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Protective Effects of Nano Zinc on Barki Ram Semen Viability

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Abstract

Sixty Barki semen ejaculates were collected using the artificial vagina. Semen samples were evaluated, pooled then subjected for processing using different concentrations of nano zinc (10, 50, 100 and 150 µg/ml). Short-term chilled storage duration, absolute survivability index, post-thaw recovery, reduction rate, live percent, membrane integrity, acrosomal reaction, DNA integrity, head & tail's diameter, and DNA % were assessed. 150 µg/ml of nano zinc can stabilize high motility % (80±0.54%) for about 11 days post dilution and chilling, then drastically dropped on day 18 (47±0.57%). Moreover, 150 µg/ml recorded the best of absolute survivability index (33096±374), motility after dilution (86.67±1.05%), after cooling (87.50±1.11%), after freezing- thawing process (56.67±1.66%). Furthermore, 150 µg/ml treated group keeping the highest records along the first thee hours post-thawing (54.17±1.53, 50.00±1.29, and 47.50±1.11%, respectively). Additionally, 150 µg/ml treated samples presented the highest viability index (180±3.47%), post-thaw recovery rate (65.47±2.26%), post-thawing live % (54.17±2.58%). While 150 µg/ml treated group showed the lowest reduction % (15.81±3.12%), acrosomal reaction (9.83±0.7%). Moreover, 150 µg/ml treated samples recorded the largest head diameter and highest DNA % (52.87±1.74 and 97.51±0.30%, respectively), but showed the lowest tail diameter and tail DNA % (6.22±0.33 and 2.48±0.30, respectively). Furthermore, 150 µg/ml treated group displayed the lowest comet %, tail moment and olive tail moment (8.950±0.02, 0.15±0.02 and 0.40±0.10, respectively) if compared with the other nano zinc treated groups. In conclusion: 150 µg/ml of ZnO-NPs improves Barki ram semen processing, particularly, chilled semen viability, motility, longevity, membrane, and DNA integrity.

KEYWORDS
Barki, Ram, Semen, Cryopreservation, ZnO NPs

INTRODUCTION

Semen preservation, either by cooling or cryopreservation is still challenged, because of their negative impacts on the sperm quality; both techniques evoke reactive oxygen species (ROS) that exerts deleterious effects on semen quality (Falchi, *et al*., 2018a). Sperm cells are extremely sensitive to damage by oxidative stress due to the large amounts of unsaturated fatty acids in their membranes (Xie *et al*., 2018). Generation and accumulation of ROS impairs the sperm plasma and mitochondrial membranes integrity and function (O'Connell *et al*., 2002); increasing the number of morphologically abnormal sperm cells (coiled tails). Therefore, reducing its viability (Critser *et al*., 1988; Nallella *et al*., 2004); affecting nuclear DNA content, increasing structural DNA damages and apoptosis (Aitken *et al*., 2009; Zribi *et al*., 2010), inactivate inner cell enzymes, decreasing the sperm motility, increasing the rate of lipid peroxidation and membrane damage (Xie *et al*., 2018). which in turn, diminishing its fertilization capacity (Yamaguchi *et al*., 2009; Schulte *et al*., 2010; Falchi *et al*., 2018a; Xie *et al*., 2018), and finally infertility. Thus, many intensive attempts to ameliorate these negative effects of semen cryopreservation steps on the spermatozoal structure and function by manipulating the chemical composition of semen extenders were achieved. In this respect, nanotechnology had been aroused to modify semen extender properties. Enriching the diluent with many of nano active components that are characterized by good surface area, charge, and binding properties (Feugang *et al*., 2019; Hashem and Sallam, 2020), improving cellular nutrients uptake and reactivity (Feugang *et al*., 2019; Hashem and Sallam, 2020). In between these new substances are the antioxidant agents which recently used at an extensive level to improve semen quality during cooling and post-thawing (Falchi *et al*., 2018b). The matter opened a new platform for biosciences innovation.

Zinc oxide nanoparticles (ZnO-NPs) are nano-sized particles of less than 100 nm diameter (Medina *et al*., 2007; Dawei *et al*., 2009) characterized by high catalytic activity and a large surface area (Shoeb *et al*., 2013). Zn is a vital element with a wide range of biological roles. It exerts its biological functions though many intracellular signaling pathways (activates the trans-membrane-adenylyl-cyclase to catalyze cAMP production) after its interaction with the Zn^{2+} sensing receptor named GPR39 which is detected in the sperm tail and acrosome (Allouche-Fitoussi and Breitbart, 2020). It plays structural, catalytic, and regulatory roles

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for many mammalian enzymes; moreover, it has great roles in maintaining or even improving mitochondrial activity (Yazdanshenas *et al*., 2016). It improved a lot of cellular processes like DNA replication, DNA repair, and cell division so ensuring the cell membranes and cell division stability (Xie *et al*., 2018). Zinc plays a basic role in sperm tail morphology and motility though its significant effects on the physical properties and membrane stability of the attached fibers, where zinc deficiency may result in poor sperm quality (Colagar *et al*., 2009); especially, on the level of motility, capacitation, and acrosomal exocytosis (El-Maddawy and Abd Elnaby, 2019). Zinc plays an essential role in nuclear chromatin decondensation after fertilization (Kvist, 1982). Furthermore, it has a great protection role against oxidative stressors (Roy *et al*., 2013). Zhang *et al*. (2015) stated that it increases antioxidase activities and the expression of copper-zinc superoxide dismutase (Cu-Zn SOD). It can maintain the malondialdehyde (MDA) levels to near normal levels (Dani and Dhawan, 2005). Therefore, it possesses the capability to reduce ROS generation and increase free radical scavenging activities (Raajsheer and Durairaj, 2017).

Supplementation of bull and ram semen extender with ZnO-NPs improved the survival percent, mitochondrial activity, and the functionality of sperm cell plasma membrane in a dose-dependent manner without any deleterious effect on their motility parameters during the freeze-thawing process (Jahanbin *et al*., 2016; Heidari *et al*., 2019). In a study on buffalo semen, zinc reported to have a direct relationship with the sperm cell survival rate and their progressive motility (Alavi-Shoushtari *et al*., 2009). As far as we know, there are no available papers about the use of Nano Zinc in the cryopreservation of Barki ram semen. Therefore, the current study aimed to discover its effects when it added to the Barki ram semen extender. Moreover, the current study tested a wide-range of ZnO-NPs levels to determine the suitable concentration that could be used safely during ram semen extension and cryopreservation processes. Therefore, the current study considered the first report about using this promising element in the cryopreservation of Barki ram semen.

MATERIALS AND METHODS

Experimental 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. They were maintained under standard environmental conditions and were fed a balanced ration to meet the NRC requirements of rams according to NRC (2007) and water ad libitum. Rams were housed free in well-ventilated semi-open shed stalls so that they were exposed to the natural photoperiod. Moreover, all vaccinations and deworming drugs were given 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 02-03-23).

Experimental procedures

Nano zinc preparation and evaluation

Materials

All chemicals (Loba Fine Chemicals, Mumbai, India) used in nano zinc preparation were of the analytical grade and were used without further purification.

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

Precursor zinc acetate dihydrate (0.1 M) was refluxed in diethylene glycol and triethylene glycol at 180°C and 220°C, respectively, to produce ZnO nanoparticles. 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 1.5 hours prior to refluxing. Following the completion of the reflux action, the samples were centrifuged at 8000 rpm for 15 minutes and washed three times with distilled water and ethanol, then dried overnight at 80°C. Nano zinc was evaluated using computerized X-ray diffractometer (CXRD); Shimadzu, XRD-10000× at NCRRT, Cairo, Egypt (Figs. 1 and 2) according to Reheem *et al*. (2016a; b).

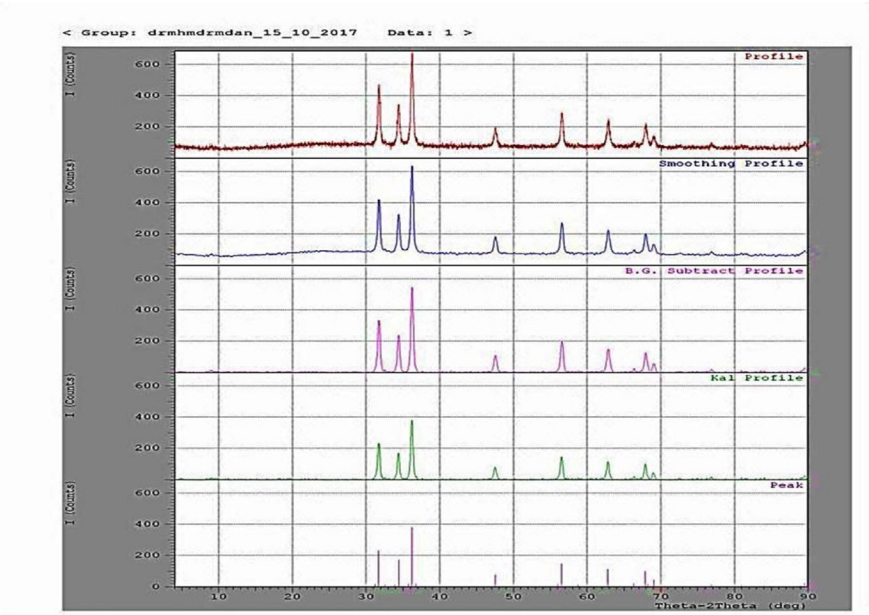

Fig.1. Computerized X-ray diffractometer (CXRD) pattern of nano Zinc; Shimadzu, XRD-10000× at NCRRT, Cairo, Egypt

Semen collection

Sixty semen ejaculates were collected using ram's artificial vagina. The pass semen samples had 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 (first to have sufficient semen to replicate and to eliminate the individual variations between the semen samples).

Barki ram semen processing

Short-term chilled storage

Semen samples were split into 5 portions then diluted at a rate of 1:19 according to Khalifa *et al*. (2004), with OpticXcell2® extender (REF: 026218, LOT: 7083001, IMV technologies, France), that supplemented with 0.00 (control), 10 µg, 50 µg, 100 µg, and 150 µg/ml Nano zinc. OpticXcell2® 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 gradually cooled to 5°C for 1h, and then stored at 5°C until its viability and motility reached to the lowest acceptable percentage (40%) to achieve perfect conception rate as stated by the World Health Organization (WHO, 2010). Sperm motility was assessed by CASA system (CEROS II, REF. 024905, Hamilton Thorne, Reference Army semen and embryo transfer lab, Military Veterinary Hospital) immediately after dilution and along 18 days (432 hours) of incubation period at 5°C. Absolute index of survivability of the incubated semen was calculated according to Ahmad *et al*. (1996).

Absolute Index of Survivability La = ∑ (T × R) where,
La = absolute index of survivability
T: Time interval between (2) observations
R: Average of motility of (2) observations

Freeze/ thaw processing of Barki ram semen

These samples were split into 5 portions and diluted at a rate of (1:19) at 30°C with OptiXcell2® extender supplemented with 0.00 (control), 10 µg, 50 µg, 100 µg, and 150 µg/ml Nano zinc (Barkhordari *et al*., 2013) then cooled to 5°C for 1 h and equilibrated for 2 h. Then the semen samples were loaded in 0.25 ml PVC mini-straws (IMV, L, Aigle, France), and arranged horizontally on freezing racks at 5°C before exposed to liquid nitrogen vapor (LN_2) inside a closed Styrofoam box (54x35x18 cm) containing about 10 liters of LN_2 according to Mohammed *et al*. (1998) for 15 min at a height 6 cm above the surface of LN_2 before plunged directly in it (Khalifa, 2001). The frozen straws then transferred into the liquid nitrogen storage container.

Evaluation of frozen-thawed semen

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 were wiped dry after thawing and unplugged by cutting off with scissors, then transferred into 2 ml pre-warmed, clean, and narrow glass test tubes, then incubated in an incubator (Memmert, Western, Germany) adjusted at 37°C for 3 hours (Abdel-Malak, 1994). The frozen-thawed semen parameters were evaluated.

Sperm motility

Motility of sperm was assessed subjectively and immediately after dilution, before freezing, and after thawing as well as after 1, 2, and 3 h of thawing by using a phase-contrast microscope (40×) equipped with a thermal stage at 37°C. The post-thaw viability index was calculated according to Milovinov (1962) according to the following formula: Percent of motile sperm at 0 h × 0.5+ Percent of motile sperm at 1 h ×1+ Percent of motile sperm at 2 h ×1 + Percent of motile sperm at 3 h ×1.

Post-thaw recovery (%)

Post-thaw recovery percentage was calculated according to Khalifa (2001) from the following formula: (Sperm motility percentage immediately after thawing/sperm motility percentage immediately after dilution) × 100.

The rate of reduction in sperm motility (%)

The rate of reduction in sperm motility was calculated according to Khalifa (2001) from the following formula: (Percent of motile sperm at 0h - Percent of motile sperm after 3 h/ percent

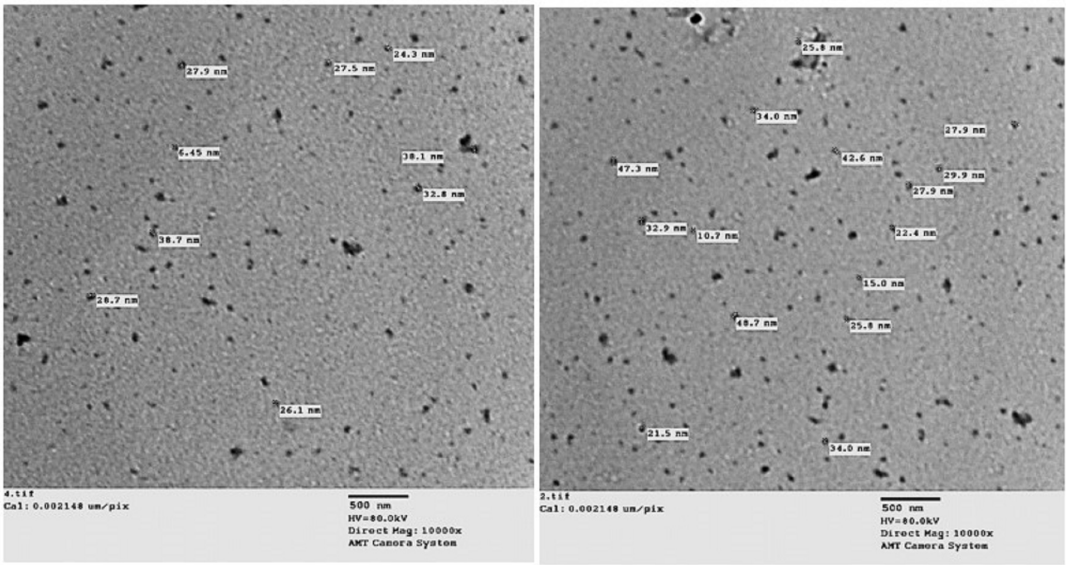

Fig. 2. Denotes the different nano zinc particles sizes, usually run below 100 nm.

of motile sperm at 0 h) ×100.

Sperm plasma membrane integrity was assessed immediately after thawing by using the following methods:

Eosin stain exclusion assay

Since the presence of glycerol (Mixner and Saroff, 1954) in frozen-thawed ram semen was found to interfere with the ability of spermatozoa to exclude the eosin dye, therefore, successful assessment of membrane integrity was accomplished by centrifugation at 30°C. Where each ZnO-NPs concentration treated, samples were handled separately. The number of unstained (viable) sperm appearing white and the number of stained (non-viable) appearing reddish yellow were evaluated until 100 spermatozoa per sample had been counted (Vazquez *et al*., 1997).

Acrosome reaction

The acrosome was determined using silver nitrate stain according to El-Amrawi and Nemetalla (1991). The percentage of complete and incomplete acrosome reactions was estimated in 100 spermatozoa.

Assessment of sperm DNA integrity using Single cell gel electrophoresis (SCGE) comet assay

The alkaline comet assay for DNA damage in spermatozoa was carried out according to Hughes *et al*. (1996) with some modifications DNA damage was detected in semen samples by centrifugation of diluted semen sample at 3000 rpm/10 minute and re-suspended in the sperm culture medium (S-TALP buffer) and stored at (-20°C) until analyzed. The DNA integrity and the incidence of DNA strand breaks or fragmentation were detected.

Staining and scoring

The stained slides were visualized with a Leica epifluorescence microscope (Green filter: N2.1 with excitation filter Bp 515-560, dichromatic mirror: 580, suppression filter: LP 590), and the images for the sperm nuclei were digitalized with Optika DFC camera DFC 280 supplied with Optika Vision Pro4.3. DFC software (Emamcapture, 2009, 4.3) with Host application program imaging to view and capture digital images. The sperm comets were visually scored according to Collins *et al*. (1995), Collins (2004), Comet Assay interest group website (2007), and Collins *et al*. (2008). For each sample, 100 randomly selected cells were photographed and scanned, respectively. We excluded those cells with small heads and large fan-like tails, under the principle that they represent comet cells. The images were analyzed with the comet score analysis system (comet score imaging analysis version IV) for each cell; the length of DNA migration (tail length) was measured in Pixel (PX) from the center of the nucleus to the end of the tail. The percentage of DNA in the tail was determined by measuring the total intensity (fluorescence) in the cells, which was taken as 100%, and determining what percentage of this total intensity corresponded to the intensity measured only in the tail. The tail moment, expressed in arbitrary units, was calculated as tail length × percentage of migrated DNA/100.

Cryopreserved Barki spermatozoa Ultrastructure analysis

Transmission electron microscopy (TEM) was used to evaluate the ultrastructure alterations in the cryopreserved Barki ram spermatozoa. Five samples from each nano zinc-treated group were used. Each sample was washed three times using the phosphate buffer saline and centrifugation at 1000 rpm/5 min. Every thawed semen sample was prefixed with phosphate buffer saline containing 2% glutaraldehyde/for 2-3 h then washed three times with phosphate buffer saline using centrifugation at 1000 rpm/ 5 min at 4°C. After that, each sample was post-fixed in (1%) osmium tetroxide/ 1-2 h at 4°C (Boonkusol *et al*., 2010). The sperm cells were dehydrated with Propylene oxide and then embedded in epon resin. Ultra-thin sections using the Ultra microtome with Leica (EM UC6) were stained with lead citrate and uranyl acetate. The stained sections were visualized by the transmission electron microscope of the Agriculture Faculty, Cairo University (JEOL-EM-100 S at TEM lab FA-CURP, Research Park CURP). Images captured by CCD camera model AMT, optronics camera with 1632 x 1632-pixel format as side mount configuration. Fields were randomly examined.

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 considered to be significant at ($P<0.05$) level and determined by superscripted letters.

RESULTS

Table 1 and Figs. 3 and 4, show highly significant difference ($p<0.0001$) between chilled motility % in relation to the different concentrations of nano zinc and storage time duration (h). Where 100 and 150µg/ml of nano zinc can stabilize and high motility percent (80±0.51 and 80±0.54 %, respectively) of Barki semen for about 11 days (264h) post dilution and chilling if compared with 50, 10 µg/ml and control groups (76.4±0.21, 74±0.41 and 70±0.38%, respectively). Although the motility percent drastically dropped on day 18 post dilution and chilling; 150ug/ml nano zinc treated group still record the highest percent of motility (47±0.57%) when compared with 100, 50, 10 µg/ml and control groups (38.5±0.36, 38±0.44, 36±0.58 and 34±0.57 %, respectively). The absolute index of survivability (La) of the different nano zinc treated groups showed also highly significant difference ($p<0.0001$) where 100 and 150µg/ml nano zinc treated groups recorded the best absolute index of survivability (32118±249 and 33096±374) if compared with 50, 10 µg/ml and control groups (30468±277, 30000±251 and 28404±249, respectively).

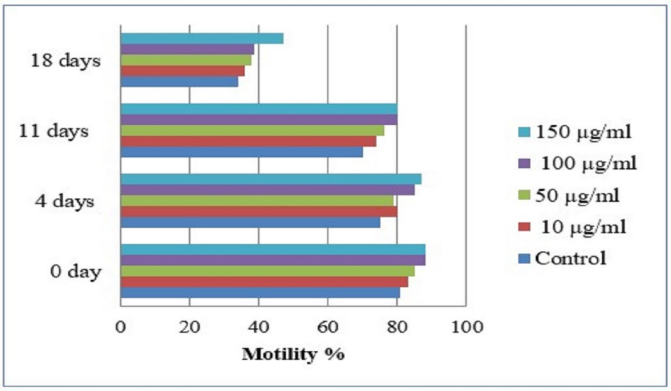

Fig. 3. Barki semen chilled motility % stability curve in relation to nano zinc concentration and storage time duration. Motility assessed on days 0, 4, 11 and 18-day post dilution and chilling by CASA system.

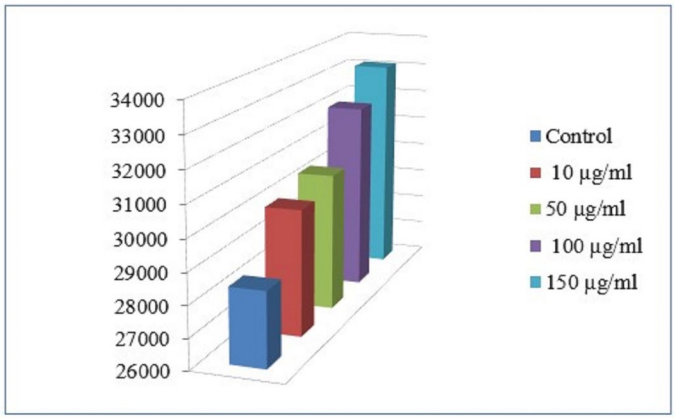

Fig. 4. Absolute index of survivability of chilld Barki ram semen treated with different concentrations of nano zinc. Absolute Index of Survivability La = ∑ (T × R).

Results in Table 2 recognized a significant difference ($p<0.0001$) between the different nano zinc treated groups (in dose dependent manner) concerning the sperm motility after dilution. Where, 150µg/ml treated groups showed the highest record of motility after dilution (86.67±1.05%), followed by 100, 50 and 10 µg/ml (85.00±1.29, 83.33±1.05, and 82.50±1.11%, respectively), while the control group recorded the lowest score of motilities after dilution (80.83±0.83%). Moreover, Table 2 clarifying more information about the changes in Barki semen motility after cooling. Where, the sperm motility after cooling was exaggerated significantly ($p<0.0001$) in 150 µg/ml nano zinc treated group (87.50±1.11%), however it was remain as it is or even slightly decreased (85.00±1.29, 83.33±1.05, and 80.83±1.53%, respectively) in the other nano zinc treated groups (100, 50, and 10 µg/ml, respectively).

Generally, the sperm motility % decreased significantly ($p<0.0001$) after sperm cells were exposed to freeze-thawing process (Table, 2). Event though, 150 µg/ml nano zinc treated group still preserve sperm motility at a high rate (56.67±1.66%), if compared with the other nano zinc treated groups (100 and 50 µg/ml) which recorded 54.17±1.53, and 51.67±1.05%, respectively. While 10 µg/ml of nano zinc and the control groups achieved the lowest un-acceptable level of the post thawing motility (48.33±1.66, and 37.50±1.11%, respectively).

Data in Table 3 denoted a highly significant differences ($p<0.0001$) in the stability patterns of the post-thawing motility for the first three hours after thawing (1h interval). Where, 150µg/ml nano zinc treated group keep the highest records along the first three hours post thawing (54.17±1.53, 50.00±1.29, and 47.50±1.11%, respectively). While, 100 µg/ml nano zinc treated group recorded (50.83±1.53, 46.67±1.05, and 40.83±1.53%, respectively), 50µg/ml nano zinc treated group showed (50.00±1.29, 45.00±1.82, and 40.00±1.82%, respectively), 10 µg/ml nano zinc treated group presented (46.67±1.05, 40.83±1.53, and 33.33±1.05%, respectively). Finally, zero nano zinc treated group (control) displayed the inferior values of motility along the first

Table 1. The stability of chilled Barki semen motility* (%) in relation to the different concentrations of nano zinc and storage time duration (h) indexed by the absolute index of survivability (La).

	Day 0 (0h)	day 4 (96h)	day 11 (264h)	days 18 (432h)	Mean±SE	Absolute Index of Survivability (La)
Control	81±0.57^{d}	75±0.51^{d}	70±0.38^{d}	34±0.57^{d}	65.00±10.58	28404±249^{d}
10µg/ml	83±0.57^{c}	80±0.48^{c}	74±0.41^{c}	36±0.58^{c}	68.00±10.80	30000±251^{c}
50µg/ml	85±0.58^{b}	79±0.53^{c}	76.4±0.21^{b}	38±0.44^{b}	69.10±10.46	30468±277^{c}
100µg/ml	88±0.58^{a}	85±0.58^{b}	80±0.51^{a}	38.5±0.36^{b}	72.88±11.58	32118±249^{b}
150µg/ml	88±0.58^{a}	87±0.55^{a}	80±0.54^{a}	47±0.57^{a}	75.50±9.66	33096±374^{a}
Mean±SE	85.00±1.37	81.20±2.15	76.08±1.89	38.70±2.22		

Results are presented as mean±SEM. *Motility estimated by CASA system.

Table 2. The effects of different concentrations of nano zinc supplementation to Barki ram semen diluent on its dilution, after cooling, and post thawing motility (%).

Conc. Nano ZN	Motility after dilution	Motility after cooling	Motility post thawing
Control	80.83±0.83^{c}	76.67±1.05^{d}	37.50±1.11^{d}
10 µg/ml	82.50±1.11^{bc}	80.83±1.53^{c}	48.33±1.66^{c}
50 µg/ml	83.33±1.05^{abc}	83.33±1.05^{bc}	51.67±1.05^{bc}
100 µg/ml	85.00±1.29^{ab}	85.00±1.29^{ab}	54.17±1.53^{ad}
150 µg/ml	86.67±1.05^{a}	87.50±1.11^{a}	56.67±1.66^{a}
Overall means	83.67±0.58	82.67±0.85	49.67±1.37

Results are presented as mean±SEM, (n.= 6 replicates/group).
Values with different superscript letters $^{(a, b, c,)}$ in the same column were significantly different at least ($P<0.001$).

Table 3. The effects of different concentrations of nano zinc supplementation to Barki ram semen diluent on motility (%) stability pattern for the first three hours post-thawing, and the viability index.

Conc Nano ZN	Post Thawing motility / 1h	Post Thawing motility / 2h	Post Thawing motility / 3h	Viability Index
Control	32.50±1.11^{d}	25.83±2.00^{d}	16.67±1.05^{d}	93.75±4.01^{d}
10 µg/ml	46.67±1.05^{c}	40.83±1.53^{c}	33.33±1.05^{c}	145.00±3.87^{c}
50 µg/ml	50.00±1.29^{bc}	45.00±1.82^{bc}	40.00±1.82^{b}	160.80±5.11^{b}
100 µg/ml	50.83±1.53^{ab}	46.67±1.05^{ab}	40.83±1.53^{b}	165.40±4.35^{b}
150 µg/ml	54.17±1.53^{a}	50.00±1.29^{a}	47.50±1.11^{a}	180.00±3.47^{a}
Overall means	46.83±1.50	41.67±1.69	35.67±2.03	149.00±5.80

Results are presented as mean±SEM, (n.= 6 replicates/group).
Values with different superscript letters $^{(a, b, c,)}$ in the same column were significantly different at least ($P<0.001$).

three hours post thawing (32.50±1.11, 25.83±2.00, 16.67±1.05%, respectively). Additionally, the viability index of Barki semen samples treated with the different concentration of nano zinc showed highly significant difference (p<0.0001). Where, the viability index of 150 µg/ml treated group presented the highest record (180.00±.3.47%), if compared with 50, 100, 10 µg/ml nano zinc treated groups (165.40±4.35, 160.80±5.11, and 145.00±3.87%, respectively), while control showed the lowest viability index (93.75±4.01%).

Table 4 shows highly significant difference (p<0.0001) between the semen samples treated with the different concentrations of ZnO-NPs concerning the post thaw recovery and reduction %. Where, 150 µg/ml treated group showed the highest post thaw recovery (65.47±2.26%) followed by 100, 50 and 10 µg/ml treated groups (63.74±1.64, 62.07±1.66, and 58.58±1.86%, respectively), while, the control group showed the lowest post recovery percent (46.38±1.25%). Moreover, the reduction (%) between groups showed also a prominent significant difference (p<0.0001); where the lowest reduction % was reported with 150 µg/ml treated group (15.81±3.12%), while 50 100 and 10 µg/ml treated groups showed and mostly resemble reduction % (22.73±2.34 %, 24.47±2.76, and 30.83±2.00%, respectively), whereas the control groups demonstrated the highest reduction % (55.36±3.12%). Furthermore, the percentage of the live spermatozoa between ZnO-NPs treated groups showed distinguishing significant difference (p<0.0001). Where, 150 µg/ml nano zinc treated group reported the highest live percent (54.17±2.58%), whereas, 100, 50 and 10 µg/ml treated group shown nearly similar values (49.00±3.15, 46.17±2.28, and 43.67±2.15%, respectively), but zero nano zinc treated group showed the lowest live percent (32.33±0.95%). Additionally, table (4) clarifying a highly significant difference (P<0.0001) between nano zinc treated groups concerning the acrosomal reaction %. Where 150µg/ml nano zinc treated group presented the lowest record of the acrosomal reaction (9.83±0.79%), if compared with 100, 50, and 10 µg/ml nano zinc treated groups (11.33±0.88, 12.00±0.44, and 13.50±0.55%, respectively). While the control groups showed the highest level of the acrosomal reaction (23.00±0.81%).

Table 5 present a highly significant difference (P<0.001) between nano zinc treated groups concerning head diameter and head's DNA %. Where, 150 µg/ml treated group recorded the largest head diameter and the highest DNA % (52.87±1.74 and 97.51±0.30%, respectively), while 100, 50 and 10µg/ml recorded [(50.61±1.24 and 96.80±0.39%), (49.20±0.40 and 96.38±0.41%) and (46.88±1.44 and 95.95±0.27%), respectively]. Moreover, control groups presented the smallest head diameter and lowest DNA % (40.94±1.65 and 94.56±0.23%, respectively). On contrary, tail length and DNA % in the sperm tail recorded a significant declined pattern (P<0.0001, P<0.001, respectively) starting from control and run toward 150µg/ml treated group. Where, the control group showed the tallest tail length and highest DNA % in the sperm tail (11.64±0.62 and 5.39±0.24%, respectively) if com-

Table 4. The effect of different concentrations of nano zinc on post thaw recovery, reduction, live spermatozoa and acrosomal reaction (%) of the diluted Barki ram spermatozoa.

Concentration Nano ZN	Post thaw recovery (%)	Rate of reduction (%)	Live spermatozoa (%)	Acrosomal reaction (%)
Control	46.38±1.25^{c}	55.36±3.12^{a}	32.33±0.95^{c}	23.00±0.81^{a}
10 ug/ml	58.58±1.86^{d}	30.83±2.00^{b}	43.67±2.15^{bc}	13.50±0.55^{b}
50 ug/ml	62.07±1.66^{ab}	22.73±2.34^{b}	46.17±2.28^{bc}	12.00±0.44^{bc}
100 ug/ml	63.74±1.64^{ab}	24.47±2.76^{bc}	49.00±3.15^{ab}	11.33±0.88^{bc}
150 ug/ml	65.47±2.26^{a}	15.81±3.12^{c}	54.17±2.58^{a}	9.83±0.79^{c}
Overall means	59.24±1.46	29.83±2.76	45.06±1.66	13.93±0.91

Results are presented as mean±SEM, (n.= 6 replicates/group).
Values with different superscript letters $^{(a, b, c,)}$ in the same column were significantly different at least (P<0.001).

Table 5. The effects of different concentrations of nano zinc supplementation to Barki ram semen diluent on its head diameter, tail length, and DNA% in head and tail.

Conc Nano ZN	Head diameter	Tail length (PX)	DNA% in head	DNA% in Tail
Control	40.94±1.65^{c}	11.64±0.62^{a}	94.56±0.20^{c}	5.39±0.24^{a}
10 µg/ml	46.88±1.44^{b}	9.19±0.40^{b}	95.95±0.27^{b}	3.84±0.41^{b}
50 µg/ml	49.20±0.40^{ab}	8.49±0.18^{bc}	96.38±0.40^{b}	3.36±0.29^{bc}
100 µg/ml	50.61±1.24^{ab}	7.93±0.06^{c}	96.80±0.39^{ab}	3.19±0.39^{bc}
150 µg/ml	52.87±1.74^{a}	6.22±0.33^{d}	97.51±0.30^{a}	2.48±0.30^{c}
Overall means	48.09±0.77	8.69±0.49	96.23±0.29	3.65±0.28

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).

Table 6. The effects of nano zinc supplementation to Barki ram semen diluent on its comet %, tail moment and olive Tail moment.

Conc Nano ZN	Comet %	Tail Moment	Oliv. Tail Moment
Control	16.47±0.28^{a}	0.62±0.01^{a}	3.37±0.13^{a}
10 µg/ml	16.20±0.23^{a}	0.35±0.02^{b}	1.36±0.24^{b}
50 µg/ml	15.84±0.08^{a}	0.28±0.02^{bc}	0.98±0.18^{bc}
100 µg/ml	12.45±0.20^{b}	0.25±0.03^{c}	0.83±0.20^{bc}
150 µg/ml	8.95±0.02^{c}	0.15±0.02^{d}	0.40±0.10^{c}
Overall means	13.98±0.77	0.33±0.04	1.39±0.28

Results are presented as mean±SEM, (n.= 6 replicates/group).
Values with different superscript letters $^{(a, b, c,)}$ in the same column were significantly different at least (P<0.001).

pared with 10, 50, 100, and 150µg/ml nano zinc treated groups [(9.19±0.40 and 3.84±0.41%); (8.49±0.18 and 3.36±0.29%); (7.93±0.06 and 3.19±0.39%) and (6.22±0.33 and 2.48±0.30%), respectively].

Results in Table 6 and Fig. 5 revealed a highly significant difference ($p < 0.0001$) between nano zinc treated groups concerning the comet percentage, tail moment and olive tail moment. Where, control group recorded the greater DNA fragmentation level that was clear in the comet % (16.47±0.28%), tail moment and olive tail moment (0.62±0.01 and 3.37±0.13, respectively). On the other hand, the DNA fragmentation level begins to steady decrease as the concentration of nano zinc increase. Where, concentration of 10µg/ml treated group recorded (16.20±0.23%, 0.35±0.02 and 1.36±0.24, respectively); 50µg/ml treated group recorded (15.84±0.08%, 0.28±0.02 and 0.98±0.18, respectively); 100µg/ml treated group recorded (12.45±0.20%, 0.25±0.03 and 0.83±0.20, respectively) for comet %, tail moment and olive tail moment. 150 µg/ml nano zinc treated group showed the lowest comet %, tail moment and olive tail moment (8.95±0.02%, 0.15±0.02 and 0.40±0.10, respectively).

DISCUSSION

The accessory sex glands secrete many elements that protect spermatozoa at the time of ejaculation (Khosronezhad *et al*., 2015). In between these elements is Zn which is a plentiful microelement found in most body fluids, especially the seminal plasma; where, found at high concentrations (Cheah and Yang, 2011), and has multifaceted roles in the sperm's functions and quality (Fallah *et al*., 2018). Semen processing deprived the sperm cells of their seminal plasma, which contains this valuable antioxidant that acts to protect the sperm cells against the generated side products (free radicals), besides it improved its viability and quality.

The most recent advances in semen cryopreservation aimed to provide the semen diluent with the appropriate antioxidant (type and dose); to protect the sperm cells from the reduction in motility, viability, or alteration in their morphology (Nallella *et al*., 2004). Consequently, offering a protection for the sperm cell membrane from damaging (Critser *et al*., 1988), as well as DNA from fragmentation (Aitken *et al*., 2009; Zribi *et al*., 2010); moreover, they improve the mitochondrial functions (O'Connell *et al*., 2002).

Zinc oxide nanoparticles were initially developed for cancer research (Rasmussen *et al*., 2010), but the current study and for the first time reported the relationship between the addition of nano zinc to Barki ram semen extender and the duration of the chilled sperm longevity. Where, it had been found that at concentrations of 100 and 150 µg/ml the diluted Barki spermatozoa remain viable and active (80±0.51 and 80±0.54%) for about 11 days post-dilution and chilling. The current study finding runs in harmony with (Alavi-Shoushtari *et al*., 2009) who reported that zinc in semen plasma had a direct relationship with the number of survivable and progressively motile buffalo sperm cells. Recent studies demonstrated that there were beneficial effects for ZnO-NPs on the post-thawing quality of dromedary camel epididymal spermatozoa (Shahin *et al*., 2020), as well as on the human sperm viability and motility before cryopreservation

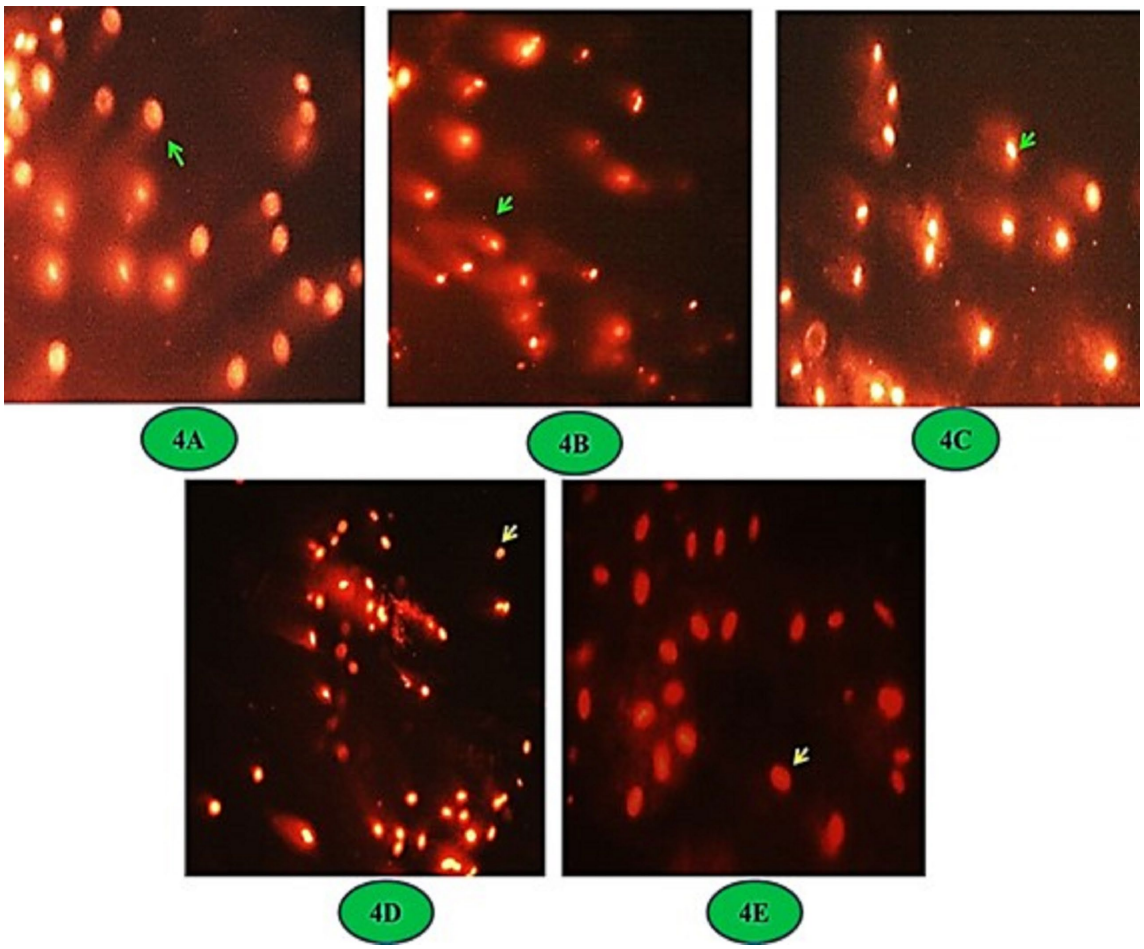

Fig. 5. Sperm DNA integrity using Single cell gel electrophoresis (SCGE) comet assay for the frozen-thawed Barki sperm cells treated with different concentrations of nano zinc. A, B and C) show high DNA fragmentation (%) that was illustrated by the huge amount of DNA in the comet tail of Barki ram spermatozoa cryopreserved in OptiXcell2® extender supplemented with 0, 10, and 50µg/ml of nano zinc, respectively. The less dark red "comet-shaped" area adjacent to the nucleus represents DNA breaks that are small enough to move in the gel (green arrows). D and E) showed low DNA fragmentation (%) that was illustrated by a limited amount of DNA in the comet tail of Barki ram spermatozoa cryopreserved in OptiXcell2® extender supplemented with 100 and 150 µg/ml of nano zinc, respectively. Each spot represented the DNA of a sperm cell. Intact DNA is large enough so does not migrate much in the electrophoretic field (yellow arrow). Level of DNA fragmentation decreased as the concentration of nano zinc in OptiXcell2® extender increased (A to E). Where, it was markedly decreased at 100µg/ml (D) and totally absent with 150 µg/ml (E).

and after thawing (Wu *et al*., 2015; Berkovitz *et al*., 2018). Zinc supplementation to the sperm diluent was found to have great roles in controlling the energy consumption in the ATP system by regulating the energy in the sperm phospholipids so exerting a direct impact on sperm motility and survivability (Orzołek *et al*., 2021). This, in turn, could be useful in preserving sperm quality during the freeze-thawing process. The enhancing roles ZnO-NPs in ram semen extender not stopped at the level of motility but the current study discovered that ZnO-NPs supplementation to Barki semen diluent has promising ameliorative roles in enhancing its post-thaw recovery and reduction rates, viability index, and live spermatozoa percent especially at 150 µg/ml. Moreover, 150µg/ml treated group showed the highest record of motility after dilution (86.67±1.05%) which significantly exaggerated after cooling in the same group (87.50±1.11%). These results came in harmony with Wu *et al*. (2015) and Berkovitz *et al*. (2018) who observed that zinc had beneficial effects on human semen before cryopreservation as well as its viability and motility after thawing. Furthermore, Berkovitz *et al*. (2018) added that when the sperm cells frozen, thawed and refrozen again in the presence of zinc there was a considerable increase in its motility.

On the other hand, it remains as it is or even slightly decreased (85.00±1.29, 83.33±1.05, and 80.83±1.53%, respectively) in the other nano zinc treated groups (100, 50, and 10 µg/ml, respectively). These results explained by Henkel *et al*. (2003) who reported that the improved release of zinc from ZnO-NPs was correlated with an increased oxygen input and sperm motility. The situation is the same with the post-thawing motility and the motility percent after 3 hours post-thawing also showed a brilliant role for ZnO-NPs in ram semen diluent. Where, ZnO-NPs exert a vital role in preserving an acceptable percentage of sperm motility after thawing especially with 100 and 150 µg/ml doses. Zn in the diluent is associated with the lipid catabolism in the sperm middle piece so providing the energy needed for sperm motility (Fallah *et al*., 2018).

ZnO-NPs positively affect sperm motility by affecting their flagellar and mitochondria functions (Henkel *et al*., 1999). Zn^{2+} found to be predominated in sperm mitochondria and along the flagella (Morisawa and Mori, 1972; Stoltenberg *et al*., 1997); Besides, it was localized predominantly in the outer dense fibers (Baccetti *et al*., 1976; Calvin, 1979); so, exerting an efficient protecting role on the sperm membrane integrity as well as its stability (Dawei *et al*., 2009).

Additionally, the current study result shows improving effects for ZnO-NPs in ram semen diluent of the percentage of healthy acrosome, especially with 100 and 150 µg/ml , where they recorded the lowest percentage of the acrosomal reaction (11.33±0.88 and 9.83±0.79%, respectively). It was reported that Zn protects sperm physiology, especially lipid flexibility, sperm membrane stabilization (Bettger and O'Dell, 1981; Chia *et al*., 2000; Kendall *et al*., 2000), and cell morphology during cryopreservation (Allouche-Fitoussi and Breitbart, 2020). In addition, Zn has a regulated role in the acrosomal reaction and sperm capacitation (Eggert-Kruse *et al*., 2002); this came in harmony with the current study results where ZnO-NPs in ram semen diluent protect its ultrastructure (Fig. 6).

The current study results reported for the first time the relationship between the addition of nano zinc to the ram semen diluent and the sperm head diameter and the head's DNA percentage. Where, the 150 µg/ml treated group recorded the largest head diameter and the highest DNA percentage (52.87±1.74 and 97.51±0.30%, respectively), while the control group presented the smallest head diameter and lowest DNA % (40.94±1.65 and 94.56±0.23%, respectively).

The situation was reversed with the tail length and DNA % in the sperm tail. Where, the control group showed the tallest tail length and highest DNA % in the sperm tail (11.64±0.62 and 5.39±0.24%, respectively) if compared with 150 µg/ml nano zinc treated groups (6.22±0.33 and 2.48±0.30%, respectively). Furthermore, the current study results show a highly significant difference between the nano zinc treated groups concerning the comet percentage, tail moment, and olive tail moment. Where, the control group showed a greater DNA degradation level that was clear in the comet percent (16.47±0.28%), tail moment, and olive tail moment (0.62±0.01 and 3.37±0.13, respectively). While, the 150µg/ml nano zinc treated group showed the lowest DNA fragmentation level (8.950±0.02, 0.15±0.02 and 0.40±0.10) for comet percentage, tail moment, and olive tail moment, respectively. These results indicate that the zinc nanoparticles in the semen diluent may act as a key regulator of various processes within the sperm. Zinc had been reported to preserve genomic integrity so preventing its damage (Tuerk and Fazel, 2009; Isaac *et al*., 2017), and stabilizing the sperm chromatin (Blazak and Overstreet, 1982; Kotdawala *et al*., 2012; Fallah *et al*., 2018). These protective effects are supposed to be linked to the creation of a protective layer of ZnONPs around the sperm cell, preventing lipid peroxidation (Isaac *et al*., 2017), moreover, it influences nuclear chromatin condensation/decondensation (Fallah *et al*., 2018). Additionally, 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. Wu *et al*.

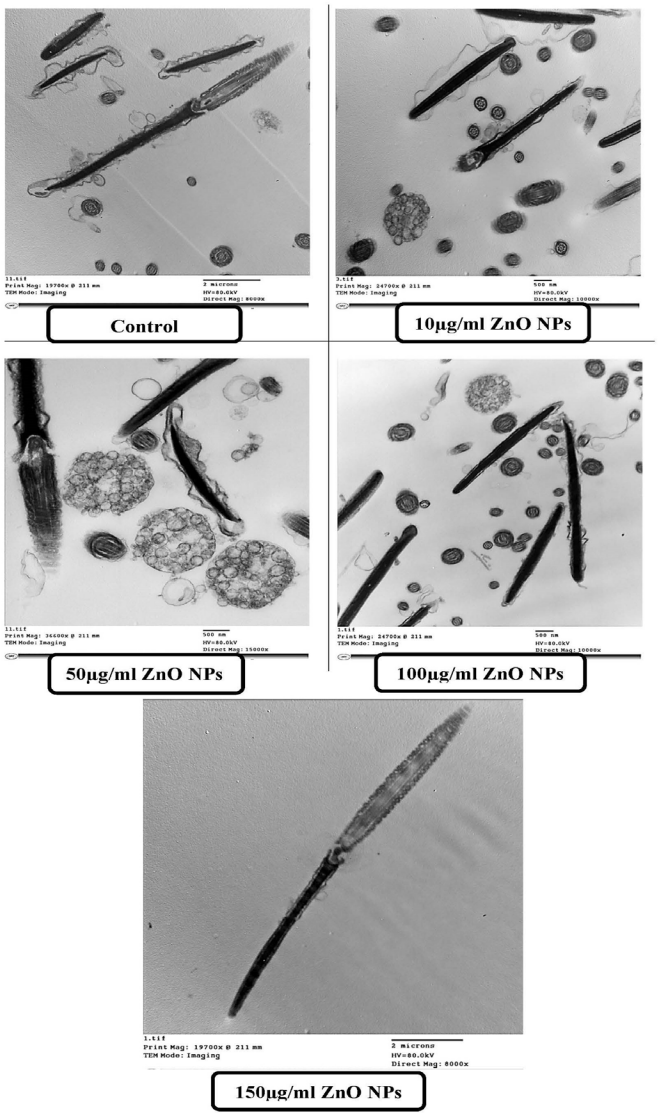

Fig. 6. Sagittal and longitudinal electron micrograph sections of the frozen-thawed Barki sperm cells treated with different concentrations of nano zinc. Control, 10, 50 µg/ml nano zinc treated groups showed swollen, degenerated and vacuolated plasma membrane, segmentation of the outer acrosomal membrane (OAM) and swollen acrosome. 10 and 50 µg/ml treated groups showed mitochondria in different orientation indicating severe degeneration (marked vacuolation in the mitochondria that contained electron-translucent spaces with complete absence of the transverse cristae and some mitochondria are completely disappeared). Despite concentrations, 100 and 150 µg/ml of nano zinc showed less degradation level in the sperm structures, where outer and inner acrosomal membranes are intact and the sub acrosomal space is evident, and the nuclear and mitochondrial contents are homogenous in the electron density.

(2015) reported that zinc supplementation to sperm diluent medium could reverse DNA disintegration. Furthermore, Jahanbin *et al*. (2021) reported that sperm treatment with zinc nanoparticles improved its plasma membrane integrity and mitochondrial activity; this came in agreement with the current study results where ZnO-NPs in Barki ram semen extender guard its ultrastructure (Fig. 6). On the other hand, Isaac *et al*. (2017) reported that the addition of different concentrations of ZnO-NPs to the cryopreserved men's semen samples did not improve the total motility and viability after the freeze thawing process whoever it reduced the rate of chromatin damage. Additionally, Orzołek *et al*. (2021) reported that turkey ejaculates supplemented with Zn-NPs and preserved for 48 h showed reduced progressive motility and mitochondrial membrane potential; they explained that on basis of high concentration of Zn-NPs used for extension. Where, Zhandi *et al*. (2020) documented positive effects for Zn-NPs on rooster spermatozoa freezability at concentrations (0.77–1.27 µg/ml); while the higher concentrations compromised post-thaw sperm quality indices.

Altogether, the current study proved that ZnO-NPs supplementation to Barki semen diluent could protect sperm viability and quality indices (total motility, vitality, DNA and plasma membrane integrity, and morphological abnormality) during low-temperature storage. The positive effects of ZnO-NPs depend upon concentration and time of exposure (Barkhordari *et al*., 2013; Han *et al*., 2016; Liu *et al*., 2016). The manner in which extracellular Zn affects sperm motility seems to be species-dependent also (Allouche-Fitoussi and Breitbart, 2020).

CONCLUSION

In vitro addition of nano zinc to Barki sperm diluent can ameliorate sperm viability and quality after dilution and during the freeze-thawing process. The current results revealed that ZnO-NPs has a promising effect on a wide range of doses starting from 10-150 µg/ml (in dose manner) concentration of 150 µg/ml ZnO-NPs has excellent effects nominating it to be the ideal dose in Barki semen processing. 150µg/ml of ZnO-NPs can improve the outcome of ram semen *in vitro* processing, particularly, chilled ram semen longevity, progressive motility, viability, and membrane and DNA integrity. Zinc supplementation during semen processing could consider a good player for *in vitro*-assisted reproduction improvement.

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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 paper.

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