites. Animals are maintained under standard environmental conditions where they are exposed to the natural photoperiod. Rams fed a balanced ration according to NRC (2007), get *ad libitum* access to water, and vaccinated and dewormed annually.

All procedures were authorized by the institutional review board for animal experiments of Faculty of Veterinary Medicine, Benha University, Egypt with ethical approval number (BUFVTM 01-03-23).

Synthesis of ZnO nanoparticles according to Mahamuni et al. (2019)

Zn acetate dihydrate precursor (0.1 M-Loba Fine Chemicals, Mumbai, India) was refluxed in diethylene glycol and triethylene glycol at 180°C and 220°C, respectively, to produce ZnO nanoparticles that are less than 100 nm in size. The reaction time varied between 2 and 3 hours with and without sodium acetate (0.01 M). The solution kept on a magnetic stirrer at 80°C for one and half hours prior to refluxing again. Following the completion of the refluxing action, the samples centrifuged at 8000 rpm for 15 minutes, and then washed 3 times with distilled water and ethanol; finally, dried overnight at 80°C (Fig. 1).

Experimental procedures

Semen collection

Fifteen semen ejaculates were collected using ram's artificial vagina. The pass semen samples should have at least 75% of the initial motility, and 2×10^9 sperm cells/ml and total abnormal sperm ≤ 15%. The chosen semen samples were evaluated and pooled (to remove the variations between semen samples and to have plenty semen to replicate).

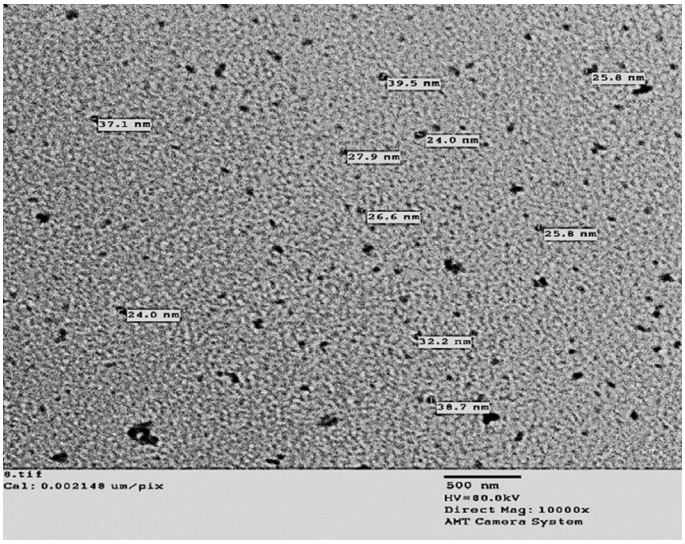

Fig.1. Different nano zinc particles sizes, usually run below 100nm.

Barki ram semen processing

Semen samples are diluted at a rate of 1:19 according to Khalifa *et al*. (2004), with OpticXcell$_2$® extender (REF: 026218, LOT 7083001, IMV technologies, France) to which 10, 50,100 and 150µg/ml of nano zinc were added (Lab. prepared, see acknowledgement section), the blank diluent considered as control (Barkhordari *et al*., 2013). OpticXcell$_2$® extender is a liposome-based formula that protects sperm without the sanitary concern associated with the use of egg yolk containing broad-spectrum antibiotics. Immediately after dilution, the extended semen samples (3 trails) gradually cooled to 5°C for 1hr and equilibrated for 2 h. At this stage, each treated semen sample divided into two halves one-half subjected directly to centrifugation at (3000rpm) for 10 minutes to collect the supernatant solution, which preserved at -20°C until measuring the antioxidant parameters. The other half of each treated semen sample loaded in 0.25 ml PVC mini straws (IMV, L, Aigle, France). Then arranged horizontally on freezing racks at 5°C before exposed to liquid nitrogen vapor (LN_2) inside a closed Styrofoam Box (54x35x18cm) containing about 10 liters of LN_2 (Mohammed *et al*.,1998); for 15 min at a height 6 cm above the surface of LN_2 before plunged directly into it (Khalifa, 2001). The frozen straws then transferred into the liquid nitrogen storage container. After two days of storage, two straws from each concentration thawed by dropping them into a water bath at 40°C for 30 seconds (Khalifa *et al*., 2004). Separately, the straws from each concentration wiped dry after thawing and unplugged by cutting off with scissors, then transferred into 2ml pre-warmed, clean, and narrow glass test tubes. The thawed semen samples subjected to centrifugation at (3000rpm) for 10 minutes to collect the supernatant solution, which preserved at -20°C until measuring the different antioxidant parameters.

Estimation of transaminases and phosphatases enzymes (U/L)

To measure Aspartate-Aminotransferase (AST); Alanine-Aminotransferase (ALT) and Alkaline Phosphatase (ALP) enzymes spectrophotometrically, the supernatant solution of each nano zinc treated semen samples (different groups) was collected after centrifugation at (800 rpm) for 10 mins. Reagent kits was supplied by Reactivos GPL, Barcelona, España for estimating AKP

(ALP LQ code No: EZ002LQ) at 405 nm wavelength, AST (GOT/AST-Code No: EZ012) at 340 nm wavelength, and ALT (GPT/ALT-LQ-code No. EZ016LQ) at 340 nm wavelength as described by Reitman and Frankel (1957), and Tietz (1976). A minimum of (3) observations tested for each enzyme in each treated group.

Superoxide Dismutase (SOD / U/ml)

The SOD activity measured spectrophotometrically at 560 nm wavelength over (5) minutes by superoxide dismutase Biodiagnostic kits (CAT. No. SD 25 21) according to Nishikimi *et al*. (1972). A minimum of 3 observations tested for SOD in each group.

Hydrogen peroxide (H_2O_2 / mM/L)

Hydrogen peroxide measured calorimetrically according to Fossati *et al*. (1980), and Aebi (1984) at 510 nm wavelength over 10 minutes by hydrogen peroxide Biodiagnostic kits (CAT. NO. HP 25). A minimum of 3 observations tested for H_2O_2 in each group.

MDA or Lipid Peroxidation (LPO / nmol/ml)

The principle of this test focused on the reactivity of MDA with Thiobarbituric acid to form thiobarbituric, the reaction should be applied at 95°C for 30 min in acidic medium. The absorbance of the resultant-colored product (pink) measured at wavelength of 534 nm by Malondialdehyde Biodiagnostic kits (CAT. No. MD 25 29) according to Satoh (1978), and Ohkawa *et al*. (1979). A minimum of 3 observations tested for MDA in each treated group.

Total Antioxidant Capacity (TAC / mM/L)

The antioxidative capacity in certain semen samples was assessed by the reaction of the antioxidants in this semen sample with fixed amount of exogenous H_2O_2. The residual H_2O_2 was measured calorimetrically through an enzymatic reaction that involves the conversion of 3, 5, dichloro-2- hydroxy benzensulphonate to a colored product.

Biodiagnostic total antioxidant capacity kits (CAT. NO. TA 25 13) was used for TAC estimation at wavelength 505nm according to Koracevic *et al*. (2001). A minimum of 3 observations tested for TAC in each treated group.

The rate of the extracellular ALT, ALP, AST, and MDA Leakage (%), and the rate of H_2O_2, SOD, and TAC production (%) was calculated according to Khalifa (2001).

Statistical analysis

Data statistically analyzed by Graph Pad Prism software 2007 version 5.03 (Graph Pad Prism, San Diego, CA) to determine the significant difference between treatment groups by one-way analysis of variance (ANOVA). Comparison of means was carried out by Duncan's Multiple Range test (LSD) using Costat Computer Program (1986), Differences were significant at ($P<0.05$) level and determined by superscripted letters.

RESULTS

Table 1 shows that there was a highly significant difference ($p<0.0001$) between nano zinc treated ram semen samples concerning the level of Alanine transaminase enzyme (ALT) that was leaked during semen processing procedures. Where, semen samples treated with 150 µg/ml revealed the lowest extracellular ALT leakage level after chilling (14.00±1.01 U/L), whereas the control group showed the highest extracellular ALT leakage level (32.67±0.58 U/L). While the other nano zinc treated samples recorded values in between. Where, 10, 50 and 100 µg/ml of nano zinc presented (26.83±0.58, 22.17±0.58 and 18.67±0.58 U/L, respectively). Moreover, this protective effect of Nano zinc on the plasma membrane of diluted chilled ram semen contin-

Table 1. Effect of different Nano zinc concentrations on alanine transaminase enzyme (ALT) level of chilled and cryopreserved Barki ram semen (Mean±S.E).

Nano ZN Conc.	After chilling U/L	After Thawing U/L	Difference U/L	ALT_{ELR} %
Control	32.67±0.58^{a}	94.50±1.01^{a}	61.83±0.58^{a}	189.00±2.88^{a}
10 µg/ml	26.83±0.58^{b}	70.85±1.54^{b}	44.02±1.01^{b}	163.30±2.02^{b}
50 µg/ml	22.17±0.58^{c}	57.17±1.54^{c}	35.00±1.01^{c}	158.00±2.30^{b}
100 µg/ml	18.67±0.58^{d}	42.58±2.54^{d}	23.91±2.10^{d}	127.70±8.68^{c}
150 µg/ml	14.20±1.01^{e}	28.58±0.58^{e}	14.38±0.58^{e}	106.00±11.93^{d}
Overall means	22.91±1.73	58.74±6.11	35.83±4.38	-

Results are presented as mean±SEM, (n= 3 replicates/group)
Values with different superscript letters ($^{a, b, c}$) in the same column were significantly different at least ($P<0.001$). ALT_{ELR} = ALT extracellular leakage rate

Table 2. Effect of different Nano zinc concentrations on aspartate transaminase enzyme (AST) of chilled and cryopreserved Barki ram semen (Mean±S.E).

Nano ZN Conc.	After chilling U/L	After Thawing U/L	Difference U/L	AST_{ELR} %
Control	66.50±1.01^{a}	112.00±1.01^{a}	45.50±1.01^{a}	68.33±2.18^{a}
10 µg/ml	59.50±1.01^{b}	96.83±2.54^{b}	37.33±1.54^{b}	63.00±1.52^{ab}
50 µg/ml	53.67±0.58^{c}	86.92±1.54^{c}	33.25±1.01^{c}	62.00±1.52^{ab}
100 µg/ml	47.25±1.01^{d}	73.50±2.02^{d}	26.25±1.01^{d}	55.67±0.88^{b}
150 µg/ml	39.67±1.54^{e}	54.83±2.33^{e}	15.16±1.54^{e}	38.33±4.17^{c}
Overall means	53.32±2.37	84.82±5.28	31.50±2.78	

Results are presented as mean±SEM, (n= 3 replicates/group)
Values with different superscript letters ($^{a, b, c}$) in the same column were significantly different at least ($P<0.001$). AST_{ELR} = AST extracellular leakage rate

ued after freeze-thawing process. Where, the level of ALT leakage level still low with 150 µg/ml treated semen sample (28.58±0.58 U/L), while the level of ALT leakage level for the control group still shows the highest records (94.50±1.01 U/L). Whereas the other nano zinc concentrations (10, 50, and 100 µg/ml) presented variable values in between (70.85±1.54, 57.17±1.54, and 42.58±2.54 U/L, respectively).

Table 2 shows that there was a highly significant difference ($p<0.0001$) between the different nano zinc treated ram semen samples concerning the level of aspartate transaminase enzyme (AST) that was leaked during semen processing procedures. Where, semen samples treated with 150 µg/ml revealed the lowest AST leakage level (39.67±1.54 U/L), whereas the control group showed the highest extracellular AST leakage value (66.50±1.01 U/L). The other nano zinc concentrations recorded values in between. Where, 10, 50 and 100 µg/ml of nano zinc presented (59.50±1.01, 53.67±0.58, and 47.25±1.01 U/L, respectively). Moreover, this protective effect of Nano zinc on the plasma membrane of chilled Barki ram semen continued after freeze-thawing process. Where the AST leakage level still low with 150µg/ml treated semen sample (54.83±2.33 U/L); while AST level of control group still display the highest extracellular leakage value (112.0±1.01U/L). Whereas the other nano zinc concentrations (10, 50, and 100) showed variable values between 96.83±2.54, 86.92±1.54, and 73.50±2.02 U/L, respectively.

Table 3 shows that there was a highly significant difference ($p<0.0001$) between the different nano zinc treated groups concerning the extracellular leaked alkaline phosphatase enzyme (ALP) of Barki ram semen. Where, 150 µg/ml treated semen samples showed the lowest ALP leakage level (174.9±1.90 U/L), whereas the control group showed the highest ALP leakage level (226.6±2.91 U/L). Moreover, this protective effect of Nano zinc on the plasma membrane of the chilled ram semen continued after freeze-thawing process. Where, ALP leakage level still low with 150µg/ml treated semen sample (316.8±1.90 U/L); while, ALP leakage level of control group revealed the highest level without any improvement (533.50±2.91 U/L). While 10, 50 and 100 µg/ml treated groups showed values that run in between control and 150 µg/ml after both chilling and freeze-thawing process (Table 3).

Table 3. Effect of different Nano zinc concentrations on alkaline phosphatase enzyme (ALP) of chilled and cryopreserved Barki ram semen (Mean±S.E).

Nano ZN Conc.	After chilling U/L	After Thawing U/L	Difference U/L	ALP_{ELR} %
Control	226.60±2.91[a]	533.50±2.91[a]	306.90±5.04[a]	135.30±3.84[a]
10 µg/ml	217.80±1.90[b]	452.10±3.81[b]	234.30±1.90[b]	107.70±0.33[b]
50 µg/ml	210.10±1.10[c]	430.10±1.10[c]	220.00±1.10[c]	104.70±0.88[b]
100 µg/ml	192.50±2.91[d]	387.20±2.91[d]	194.70±3.81[d]	101.30±3.18[b]
150 µg/ml	174.90±1.90[e]	316.80±1.90[e]	141.90±3.81[e]	81.33±3.18[c]
Overall means	204.38±5.02	423.94±19.17	219.56±14.44	

Results are presented as mean±SEM, (n= 3 replicates/group)
Values with different superscript letters ([a, b, c,]) in the same column were significantly different at least ($P<0.001$). ALP_{ELR} = ALP extracellular leakage rate.

Table 4. Effect of different Nano zinc concentrations on hydrogen peroxide (H_2O_2) of chilled and cryopreserved Barki ram semen (Mean±S.E).

Nano ZN Conc.	After chilling mM/L	After Thawing mM/L	Difference mM/L	$H_2O_{2\ EGR}$ %
Control	0.078±0.0006[a]	0.167±0.001[a]	0.089±0.0006[a]	113.70±0.88[a]
10 µg/ml	0.076±0.0006[a]	0.161±0.001[a]	0.085±0.001[a]	111.70±0.88[a]
50 µg/ml	0.070±0.001[b]	0.141±0.001[b]	0.071±0.0006[b]	101.00±2.64[b]
100 µg/ml	0.058±0.002[c]	0.111±0.0016[c]	0.053±0.0006[c]	91.67±3.528[b]
150 µg/ml	0.042±0.002[d]	0.065±0.005[d]	0.023±0.002[d]	55.00±5.00[c]
Overall means	0.065±0.003	0.129±0.01	0.064±0.006	

Results are presented as mean±SEM, (n= 3 replicates/group)
Values with different superscript letters ([a, b, c,]) in the same column were significantly different at least ($P<0.001$). $H_2O_{2\ EGR}$ = H_2O_2 extracellular generation rate.

Table 5. Effect of different Nano zinc concentrations on malondialdehyde or lipid peroxide (MDA) of chilled and cryopreserved Barki ram semen (Mean±S.E).

Nano ZN Conc.	After chilling nmol/ml	After Thawing nmol/ml	Difference nmol/ml	MDA_{EGR} %
Control	6.53±0.10[a]	10.31±0.11[a]	3.78±0.03[a]	58.00±1.15[a]
10 µg/ml	6.27±0.04[b]	9.27±0.11[b]	3.00±0.06[b]	47.67±0.66[b]
50 µg/ml	6.06±0.01[c]	8.59±0.03[c]	2.53±0.02[c]	41.67±0.33[c]
100 µg/ml	5.89±0.07[c]	7.23±0.05[d]	1.34±0.07[d]	23.00±1.52[d]
150 µg/ml	5.45±0.02[d]	6.21±0.01[e]	0.76±0.03[e]	14.00±0.57[e]
Overall means	6.04±0.09	8.32±0.38	2.28±0.29	

Results are presented as mean±SEM, (n= 3 replicates/group)
Values with different superscript letters ([a, b, c,]) in the same column were significantly different at least ($P<0.001$). MDA_{EGR} = MDA extracellular generation rate.

Table 4 shows that there was a highly significant difference ($p<0.0001$) between the different nano zinc treated groups concerning the generated hydrogen peroxide (H_2O_2) level especially after chilling. Where, semen sample treated with a diluent containing 150µg/ml of nano zinc revealed the lowest H_2O_2 value (0.042±0.002 mM/L), whereas the control group showed the highest record (0.078±0.0006 mM/L). While the other nano zinc treated semen samples (10, 50, and 100µg/ml) revealed steadily decreased values in between as follow (0.076±0.0006, 0.070±0.001 and 0.058±0.002 mM/L, respectively). Moreover, this scavenging effect of nano zinc in the chilled ram semen continued to some extent after the freeze-thawing process. Where, the H_2O_2 level still low with 150 µg/ml treated semen sample (0.065±0.005 mM/L); although the record shows a slight elevation if compared with the same parameter level directly after chilling, while H_2O_2 level of the control group remain at the highest value of H_2O_2 (0.167±0.001 mM/L). Semen samples treated with 10, 50,100 µg/ml showed slight rise in H_2O_2 level after the freeze-thawing process, but their values still run in between control and 150µg/ml treated groups (Table 4).

Table 5 shows that there was a highly significant difference ($p<0.0001$) between the different nano zinc treated semen samples concerning level of produced malondialdehyde or lipid peroxide (MDA). Where, semen sample treated with 150µg/ml revealed the lowest MDA level (5.45±0.02 nmol/ml), whereas the control group showed the highest MDA level (6.53±0.10 nmol/ml). Moreover, this protective effect of nano zinc on the plasma membrane of chilled ram semen continued after freeze-thawing process. Where, the MDA level still low with 150 µg/ml treated semen sample (6.21±0.01 nmol/ml); while MDA level of control group remain at the highest concentration (10.31±0.11 nmol/ml).

Table 6 shows that there was a highly significant difference ($p<0.0001$) between the different nano zinc treated groups concerning the level of generated superoxide dismutase (SOD). Where, semen sample treated with 100 and 150 µg/ml showed the highest SOD level (7318±15.90 and 7417±16.67 U/ml, respectively), While the control group displayed the lowest SOD value (6318±143.8 U/ml). The other nano zinc treated semen samples (10, 50µg/ml) revealed values in between as follow (6833±72.65 and 7200±28.87 U/ml, respectively). Moreover, this ameliorative nexus effect of nano zinc on the redox state of chilled Barki ram semen continued after freeze-thawing process. Where, SOD level improved significantly with 100 and 150 µg/ml treated semen samples (8150±76.38 and 8600±76.38 U/ml, respectively). The control group showed a slight elevation in SOD value (6650±144.3 U/ml) if compared with the same parameter level directly after chilling in the same group, even though the control group still attained the lowest values if compared with other nano zinc treated groups. Furthermore, 10, 50 µg/ml showed significant rise in SOD level after the freeze-thawing process (7317±44.10 and 7817±33.33 U/ml) but their values still in between control, 100, and 150µg/ml treated groups. From Table 6, it was clear that there was significant difference ($p<0.0001$) between nano zinc treated groups concerning the rate difference in SOD level both after chilling and thawing where 100 and 150µg/ml of nano zinc potentially increase the level of SOD production after thawing (831.7±65.85 and 1183±66.67 U/ml, respectively).

Table 7 shows that there was a highly significant difference ($p<0.0001$) between the different nano zinc treated semen samples concerning the level of the total antioxidant capacity (TAC). Where, 150 µg/ml treated semen sample revealed the highest TAC value (1.62±0.006 mM/L), whereas the control group showed the lowest TAC value (1.55±0.001 mM/L). Whereas the other nano zinc treated groups recorded values in between. Where, 10, 50 and 100 µg/ml nano zinc treated groups presented (1.57±0.001, 1.59±0.006, and 1.61±0.006 mM/L, respectively). Moreover, this ameliorative effect of nano zinc on chilled ram semen continued after the thawing process. Where, 150µg/ml treated semen sample showed improved TAC value (1.70±0.005 mM/L), while TAC level of control group is the lowest value (1.57±0.005). Whereas the other nano zinc concentrations (10, 50, and 100 µg/ml) showed variable values in between (1.60±0.005, 1.63±0.003, and

Table 6. Effect of different Nano zinc concentrations on superoxide dismutase (SOD) of chilled and cryopreserved Barki ram semen (Mean±S.E).

Nano ZN Conc.	After chilling U/ml	After Thawing U/ml	Difference U/ml	SOD_{EPR} %
Control	6318±143.80^{e}	6650±144.30^{e}	332.00±15.90^{d}	5.33±0.33^{d}
10 µg/ml	6833±72.65^{b}	7317±44.10^{d}	484.00±33.33^{cd}	7.00±0.57^{cd}
50 µg/ml	7200±28.87^{a}	7817±33.33^{c}	617.00±44.10^{c}	8.66±0.66^{c}
100 µg/ml	7318±15.90^{a}	8150±76.38^{b}	832.00±65.85^{b}	11.33±0.66^{b}
150 µg/ml	7417±16.67^{a}	8600±76.38^{a}	1183.00±66.67^{a}	16.00±1.00^{a}
Overall means	7017.20±110.87	7706.80±183.08	689.60±81.46	

Results are presented as mean±SEM, (n= 3 replicates/group)
Values with different superscript letters ($^{a, b, c,}$) in the same column were significantly different at least ($P<0.001$). SOD_{EPR} = MDA extracellular production rate.

Table 7. Effect of different Nano zinc concentrations on total antioxidant capacity (TAC) of chilled and cryopreserved Barki ram semen (Mean±S.E).

Nano ZN Conc.	After chilling mM/L	After Thawing mM/L	Difference mM/L	TAC_{PR} %
Control	1.55±0.001^{e}	1.57±0.005^{e}	0.02±0.007^{d}	1.00±0.57^{d}
10 µg/ml	1.57±0.001^{d}	1.60±0.005^{d}	0.03±0.005^{c}	1.66±0.33^{cd}
50 µg/ml	1.58±0.006^{c}	1.62±0.003^{c}	0.04±0.005^{c}	2.66±0.33^{bc}
100 µg/ml	1.60±0.006^{b}	1.66±0.009^{b}	0.06±0.003^{b}	3.33±0.33^{ab}
150 µg/ml	1.62±0.006^{a}	1.69±0.005^{a}	0.07±0.003^{a}	4.33±0.33^{a}
Overall means	1.58±0.007	1.63±0.012	0.044±0.005	

Results are presented as mean±SEM, (n= 3 replicates/group)
Values with different superscript letters ($^{a, b, c,}$) in the same column were significantly different at least ($P<0.001$). TAC_{PR} = TAC production rate.

1.66±0.009 mM/L, respectively).

DISCUSSION

Zn is a trace mineral playing vital roles in cellular functions and integrity. It acts as a cofactor for many DNA and RNA polymerases (Root *et al*., 1979; Prasad, 2009). Moreover, it acts as co-factor in many enzymes that are involved in protein, lipid, and carbohydrate metabolism as well as DNA transcription and protein synthesis (Cummings and Kovacic, 2009). Besides, it exerts positive roles on semen quality by increasing its total antioxidant capacity (TAC) to combat the excessively generated ROS during the processes of dilution, cooling, and cryopreservation (Zhandi *et al*., 2019; Afifi *et al*., 2015; Khoobbakht *et al*., 2018). Disrupted redox state, DNA fragmentation, lipid peroxidation, and protein oxidation are the main deleterious effects of the generated ROS during semen processing (El-Maddawy and Abd Elnaby, 2019). Moreover, ROS accelerates the mitochondrial dependent apoptosis by stimulating mitogen activated protein kinase signaling and down regulating the expression of many antioxidant enzymes like SOD2 and peroxiredoxin-1 (Liu *et al*., 2016). Essentially, the sperm mitochondrial sheath (Kerns *et al*., 2018; Song and Sutovsky, 2019) and sperm chromatin (Kvist, 1980; Roomans *et al*.,1982) are stabilized by zinc bridges.

Analysis of the sperm extracellular enzymatic activity usually used as bio-diagnostic markers that determine the suitability of cryopreserved sperm cells for fertilization as it measures directly the potency of the sperm cell membrane.

Transaminases and phosphatases enzymes (AST, ALT, and ALP) are vital intracellular enzymes that share efficiently in the sperm metabolism to generate the sufficient energy that is required for sperm motility and survivability (Sirat *et al*., 1996; Duan and Goldberg, 2003; Perumal, 2014). Moreover, transaminases and phosphatases levels in extracellular are correlated directly to the degree of sperm membrane stability (Sirat *et al*., 1996; Ciereszko *et al*., 1992; Dogan *et al*., 2009), acrosomal damage (Sharma *et al*., 2001; Okab, 2007) and consequently the semen quality.

The current study (Tables 1, 2 and 3) revealed that nano zinc supplementation to Barki ram semen extender can modulate the extracellular ALT, ALP, AST levels after both chilling, and freeze-thawing processes in dose dependent manner. Although, these positive effects were clear in 150 µg/ml treated group that showed the lowest extracellular ALT, ALP, AST records (14.00±1.01 and 28.58±0.58 U/L), (174.90±1.90 and 316.80±1.90 U/L), and (39.67±1.54 and 54.83±2.33 U/L) after both chilling and free-thawing processes, respectively. Furthermore, from Tables 1, 2 and 3, the addition of nano zinc to Barki ram semen processing has a powerful ability to preserve Barki sperm cells integrity also in dose dependent manner. This integrity preserving effect was clear from the rate of difference in the level of extracellular ALT and ALP both after chilling and thawing processes. Where, 150µg/ml treated samples presented the lowest difference in the extracellular ALT, ALP, and AST (14.58±0.58, 141.90±3.81 and 15.16±1.54 U/L, respectively); which it means the lowest level of membrane damage and enzymatic leakage on using 150µg/ml of nano zinc during the process of Barki ram semen extension. The current study results came in agreement with Daader *et al*. (1993) who reported that there was a negative correlation between the extracellular AST/ALT ratio and semen quality. Where, the higher extracellular transaminases and phosphatases activities in control, 10, 50, and 100 µg/ml treated semen samples (Tables, 1, 2 and 3) indicating an enzymatic leakage due to structural damage on the level of sperm mitochondria (Strzegek, 1988). Besides, it indicates disrupted membrane integrity and stability (Katila, 2001; Gündoğan, 2006; Attia and Kamel, 2012; Chaudhary *et al*., 2018; Alamaary *et al*., 2020). In other words, the extracellular transaminases and phosphatases leakage is considered as an index for the optimization of the semen cryopreservation process (Upreti *et al*., 1996; Sharma *et al*., 2003; Tejaswi *et al*., 2016). Moreover, the current results came in harmony with Cocchia *et al*. (2011) and Abdel-Wareth *et al*. (2020) who reported that ALT and AST in the seminal plasma of Awassi rams significantly decreased during cooling periods by the powerful antioxidant roles of Zn-ONPs.

The level of H_2O_2 significantly decreased in the extender containing Zn-ONPs than control in a dose dependent manner. Where, 150 µg/ml treated semen samples recorded the lowest H_2O_2 level (0.042±0.002 and 0.065±0.005 mM/L) after both chilling and freeze-thawing processes, respectively. Wyck *et al*. (2018) reported that exposing of bull spermatozoa to H_2O_2 resulted in increased DNA damage through impairment of DNA demethylation during epigenetic reprogramming in early embryonic development.

Zn is an important factor that has a vital ability to stabilize the sperm plasma membrane through its interaction with the sulfhydryl groups (Dawei *et al*., 2010). It was assumed that supplementation of semen extenders with Zn-ONPs can enhance its total antioxidant capacity leading to a significant reduction in the plasma membrane lipid peroxidation resulting in higher stability in the sperm acrosome and plasma membrane after thawing (Thys *et al*., 2009). In harmony with these previous reports, the current study results revealed that the addition of Zn-ONPs to the Barki ram semen diluent significantly reduced its lipid peroxidation rate (LPOR) in dose dependent manner. Where, 100 and 150 µg/ml Zn-ONPs treated semen samples recorded the lowest values of malondialdehyde or lipid peroxide (MDA) for both chilled and cryopreserved Barki ram semen samples (5.89±0.07 and 7.23±0.05 nmol/ml), and (5.45±0.02 and 6.21±0.01 nmol/ml), respectively. This structural protecting effect for nano zinc when added to Barki semen extender was obvious from the rate of difference in the level of MDA after both chilling and thawing processes. Where, 100 and 150 µg/ml treated semen samples showed the lowest rate of difference in MDA after both chilling and thawing (1.34±0.07 and 0.762±0.03 nmol/ml, respectively). The current study results came in agreement with Dawei *et al*. (2009); Zanganeh *et al*. (2013), and Ma *et al*. (2020) who reported that supplementation of semen diluent with Zn-ONPs reduce MDA levels thus preserving the sperm plasma membrane integrity and improved mitochondrial activity during cooling process. Even though, the current results came partially in agreement with Isaac *et al*. (2017) who reported that Zn-ONPs slightly not significantly decreased the LPO in ram semen, they add that the slight insignificant decrease in MDA also proves the efficient ability of Zn-ONPs to scavenge the produced ROS during freeze-thawing process.

SOD is the most important sperm antioxidant enzyme that has a highly significant correlation with semen quality. The intracellular SOD activity in a given semen sample is considered a good predictor for their survival time (Perumal, 2014). Additionally, a negative correlation between sperm DNA fragmentation and SOD activity was detected suggesting the crucial role of SOD in combating ROS that accelerates the process of apoptosis (Agarwal *et al*., 2003; Perumal., 2014; Yan *et al*., 2014). SOD prevents premature sperm hyper-activation and capacitation induced by the free radicals (de-Lamirande and Gagnon,1995), maintains acrosomal integrity (Urata *et al*., 2001). Furthermore, it stabilizes the sperm plasma membrane integrity during sperm liquid storage so increases the motility (Holt, 2000; Bilodeau *et al*., 2001; El-Sisy *et al*., 2008; Cocchia *et al*., 2011; Asadpour *et al*., 2012; Perumal., 2014), protecting the flagella of the sperm and reducing MDA production (Asadpour *et al*., 2012). In addition, it preserves the functional integrity of the mitochondrial membrane by Mn-SOD enzyme (Yan *et al*., 2014), therefore, increasing the sperm cryo-resistance. Zn is a part of superoxide dismutase (SOD) (Narasimhaiah *et al*., 2018). In accordance with the previous finding, the current study results revealed that nano zinc supplementation to Barki semen extender has a potent enhancing effect on SOD production in dose dependent manner. This zinc motivated effect on SOD production was profound, especially with 100 µg/ml (7318±15.90 and 8150±76.38 U/ml, respectively), and 150 µg/ml (7417±16.67 and 8600±76.38 U/ml, respectively) after both

chilling and thawing processes. Furthermore, Table 6 clarifies that nano zinc supplementation to Barki ram semen diluent provides excellent protection against ROS effects especially after thawing process by activating the intrinsic antioxidant enzymes (production/function) like SOD also in dose dependent manner. Where, 100µg/ml and 150µg/ml achieved the highest difference in SOD level between the process of chilling and thawing (831.70±65.85 and 1183.00±66.67 U/ml, respectively) meaning more structural and functional protection (cryo-tolerance) for Barki ram spermatozoa during cryopreservation.

On the contrary, Abaspour Aporvari *et al*. (2018) reported that there was a significant drop-in SOD activity with increasing Zn-ONPs to the ram semen and this drop significantly exerts negative correlations with semen quality. This disagreement with Abaspour Aporvari *et al*. (2018) was attributed to the dose difference, and how Zn-ONPs used where the current study used it *in vitro* (on the semen extender) but the former used Zn-ONPs in vivo (orally).

Finally, the current study showed that the total antioxidant capacity (TAC) of Barki ram semen was improved when treated with different concentrations of nano zinc (10, 50, 100, 150 µg/ml) in dose dependent manner. Where, 100 (1.60±0.006 and 1.66±0.00 mM/L) and 150µg/ml (1.62±0.006 and 1.69±0.005 mM/L) treated groups recorded the highest level of TAC after both chilling and thawing processes if compared with other nano zinc treated groups. The improving effects of Zn-ONPs on the total antioxidant potential indicating ideal protecting effects of ZnONPs on Barki ram sperm cell redox state, which directly affects its structural and functional integrity, and indirectly affecting its fertilizing capacity and rate of embryo development.

CONCLUSION

In vitro supplementation of Zn-ONPs to Barki sperm diluent can modulate the sperm redox hemostasis in a dose dependent manner by reducing the extracellular transaminases and phosphatases leakage by enhancing sperm membrane stability, keeping the sperm plasma membrane functional and structural integrity by reducing the rate of lipid peroxidation. Zn-ONPs in Barki ram semen diluent is an excellent promising antioxidant that acts on the level of scavenging the generated ROS (decreasing H_2O_2 production), as well as it acts on enhancing the production and function of the intrinsic antioxidant enzymes like SOD which modifies the total antioxidant potential of the cryopreserved Barki ram semen. Finally, Zn-ONPs supplementation to Barki sperm extender at dose of 150 µg/ml means efficient sperm quality and more sperm cryotolerance.

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CONFLICT OF INTEREST

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence the content of the current paper.

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. Archives of Razi Institute 73, 121-129. 10.22092/ari.2018.120225.1187. DOI:10.22092/ari.2018.120225.1187.

Abdel-Wareth, A., Al-Kahtani, M.A., Alsyaad, K.M., Shalaby, F.M., Saadeldin, I.M., Alshammari, F.A., Ahmed, A.E., 2020. Combined supplementation of nano-zinc oxide and thyme oil improves the nutrient digestibility and reproductive fertility in the male Californian rabbits. Animals 10, 2234. doi: 10.3390/ani10122234.

Aebi, H., 1984. Catalase *in vitro*. Methods Enzymology 105, 121-126. http://dx.doi.org/10.1016/S0076-6879(84)05016-3

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. Bio Med Research International ID153573, http://dx.doi.org/10.1155/2015/153573.

Agarwal, A., Said, T.M., Bedaiwy, M.A, Banerjee, J., Alvarez, J.G., 2006. Oxidative stress in an assisted reproductive techniques setting. Fertil. Steril. 86, 503-512.

Agarwal, A., Salah, R.A., Bedaiwy, M.A., 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril. 79, 829-43.

Aitken, R.J., 2018. Not every sperm is sacred; a perspective on male infertility. MHR Basic Sci. Reprod. Med. 24, 287-298.

Alamaary, M.S., Haron, A.W., Hiew, M.V.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. Veterinary Medicine and Science 6, 666-672.

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.

Asadpour, R., Jafari, R., Tayefi-Nasrabadi, H., 2012. The effect of antioxidant supplementation in semen extenders on semen quality and lipid peroxidation of chilled bull spermatozoa, Iranian Journal of Veterinary Research 13, 246-249.

Atig, F., Raffa, M., Ali, H.B., Abdelhamid, K., Saad, A., Ajina, M., 2012. Altered antioxidant status and increased lipid per-oxidation in sem¬inal plasma of Tunisian infertile men. Int. J. Biol. Sci. 8,139-149.

Attia, Y.A., Kamel, K.I., 2012. Semen quality, testosterone, seminal plasma biochemical and antioxidant profiles of rabbit bucks fed diets supplemented with different concentrations of soybean lecithin. Animal 6:5, 824-833. doi:10.1017/S1751731111002229.

Barkhordari, A., Hekmatimoghaddam, S., Jebali, A., Khalli, M.A., Talebi, A., Noorani, M., 2013. Effect of zinc oxide nanoparticles on viability of human spermatozoa. Iran J. Reprod. Med. 11, 767-771.

Bilodeau, J.F., Blanchette, S., Gagnon, C., Sirard, M.A., 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology 56, 275-286.

Buhr, M., Curtis, E.F., Kakuda, N.S., 1994. Composition and behavior of head membrane lipids of fresh and cryopreserved boar sperm. Cryobiology 31, 224-238.

Chaudhary, P.J., Dhami, A.J., Chaudhari, D.V., Pathan, M.M., 2018. Leakage of transaminases during cryopreservation of Cattle and buffalo semen in egg yolk tris and soya bean milk Based extenders. Indian Journal of Animal Reproduction. 39, 32-35.

Chetsawang, B., Putthaprasart, C., Phansuwan-Pujito, P., Govitrapong, P., 2006. Melatonin protects against hydrogen peroxide-induced cell death signaling in SH-SY5Y cultured cells: involvement of nuclear factor kappa B, Bax and Bcl-2. Journal of Pineal Research 41, 116-123. https://doi.org/10.1111/j.1600-079X.2006.00335.x

Ciereszko, A., Glogowski, J., Strzezek, J., Demianowicz, W., 1992. Low stability of aspartate aminotransferase activity in boar semen. Theriogenology 37, 1269-1281.

Cocchia, N., Pasolini, M.P., Mancini, R., Petrazzuolo, O., Cristofaro, I., Rosapane, I., Sica, A., Tortora, G., Lorizio, R., Paraggio, G., Mancini, A., 2011. Effect of sod (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa, Theriogenology 75,1201-1210.

Cummings, J.E., Kovacic, J.P., 2009. The ubiquitous role of zinc in health and disease. Journal of Veterinary Emergency and Critical Care 19, 215-240. https://doi.org/10.1111/j.1476-4431.2009.00418.x.

Daader, A.H., Merai, I.F., Nasr, A.S., 1993. Transaminasic and fructolytic activities of buffalo bull semen in relation to some physiological attributes of spermatozoa. In Prospects of buffalo production in the Mediterranean and the Middle East. Proc. Int. symposium. EAAP publication. 62,198-209.

Dawei, A., Zhisheng, W., Anguo, Z., 2009. Protective effects of nano-ZnO on the primary culture mice intestinal epithelial cells *in vitro*

against oxidative injury. Journal of Animal and Veterinary Advance. 8, 1964-1967.

Dawei, A.I., Zhisheng, W., Anguo, Z., 2010. Protective Effects of Nano-ZnO on the Primary Culture Mice Intestinal Epithelial Cells in *in vitro* Against Oxidative Injury. World Journal of Agricultural Sciences 6, 149-153.

de-Lamirande, E., Gagnon, C., 1995. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Human Reproduction 10, 15-21. DOI:10.1093/HUMREP/10.SUPPL_1.15.

Dogan, I., Polat, U., Nur, Z., 2009. Correlations between seminal plasma enzyme activities and semen parameters in seminal fluid of Arabian horses. Iranian J. Vet. Res. 10, 119-124.

Duan, C., Goldberg, E., 2003. Inhibition of lactate dehydrogenase C4 (LDHC4) blocks capacitation of mouse sperm *in vitro*. Cytogenetic and Genome Research 103, 352-359.

El-Maddawy, Z.K., Abd-Elnaby, W.S.H., 2019. Protective effects of zinc oxide nanoparticles against doxorubicin induced testicular toxicity and DNA damage in male rats. Toxicology Research 8, 654-662. DOI: 10.1039/c9tx00052f.

Elokil, A.A., Bhuiyan, A.A., Liu, H.Z., Hussein, M.N., Ahmed, H.I., Azmal, S.A., Yang, L., Li, S., 2019. The capability of L-carnitine mediated antioxidant on cock during aging: Evidence for the improved semen quality and enhanced testicular expressions of GnRH1, GnRHR, and melatonin receptors MT 1/2. Poult. Sci. 98, 4172-4181.

El-Sisy, G.A., El-Nattat, W.S., El-Sheshtawy, R.I., 2008. Effect of superoxide dismutase and catalase on viability of cryopreserved buffalo spermatozoa. Global Veterinaria 2, 65-61.

Fayyad, A.H., Mahmood, F.A., 2019. Effect of the addition of guanidine acetic acid to the dilute tris in some of the biochemical characteristics of the semen of the rams. Plant Archives 19,1464-1466.

Ferreira, A.L.A., Matsubara, L.S., 1997. Free radicals: concepts, associated diseases, defense system and oxidative stress (Portuguese). Rev. Assoc. Med. Bras. 43, 61-68. DOI: 10.1590/s0104-42301997000100014.

Forouzanfar, M., Sharafi, M., Hosseini, S.M., Ostadhosseini, S., Hajian, M., Hosseini, L., Abedi, P., Nili, N., Rahmani, H.R., Nasr-Esfahani, M.H., 2010. *In vitro* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. Theriogenology 73, 480-487. https://doi.org/10.1016/j.theriogenology.2009.10.005.

Fossati, P., Prencipe, L., Berti, G., 1980. Use of 3, 5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. Clinical Chemistry 26, 227-31.

Fraczek, M., Kurpisz, M., 2005. The redox system in human semen and peroxidative damage of spermatozoa. Postepy Hig. Med. Dosw. 59, 523-534.

Griveau, J.F., Renard, P., Le Lannou, D., 1994. An *in vitro* promoting role for hydrogen peroxide in human sperm capacitation. Int. J. Androl. 17, 300-307. https://onlinelibrary.wiley.com/doi/10.1111/j.1365-2605.1994.tb01260.x.

Gündoğan, M., 2006. Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi rams. Turkish J. Vet. Anim. Sci. 30, 95-100.

Hammadeh, M.E., Filippos, A., Hamad, M.F., 2009. Reactive oxygen species and antioxidant in seminal plasma and their impact on male fertility. Int. J. Fertil. Steril. 3, 87-110.

Harris, R., Lecumberri, E., Mateos-Aparicio, I., Mengibar, M., Heras, A., 2011. Chitosan nanoparticles and microspheres for the encapsulation of natural antioxidants extracted from Ilex paraguariensis. Carbohydrate Polymers 84, 803-806.

Holt, W.V., 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53, 47-58.

Isaac, A.V., Kumari, S., Nair, R., Urs, D.R., Salian, S.R., Kalthur, G., Adiga, S.K., Manikkath, J., Mutalik, S., Sachdev, D., Pasrichaet, R., 2017. Supplementing zinc oxide nanoparticles to cryopreservation medium minimizes the freeze-thaw-induced damage to spermatozoa. Biochem. Biophys. Res. Commun. 494, 656-662. https://doi.org/10.1016/j.bbrc.2017.10.112.

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.

Katila, T., 2001. *In vitro* evaluation of frozen-thawed stallion semen: A review. Acta Veterinaria Scandinavica 42, 199-217.

Kerns, K., Zigo, M., Drobnis, E.Z., Sutovsky, M., Sutovsky, P., 2018. Zinc ion flux during mammalian sperm capacitation. Nat. Commun. 9, 2061. doi: 10.1038/s41467-018-04523-y.

Khalifa, T.A.A., 2001. Effect of some antioxidants on viability of preserved buffalo and ram semen. Ph. D., Fac. Vet. Med. Cairo Univ., Egypt.

Khalifa, T.A.A., Abdel-Malak, M.G., El-Menoufy, A.A., Ayoub, M.M., 2004. Impact of antioxidants on the functional competence of preserved ram spermatozoa. Egypt J. Basic. and Appl. Physiol. 3, 411.

Khoobbakht, Z., Mohammadi, M., Mehr, M.R.A., Mohammadghasemi, F., Sohani, M.M., 2018. Comparative effects of zinc oxide, zinc oxide nanoparticle and zinc-methionine on hatchability and reproductive variables in male Japanese quail. Anim. Reprod. Sci. 192, 84-90.

Koracevic, D., Koracevic, G., Djordjevic, V., Andrejevic, S., Cosic, V., 2001. Method for the measurement of antioxidant activity in human fluids. Journal of Clinical Pathology 54, 356-361.

Kvist, U., 1980. Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation ability in man. Acta Physiol. Scand. 109, 79-84. https://onlinelibrary.wiley.com/doi/10.1111/j.1748-1716.1980.tb06567.x

Liu, Q., Xu, C., Ji, G., Liu, H., Mo, Y., Tollerud, D.J., Gu, A., Zhang, Q., 2016. Sub lethal effects of zinc oxide nanoparticles on male reproductive cells. Toxicology *in vitro*. 35, 131- 138. https://doi.org/10.1016/j.tiv.2016.05.017.

Ma, J., Han, R., Li, Y., Cui, T., Wang, S., 2020. The Mechanism of Zinc Sulfate in Improving Fertility in Obese Rats Analyzed by Sperm Proteomic Analysis. Bio. Med. Research International Article ID 9876363, 10 pages https://doi.org/10.1155/2020/9876363.

Maret, W., 2000. The function of zinc metallothionein: a link between cellular zinc and redox state. J. Nutr. 130,1455S-1458S. https://doi.org/10.1093/jn/130.5.1455S.

Mahamuni, P.P., Patil, P.M., Dhanavade, M.J., Badiger, M.V., Shadija, P.G., Lokhande, A.C., Bohara, R.A. 2019. Synthesis and characterization of zinc oxide nanoparticles by using polyol chemistry for their antimicrobial and antibiofilm activity. Biochem. Biophys. Rep. 17, 71-80. https://doi.org/10.1016/j.bbrep.2018.11.007

Medeiros, C., Forell, F., Oliveira, A., Rodrigues, J., 2002. Current status of sperm cryopreservation: why isn't it better? Theriogenology 57, 327-344. https://doi.org/10.1016/S0093-691X(01)00674-4

Mohammed, K.M.E., Ziada, M.S., Darwish, G.M., 1998. Practical trials for freezing semen of buffalo 634 and Friesian bulls: effect of various regimens of freezing, different milk extenders and types of straws packages on post-thawing semen characters. Assiut Veterinary Medical Journal 39, 70-93.

Mousavi, S.M., Towhidi, A., Zhandi, M., Amoabediny, G., Mohammadi-Sangcheshmeh, A., Sharafi, M., Hussaini, S.M.H., 2019. Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. Anim. Reprod. Sci. 209, 106171-10679. https://doi.org/10.1016/j.anireprosci.2019.106171.

NRC, 2007. Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids. (China Legal Publishing House).

Narasimhaiah, M., Arunachalam, A., Sellappan, S., Mayasula, V.K., Guvvala, P.R., Ghosh, S.K., Kumar, H., 2018. Organic zinc and copper supplementation on antioxidant protective mechanism and their correlation with sperm functional characteristics in goats. Reprod. Domest. Anim. 53, 644-654.

Nishikimi, M., Roa, N.A., Yogi, K., 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen Biochem. Bioph. Res. Common. 46, 849-854.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351-358. https://doi.org/10.1016/0003-2697(79)90738-3.

Okab, A.B., 2007. Semen characteristics and plasma testosterone of Newzealand male rabbits as affected by environmental temperatures. Slovak. J. Anim. Sci. 40, 161-167.

Orr, W.C., Sohal, R.S., 1994. Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. Science 263, 1128-1130. https://www.science.org/doi/10.1126/science.8108730

Partyka, A., Łukaszewicz, E., Nizanski, W., 2012b. Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. Theriogenology 77, 1497-1504. Doi: 10.1016/j.theriogenology.2011.11.006

Partyka, A., Lukaszewicz, E., Niżański, W., 2012a. Lipid peroxidation and antioxidant enzymes activity in avian semen. Anim. Reprod. Sci. 134,184-190. DOI: 10.1016/j.anireprosci. 07.007

Perumal, P., 2014. Effect of Superoxide Dismutase on Semen Parameters and Antioxidant Enzyme Activities of Liquid Stored (5°C) Mithun (Bos frontalis) Semen. Animals Article ID 821954, 9 pages. https://doi.org/10.1155/2014/821954.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla. D., Bitto, A., Bitto, A., 2017. Oxidative

stress: Harms and benefits for human health. Oxidative Medicine and Cellular Longevity Article ID 8416763, 13 pages https://doi.org/10.1155/2017/8416763.

Prasad, A.S., 2009. Zinc: Role in immunity, oxidative stress and chronic inflammation. Curr Opin Clin. Nutr. Metab. Care. 12, 646-652. DOI: 10.1097/MCO.0b013e3283312956

Reitman, S., Frankel, S., 1957. Colorimetric method for the determination of serum transaminase activity. Am. J. Clin. Path 28, 56.

Rogalska, J., Brzoska, M.M., Roszczenko, A., Moniuszko-Jakoniuk, J., 2009. Enhanced zinc consumption prevents cadmium-induced alter¬ations in lipid metabolism in male rats. Chem. Biol. Interact. 177, 142-152. https://doi.org/10.1016/j.cbi.2008.09.011

Roomans, G.M., Lundevall, E., Bjorndahl, L., Kvist, U., 1982. Removal of zinc from subcellular regions of human spermatozoa by EDTA treatment studied by X-ray microanalysis. Int. J. Androl. 5, 478-486. https://onlinelibrary.wiley.com/doi/10.1111/j.1365-2605.1982.tb00279.x

Root, A.W., Duckett, G., Sweetland, M., Reiter, E.O., 1979. Effects of zinc deficiency upon pituitary function in sexually mature and immature male rats. J. Nutr. 109, 9-58. https://doi.org/10.1093/jn/109.6.958

Samadian, F., Towhidi, A., Rezayazdi, K., Bahreini, M., 2010. Effects of dietary n-3 fatty acids on characteristics and lipid composition of ovine sperm. Animal 4, 2017-2022.

Satoh, K., 1978. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clinica Chimica Acta 90, 37-43. https://doi.org/10.1016/0009-8981(78)90081-5.

Seifi-Jamadi, A., Kohram, H., Zareh-Shahne, A., Dehghanizadeh, P., Ahmad, E., 2016. Effect of various concentrations of butylated hydroxyanisole and butylated hydroxytoluene on freezing capacity of Turkman stallion sperm. Anim. Reprod. Sci. 170, 108-113. https://doi.org/10.1016/j.anireprosci.2016.04.010.

Sharma, R., Pareek, P.K., Purohit, G.N., Tailor, S.P., 2001. Effect of cryopreservation on seminal enzyme (GOT, GPT and LDH) profile and its relationship with acrosomal integrity in Surti buffalo semen. In Proc. XVII Ann. Conven. Nat. Seminar on Fert. Manage. Farm Anim. under adverse agro-climatic conditions, Jodhpur.

Sharma, R., Pareek, P.K., Tailor, S.P., Purohit, G.N., 2003. Studies on Seminal Enzyme (GOT, GPT and LDH) Profile and its Relationship with Acrosomal Integrity in Surti Buffalo Bull Semen. The Indian journal of Animal Reproduction 24,75-76.

Sirat, M.P., Sinha, A.K., Singh, B.K., Prasad, R.L., 1996. Effect of cryoprotectants on release of various enzymes from buck spermatozoa during freezing. Theriogenology 45, 405-416. doi:10.1016/0093-691X (95)00377-K.

Song, W.H., Sutovsky, P., 2019. Porcine Cell-Free System to Study Mammalian Sperm Mitophagy. Methods Mol. Biol. 1854, 197-207.

Strzegek, J., 1988. Morphological structures of spermatozoa and their biological functions. Biul. Inf. ART (Akademia Rolniczo-Techniczna) Olsztyn. 25, 67-72.

Surai, P., Blesbois, E., Grasseau, I., Chalah, T., Brillard, J.P., Wishart, G., Cerolini, S., Sparks, N., 1998. Fatty acid composition, glutathione peroxidase and superoxide dismutase activity and total antioxidant activity of avian semen. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 120, 527-533.

Swain, P.S., Roa, S.B.N., Rajendran, D., George, D., Sellappan, S., 2016. Nano zinc, an alternative to conventional zinc as animal feed supplement: A review. Animal Nutrition 2, 134-141. https://doi.org/10.1016/j.aninu.2016.06.003

Talebi, A.R., Khorsandi, L., Moridian, M., 2013. The effect of zinc oxide nanoparticles on mouse spermatogenesis. J. Assist. Reprod. Gen. 30, 1203-1209.

Tejaswi, V., Narayana-Swamy, M., Yathiraj, S., Honnappa, T.G., Isloor, S., 2016. Enzymatic activities in fresh seminal plasma and extended refrigerated semen in Nari Suvarna rams. Theriogenology Insight 6, 27-33. DOI 10.5958/2277-3371.2016.00003.6.

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.

Tietz, N.W., 1976. Fundamentals of Clinical Chemistry WB, Saunders Co., Phila, PA, 2nd Ed.

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.

Upreti, G.C., Payne, S.R., Duganzich, D.M., Oliver, J.E., Smith, J.F., 1996. Enzyme leakage during cryopreservation of ram spermatozoa. Anim. Reprod. Sci. 41, 27-36.

Urata, K., Narahara, H., Tanaka, Y., Egashira, T., Takayama, F., Miyakawa, I., 2001. Effect of endotoxin-induced reactive oxygen species on sperm motility, Fertility and Sterility 76, 163-166.

Vichas, L., Tsakmakidis, I.A., Vafiadis, D., Tsousis, G., Malama, E., Boscos, C.M., 2018. The effect of antioxidant agents' addition and freezing method on quality parameters of frozen thawed ram semen. Cell Tissue Bank 19,113-121. DOI 10.1007/s10561-017-9633-6.

Vishwanath, R., Shannon, P., 2000. Storage of bovine semen in liquid and frozen state. Anim. Reprod. Sci. 62, 23-53. https://doi.org/10.1016/S0378-4320(00)00153-6.

Wyck, S., Herrera, C., Requena, C.E., Bittner, L., Hajkova, P., Bollwein, H., Santoro, R., 2018. Oxidative stress in sperm affects the epigenetic reprogramming in early embryonic development. Epigenet. Chromatin 11, 60.

Yan, L., Liu, J., Wu, S., Zhang, S., Ji, G., Gu, A., 2014. Seminal superoxide dismutase activity and its relationship with semen quality and SOD gene polymorphism. Journal of Assisted Reproduction and Genetics 31, 549-554.

Zago, M.P., Oteiza, P.I., 2001. The antioxidant properties of zinc: interactions with iron and antioxidants. Free Radic. Biol. Med. 31, 266-274. https://doi.org/10.1016/S0891-5849(01)00583-4.

Zanganeh, Z., Zhandi, M., Zare-Shahneh, A., Najafi, A., Nabi. M.M., Mohammadi-Sangcheshmeh, A., 2013. Does rosemary aqueous extract improve buck semen cooling periods? Small Ruminant Research 114, 120-125.

Zhandi, M., Seifi-Ghajalo, E., Shakeri, M., Yousefi, A.R., Sharafi, M., Seifi-Jamadi, A., 2020. Effect of glutathione supplementation to semen extender on post-thawed rooster sperm quality indices frozen after different equilibration times. Cryo Letters 41, 92.

Zhandi, M., Talebnia-Chalanbar, A., Towhidi, A., Sharafi, M., Yousefi, A.R., Hussaini, S.M.H., 2019. The effect of zinc oxide on rooster semen cryopreservation. British Poult. Sci. 61 188-194. https://doi.org/10.1080/00071668.2019.1686125.