Biological Trace Element Research
https://doi.org/10.1007/s12011-020-02153-4

In Vivo and In Vitro Evaluation of Bull Semen Processed with Zinc (Zn) Nanoparticles

Rana Jahanbin[1] · Parisa Yazdanshenas[1] · Maryam Rahimi[1] · Atieh Hajarizadeh[1] · Eva Tvrda[2] · Sara Ataei Nazari[1] · Abdollah Mohammadi-Sangcheshmeh[1] · Nasser Ghanem[3]

Received: 11 December 2019 / Accepted: 8 April 2020

Abstract

Defective sperms cause fertilization failure under both in vivo and in vitro conditions. Therefore, providing optimal conditions during semen storage is a prerequisite for maintaining viability. The current study investigated bull semen quality in vitro and in vivo when zinc (Zn) nanoparticles were used as antioxidant during semen processing and cryopreservation. In total, 32 ejaculates were collected from four Holstein bulls. All ejaculates were pooled and diluted with Bioxcell-extender containing 0 (control group), 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M of Zn nanoparticles. Several physical and biochemical sperm parameters were determined after freeze-thawing process. In vitro embryo development rate and pregnancy rate were monitored after in vitro fertilization or artificial insemination using semen treated with Zn nanoparticles. Plasma membrane integrity was improved ($P < 0.05$) in bull semen treated with 10^{-6} M (69.3%), and 10^{-2} (62.4%) of Zn nanoparticles compared to untreated group (51.3%). In addition, proportions of live spermatozoa with active mitochondria were increased ($P < 0.05$) in semen supplemented with Zn nanoparticles at concentration of 10^{-6} M (67.3%), and 10^{-2} (85.3%) compared to control group (49.8%). Moreover, the level of MDA was lower ($P < 0.05$) in semen with Zn nanoparticles at 10^{-6} M (2.97 mol/mL) and 10^{-2} (2.7 mol/mL) concentrations than control semen samples (3.77 mol/mL). However, sperm total and progressive motility, sperm viability, DNA fragmentation, and pregnancy rate were not affected by treatment of semen with Zn nanoparticles. On the other hand, supplementation of in vitro maturation media with 10^{-6} M Zn nanoparticles has increased blastocyst rate ($P < 0.05$) compared to other experimental groups, while addition of Zn nanoparticles-treated sperm during in vitro fertilization did not affect embryo development rate. In conclusion, supplementation of Zn nanoparticles to semen has improved its quality without affecting embryo development rate in vitro. However, in vitro embryo development rate was increased when Zn nanoparticles were supplemented to IVM media. This support the notion of Zn nanoparticles beneficial action on improving bovine gametes quality without affecting pregnancy rate.

Keywords Zinc nanoparticles · Bovine · Spermatozoa · Antioxidant enzymes · IVP · Fertility

Introduction

In modern cattle breeding, where artificial insemination (AI) is the most widely applied tool facilitating extensive utilization of frozen semen from genetically superior sires, cryopreservation has been an invaluable technique. Semen freezing is a technique to maintain sperm for long time and using them for AI [1]. However, use of these processing technologies induces some irreversible damage to sperm in and reducing the percentage of viable sperm cells [2]. These damages can compromise anatomical and structural integrity of sperm. Additionally, there is substantial evidence that cryopreservation results in increase DNA damage, aneuploidy, and chromosome fragmentation [3, 4]. Consequently, physical characteristics of semen like sperm DNA integrity and plasma membrane integrity have a crucial role in sperm survival after insemination, transmission of genetic material, and finally early embryonic development [5–7].

Mammalian spermatozoa membranes are rich in polyunsaturated fatty acids and are sensitive to oxygen induced damage mediated by lipid peroxidation [8]. In both human and animal studies, the role of oxidative stress within sperm was

These two authors share equally senior authorship of this work.

✉ Nasser Ghanem
nassergo@agr.cu.edu.eg

Abdollah Mohammadi-Sangcheshmeh
amohammadis@ut.ac.ir

[1] Department of Animal and Poultry Science, College of Aburaihan, University of Tehran, Pakdasht, Tehran, Iran

[2] Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Nitra, Slovakia

[3] Department of Animal Production, Faculty of Agriculture, Cairo University, Giza 12613, Egypt

Published online: 03 June 2020

emphasized, resulting in DNA damage [9, 10], reduced sperm parameters [11, 12], and gene expression [13, 14], and defective membrane integrity [1]. In some situations, the damage caused by oxidants may be repaired. Unfortunately, spermatozoa are unable to restore the damage induced by oxidative stress because they lack the necessary cytoplasmic-enzyme repair systems [15]. This is one of the features that make spermatozoa unique in their susceptibility to oxidative insult [8] which increases sperm deformations [5, 8].

On the basis of these, several studies [1, 16–23] have shown the beneficial effect of antioxidant therapy on oxidative stress in mammalian spermatozoa. Nanoparticles, with at least one dimension at 1–100 nm range, have become much prevalent in life over the past two decades, and are now widely used in a variety of industries and medical fields [24]. Kobyliak et al. [25] have reported that cerium dioxide nanoparticles administrated for 10 days into male rats have decreased the serum level of lipid peroxidation product and increased catalase and superoxide dismutase activity, coupled with an increase in semen physical parameters (count, motility, viability, and percentage of live spermatozoa) [25]. On the other hand, it has been indicated that sperm motility was decreased after exposure to gold nanoparticles, compared with normal group [26]. Other study revealed that titanium dioxide (TiOR2R) nanoparticles decrease human sperm motility, and silica nanoparticles are toxic for mouse sperms [27]. In 2011, a study on toxicity of TiOR2R and silver nanoparticles on reproductive system showed that a single oral 100 mg/kg dose of TiOR2R nanoparticles decreased mice developmental process, and increased fetal deformities and mortality [28].

Trace mineral like zinc is essential for media used for in vitro embryo production. In this regard, supplementation of IVM media with zinc sulphate has improved bovine COCs quality by reducing DNA damage, increased cleavage rate, blastocyst rate, and total cell number compared to control group [29]. The beneficial effect of zinc on oocyte quality during IVM was also confirmed in two different investigations [30, 31]. Moreover, supplementing embryo culture media with zinc sulphate did not affect embryo development, however increased total cell number and inner cell mass of blastocyst [32]. All above-mentioned studies were done using Zn in normal form.

Research is limited on the use of nanoprticles on male fertility. In addition, to our knowledge, there is no study on impact of Zinc (Zn) nanoparticles on sperm, but it has been shown that elements such as Zn and Se are essential for testicular development and spermatogenesis [33–35]. Therefore, it was hypothesized that insufficient intake of Zn impairs antioxidant defense system [36, 37]. This may be an important risk factor in oxidant release and makes the spermatozoa more susceptible to lipid peroxidation [36, 37].

The current study was performed to evaluate Zn nanoparticles as an antioxidant in semen extender. Therefore, Zn nanoparticles were added to Bioxcell® extender in a dose-dependent manner, and after freeze-thawing process, semen quality was assessed using computer-assisted semen analysis (CASA). In addition, plasma membrane functionality (hypo-osmotic swelling test; HOS), mitochondrial activity (Rhodamine-123), abnormal morphology (Hancock solution), and DNA fragmentation (Acridine orange) were determined after 72 h of storage. In vitro embryo development rate was evaluated after adding Zn nanoparticles to in vitro maturation media and after in vitro fertilization using Zn nanoparticles-treated semen. Finally, pregnancy rate was monitored at day 30 and 60 after artificial insemination using semen treated with Zn nanoparticles.

Material and Methods

Chemicals

Unless otherwise mentioned, all the chemicals, reagents, media, biologics, and media constituents were purchased from Sigma-Aldrich Chemicals, USA.

Semen Collection

Semen was collected from four tested bulls of Holstein breed with artificial vagina. Four ejaculates were pooled and diluted with Bioxcell® extender containing 0 (control group), 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M of Zn nanoparticles. The semen samples were frozen for 72 h before used for further analysis. The wide range of Zn nanoparticles concentration selected in this study was due to lack of information in appropriate concentration of nanoparticles that could be added to semen extender; in addition, we followed the guidelines of study that was done by Barkhordari et al. [24].

For cryopreservation, semen samples from different groups were filled and packed in 0.5-mL straws. After that, the temperature was brought to 4–6 °C, and after three and a half hours, the straws were transferred to the cryopreservation device. After 10 min, when the temperatures of straws reached almost − 140 °C, samples were transferred into liquid nitrogen at − 196 °C.

Sperm Motility

Progressive motility (PM), as well as total motility (TM), was evaluated using CASA program (Animal Version 12.3 CEROS, Hamilton-Thorne Biosciences, and Beverly, MA, USA).

Sperm Viability

A drop of semen (10 μL) were mixed with eosin-nigrosin and spread on a clean glass slide. The slides were air dried and

examined under microscope. In total, 200 sperms were counted and the percentage of stained sperms (dead) and unstained sperms (live) were calculated.

Sperm Morphology

A volume of 30 μL of semen sample was added to 300 mL of Hancock solution and mixed well by repeat pipetting. A drop of the mixed solution was spread on a glass slide then covered with lamella. In total, 200 spermatozoa were examined under an inverted microscope, for calculating the percentage of abnormal sperm (Fig. 8).

Plasma Membrane Functionality

The plasma membrane functionality was assessed by hypo-osmotic swelling (HOS) test after freeze-thawing process. The idea of HOS test is based on the membrane resistance to lose permeability barriers under stress condition (stretching in a hypo-osmotic medium). The assay was done by mixing 30 μL of semen with a 300-μL hypo-osmotic solution (9 g/L fructose and 4.9 g/L sodium citrate, 100 mOsm/kg). The mixture was incubated at 37 °C for 30 min, and a drop (0.2 mL) of the mixture was spread on a glass slide and covered with cover slip. The slides were evaluated under phase-contrast microscope (CKX41; Olympus, Tokyo, Japan) on × 400 magnification. In total, 200 spermatozoa with swollen and not-swollen tails were recorded.

Mitochondrial Activity Assessment

Assessment of mitochondrial activity was performed by dual staining of Rhodamine-123 (R123; Invitrogen TM, Eugene, OR, USA) and propidium iodide (PI) dyes according to Zou et al. [38]. Briefly, 10 μL of R123 solution (0.01 mg/mL distillated water) was mixed well with 500 μL of diluted semen sample (50×10^6 spermatozoa/mL), and kept at room temperature for 30 min in the dark. Afterwards, samples were centrifuged at 500×*g* for 3 min and the sperm pellet was re-suspended in 500 μL Tris buffer. Then, 10 μL of PI dye was added to sample before flow cytometry. The percentage of live spermatozoa with active mitochondria was identified by recording the fluorescence intensities of Rh123 and PI of 10,000 spermatozoa in each semen sample. The flow cytometry analysis was carried out using a FACS Calibur flow cytometer (Becton Dickinson, San Khosoz, CA, USA). The fluorescence of R123 and PI was detected on FL1 and FL3 detectors.

DNA Fragmentation Assay

Sperm chromatin structure assay (SCSA) is dependent on measuring the fluorescence emission of acridine orange dye to evaluate DNA damage. Flow cytometry was used to detect and count a large number of sperm with DNA damage. Acridine orange is a metachromatin dye, which binds to DNA and denature. Therefore, it becomes red in color when it binds to DNA of sperm with natural form, while it indicates green color in case of sperm with DNA damage.

Malondialdehyde (MDA) Concentration

The level of lipid peroxidation was indicated by measuring the level of MDA in semen that was cryopreserved in presence of Zn nanoparticles as previously described [39]. Briefly, 1 mL of semen samples was added to 1 mL trichloroacetic acid and the precipitate was centrifuged (960×*g* for 15 min). Equal volume of the supernatant was added to thiobarbituric acid at 100 °C for 10 min and the absorbance of the resultant substance was measured at 532 nm.

Embryo Development In Vitro

Ovaries were collected from local abattoir and kept in physiological saline at 37 °C till transported to lab. Cumulus-oocyte complexes (COCs) were recovered by aspiration using 10 g syringe. Good-quality COCs (grade 1 and 2) were selected based on number of cumulus cells and cytoplasmic appearance of the oocytes. The recovered COCs were handled for in vitro maturation, fertilization, and embryo culture in five biological replicates each with 22–25 COCs for all experimental groups as previously described by our research group [40]. First, COCs were cultured in maturation medium (bicarbonate-buffered TCM-199, 5.5 mg mL − 1 sodium pyruvate, 2 mM L-glutamine, 10% fetal bovine serum, 0.5 μg mL^{-1} FSH, 5.0 μg mL^{-1} LH, 1 μg mL^{-1}estradiol, and 25 μg mL^{-1} gentamycin sulphate) for 24 h at 39 °C, 5% CO_2 in a humidified atmosphere.

Parthenogenetic activation of bovine cumulus-oocytes complexes was performed as described previously [40]. For in vitro fertilization, mature COCs were washed twice in HSOF buffer (HEPES-buffered synthetic oviductal fluid), then kept with sperms at concentration of 2×10^6 spermatozoa mL − 1 in fertilization medium (SOF supplemented with 4 IU mL − 1 heparin, 10 μM hypotaurine, 20 μM penicillamine, 1 μM epinephrine, and 2% (v/v) fetal bovine serum) for 18 h at 39 °C, 5% CO2 in a humidified atmosphere. The presumptive zygotes were denuded by repeat pipetting and washed thrice in HSOF then cultured in SOFaa medium [(1% MEM-nonessential amino acids, 2% BME-essential amino acids, 0.34 mM tri-sodium citrate, 1 mM glutamine, 8 mg mL − 1 bovine serum albumin, and 2.77 mM myo-inositol] at the same previously mentioned conditions till

blastocyst stage (192 h post insemination). Embryo culture medium was renewed every 3 days and cleavage rate was recorded at 48 h post insemination.

Estrus Synchronization, Detection and AI

Twenty-four cyclic and healthy Holstein cows at the third parity on average were kept under the same managerial conditions. The animals were checked for estrus thrice a day based on visual observation of heat signs after estrus synchronization using twice PGF2α injections at 11 days interval. Standard artificial insemination procedure was applied for all females (8 cows in each group) to evaluate the in vivo fertilizing ability of treated semen with Zn nanoparticles.

Pregnancy Detection

Expert veterinarian performed pregnancy detection using ultrasound examination. The inseminated cows of different groups ($n = 24$) were checked for pregnancy detection at 30 and 60 days after insemination.

Statistical Analysis

The data were analyzed using Proc Mixed of SAS 9.1 (version 9.1; SAS Institute, 2002, Cary, NC, USA). The results were expressed as mean ± SEM. Tukey's test was used to compare least squares means.

Results

Sperm Motility and Motion Parameters

The data dealing with effects of different levels of Zn nanoparticles on sperm motility parameters of bull spermatozoa after freeze-thawing process is shown in Table 1. The results demonstrated that different concentrations of Zn nanoparticles had no effect on TM and PM compared to control group and the differences between the groups were not significant ($P \geq 0.05$).

Sperm Viability

The results of sperm viability treated with different concentrations of Zn nanoparticles are shown in Fig. 1. Adding different levels of Zn nanoparticles to Bioxcell® extender had no significant effect on sperm viability ($P \geq 0.05$). Sperm viability was 59.5, 69.5, and 66% in 10^{-6} M, 10^{-2} M of Zn nanoparticles and control groups, respectively.

Table 1 Effect of extenders with different concentrations of nano zinc on the post-thawed bull sperm motility parameters (cumulative results of four replicates)

Groups	Parameters	
	TM (Lsmean ± SEM)	PM (Lsmean ± SEM)
Control	87.31 ± 3.72	70.48 ± 4.5
nZinc 10^{-6}	86.4 ± 3.28	71.85 ± 8.33
nZinc 10^{-5}	86.11 ± 5.36	73.43 ± 7.37
nZinc 10^{-4}	86.42 ± 3.20	69.65 ± 5.73
nZinc 10^{-3}	86.28 ± 4.09	68.15 ± 6.96
nZinc 10^{-2}	85.09 ± 5.5	75.05 ± 6.57

No difference ($P \geq 0.05$) was observed among treated and untreated groups

nZinc 10^{-6}: Extender containing 10^{-6} M nano zinc; nZinc 10^{-5}: Extender containing 10^{-5} M nano zinc; nZinc 10^{-4}: Extender containing 10^{-4} M nano zinc; nZinc 10^{-3}: Extender containing 10^{-3} M nano zinc; nZinc 10^{-2}: Extender containing 10^{-2} M nano zinc

TM, total motility; *PM*, progressive motility

Sperm Morphology

Effect of Zn nanoparticles on morphology of bovine spermatozoa was not significant ($P \geq 0.05$) after freeze-thawing process (Fig. 2). The percentage of normal sperm was almost the same in control (57.7%), 10^{-6} M (66.2%) and 10^{-2} M (60%) of Zn nanoparticles. There are some abnormalities detected in all groups like cytoplasmisc droplet and coiled-tail sperms as indicated in Fig. 8.

Plasma Membrane Functionality

Results showed that plasma membrane functionality (Fig. 3) in 10^{-6} and 10^{-2} M of Zn nanoparticles was improved (69.3 and 62.4%) significantly ($P < 0.05$) compared to control (51.3%) group.

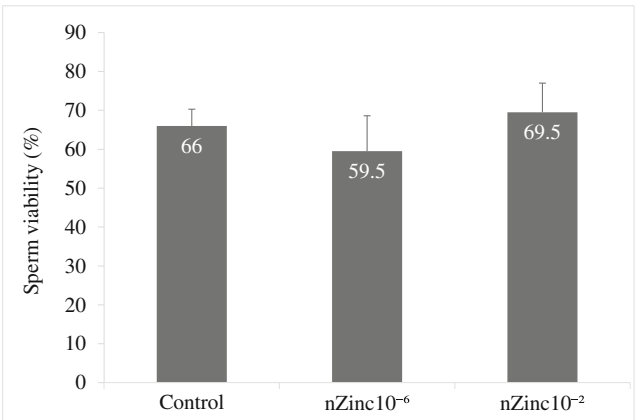

Fig. 1 Percentage of viable spermatozoa following treatment with Zn nanoparticles. No difference ($P \geq 0.05$) was observed among the groups

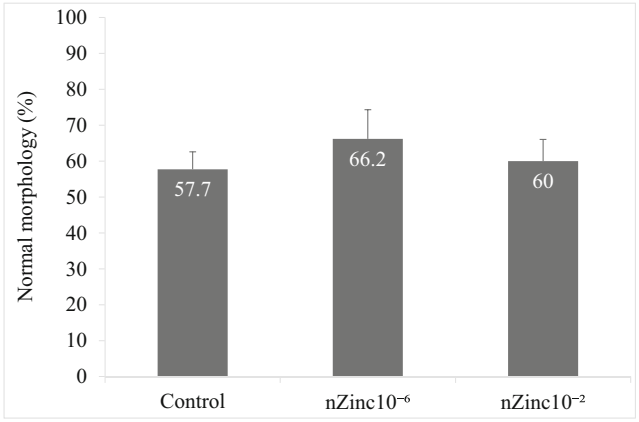

Fig. 2 Percentage of normal sperm morphology after treatment with Zn nanoparticles. No difference ($P \geq 0.05$) was observed among the groups

Mitochondrial Activity

The proportions of live spermatozoa with active mitochondria of each experimental group are shown in Fig. 4. The results indicated that 10^{-6} and 10^{-2} M of Zn nanoparticles increased ($P < 0.05$) the percentage of live spermatozoa with active mitochondria (85.3 and 67.3%) compared to untreated group (49.8%).

DNA Fragmentation

The results showed that adding different concentrations of Zn nanoparticles (10^{-6} and 10^{-2} M) had no effect ($P \geq 0.05$) on DNA fragmentation (4.4 and 4.1%) compared to control group (4.1%) as shown in Fig. 5.

Malondialdehyde (MDA) Concentration

Bull semen containing 10^{-6} and 10^{-2} M of Zn nanoparticles had significantly ($P < 0.05$) lowered the level of MDA (2.9 and 2.7 mol/mL) than control group (3.7 mol/mL) as shown in Fig. 6.

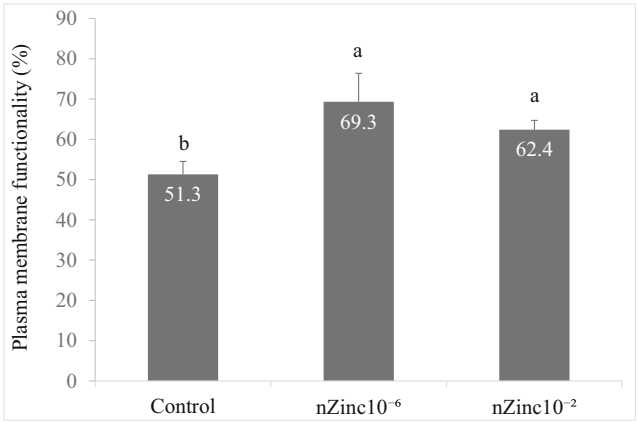

Fig. 3 Percentage of sperm plasma membrane functionality after treatment with Zn nanoparticles. Bars with different superscripts (a, b) are significantly different at $P < 0.05$

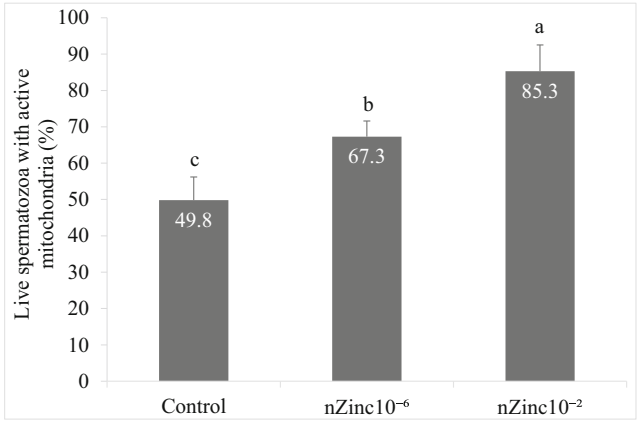

Fig. 4 Percentage of live spermatozoa with active mitochondria after treatment with Zn nanoparticles. Bars with different superscripts (a, b, c) are significantly different at $P < 0.05$

Embryo Development In Vitro

The addition of different concentrations of Zn nanoparticles to in vitro maturation media indicated that blastocyst rate was increased significantly ($P < 0.05$) when Zn nanoparticles were added at concentration of 10^{-6} M compared to other experimental groups (Table 2).

Data of early embryo development after using Zn nanoparticles in semen cryopreservation was shown in Table 3. The addition of Zn nanoparticles in semen extender did not ($P \geq 0.05$) influence early embryonic development. Cleavage, as well as blastocyst rates, was similar in 10^{-6} M (69.8 and 31.1%), 10^{-2} M (77.1 and 30.5%) Zn nanoparticles, and control (76.7 and 31.4%) groups. Thus, it seems that supplementation of IVM with Zn nanoparticles positively affected in vitro embryo, while there is no effect of Zn nanoparticles on embryo development rate when supplemented to semen extender.

Pregnancy Outcome

As shown in Fig. 7, there were no differences ($P \geq 0.05$) in pregnancy rate at day 30 and 60 of AI when 10^{-6} M (58.1 and

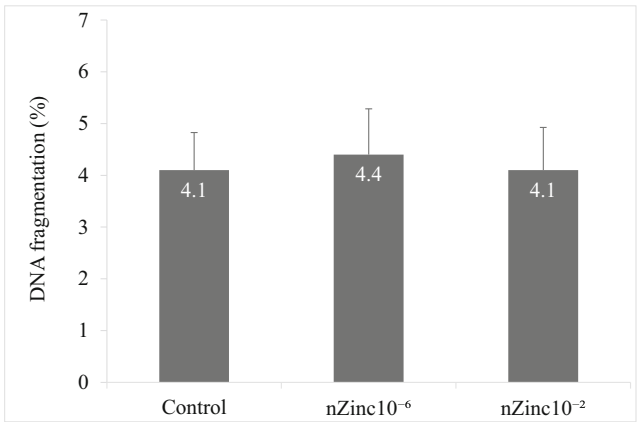

Fig. 5 Percentage of sperm DNA fragmentation after treatment with Zn nanoparticles. No difference ($P \geq 0.05$) was observed among the groups

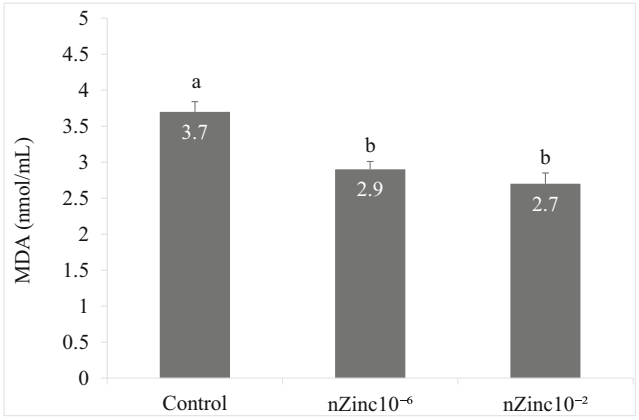

Fig. 6 Level of MDA after treatment with Zn nanoparticles. Bars with different superscripts (a, b) are significantly different at $P < 0.05$

51.4%), 10^{-2} M (53.3 and 49.5%) Zn nanoparticles were used in semen extender compared to control (56.4 and 52.7%).

Discussion

The results of adding different levels of Zn nanoparticles to Bioxcell® extender after freeze-thawing process showed partial improvement in some sperm quality parameters. In this regard, there are contradictory results in the use of antioxidants of different forms and by different ways to treat infertility or to improve fertility. It has been stated that the high Zn in semen reduces sperm motility in men [41], while our results indicated insignificant effect of Zn in this parameter. However, some other authors have confirmed that Zn improves sperm motility [42–44] and normal morphology [33, 45]. According to obtained result, this is the first study that evaluates the effect of Zn in nanoparticle form on bovine sperm quality after freeze-thawing process and this makes it difficult to compare results with previous findings that used Zn in normal form.

Indeed, Zn is an essential element in different biological processes as it could be bound to some proteins and mediates their functional activities. Additionally, Zn was localized in sperm mitochondria, flagella of rat epididymis [46], spermatogonia, and primary spermatocytes in mouse testis [47]. Yamaguchi et al. [48] have reported the presence of Zn in mitochondria of Japanese eel testis (spermatogonia, spermatids, and spermatozoa). Zn transporter protein namely ZnT-1 was found to be expressed in the mitochondria of mice spermatozoa [49]. Consistent with previous studies, our results revealed that Zn nanoparticles on both levels (10^{-6} and 10^{-2}) increased mitochondrial activity of bovine spermatozoa after freeze-thawing process. This supports the notion that increased Zn accumulation in testis may have crucial role in germ cells proliferation, meiosis, and spermiation [47, 48].

Plasma membrane integrity is a vital indicator of sperm survival and fertilizing capability when used for insemination [7]. Accordingly, results of the current study indicated that Zn nanoparticles in both levels (10^{-6} and 10^{-2}) enhanced membrane integrity of bull spermatozoa after freeze-thawing process. In support to the obtained results, bioactive Zn in seminal plasma keeps sperm membrane stability process. Moreover, sperm uses ATP as the basic energy source for maintaining cell functionality, viability, acrosome reaction, and mobility required to reach and penetrate the oocyte [17]. Therefore, it seems that Zn inclusion nanoparticles in semen extender have a beneficial influence on sperm structural (membrane integrity) and cellular (mitochondria) activities (Figs 3, 4 and 9).

In contrast, the improvement in the last two semen quality parameters, the level of MDA was significantly reduced in semen samples that had Zn nanoparticles compared with control group. This could be due to the antioxidant effect of Zn on lipid peroxidation process. In support with this hypothesis, Zn was successfully used as a fertility therapeutic agent in men with asthenozoospermia [50]. The treatment with Zn was associated with reduction of both MDA by twofold and tumor necrosis factor (TNF) by about fivefold in addition to increased total antioxidant capacity [50]. The improvement of total antioxidant capacity and plasma membrane integrity is a clear indicator of reduced incidence of lipid peroxidation,

Table 2 Embryo development at day 9 after parthenogenetic activation of bovine cumulus-oocytes complexes treated with nano zinc during in vitro maturation (cumulative results of four replications)

Groups	No. oocyte	PB n (%)	Cleaved 48 hpa n (%)	Blastocyst 192 hpa n (%)
Control	88	72 (82.6 ± 3.5)	63 (72.1 ± 2.9)	26 (29.6 ± 2.1)^{b}
nZinc 10^{-6}	91	75 (83.0 ± 3.5)	64 (70.7 ± 3.3)	32 (36.6 ± 2.0)^{a}
nZinc 10^{-5}	82	70 (85.4 ± 2.2)	58 (71.6 ± 2.4)	27 (32.1 ± 2.0)^{ab}
nZinc 10^{-4}	90	77 (85.9 ± 3.5)	66 (73.7 ± 3.4)	28 (32.4 ± 4.4)^{ab}
nZinc 10^{-3}	91	78 (85.4 ± 2.4)	68 (74.1 ± 2.0)	28 (31.1 ± 2.3)^{ab}
nZinc 10^{-2}	83	69 (84.1 ± 3.9)	59 (69.6 ± 4.1)	26 (31.3 ± 3.6)^{ab}

Differences ($P < 0.05$) were observed among treated and untreated groups

PB, polar body observed

hpa means hours post activation

Table 3 Effect of semen extenders with different concentrations of zinc nanoparticles on embryonic developmental potential of bovine oocytes after IVF (cumulative results of four replicates)

		Embryo development	
Groups	No. oocytes	Cleaved 48 hpi n (mean ± SEM)	Blastocyst 192 hpi n (mean ± SEM)
Control	113	86 (76.7 ± 3.8)	35 (31.4 ± 4.5)
nZinc 10^{-6}	125	86 (69.8 ± 6.9)	38 (31.1 ± 3.2)
nZinc 10^{-2}	122	94 (77.1 ± 3.1)	37 (30.5 ± 2.1)

No difference ($P \geq 0.05$) was observed among treated and untreated groups

nZinc 10^{-6}: Extender containing 10^{-6} M nano zinc; nZinc 10^{-2}: Extender containing 10^{-2} M nano zinc

hpi means hours post insemination

because of Zn nanoparticle supplementation to extender. Moreover, increased total antioxidant capacity could indicate enhanced activity antioxidant enzymes; however, MDA was reduced and the only one enzyme that measured. Therefore, future investigations have to consider the activity of several antioxidant enzymes to give a clear and deep insight into sperm biochemical defense mechanism.

The results of adding different levels of Zn nanoparticles to Bioxcell® extender showed that there were no significant differences in sperm viability between control and treated groups after freeze-thawing process. However, some researchers have reported that compounds with antioxidant properties such as rosemary can improve the viability of ram spermatozoa after freeze-thawing process [21]. Contrasting results with our study were observed by the other researchers through the addition of other antioxidants such as taurine, hypotaurine, glutamine, cysteine, trehalose, and hyaluronan to the diluents before freeze-thawing process [16]. The use of oral antioxidants such as vitamin E and selenium has improved sperm PM in stallion [51]. The differences in the molecular nature of listed antioxidant compounds, dose used, and its interaction with species under investigation could be a reason behind these conflicting results compared to ours.

The effects of Zn on sperm motility have been examined in a variety of species. Sperm motility was reduced in response to increased concentrations of Zn in the men seminal plasma [34]. In another study, it was reported that Zn in the seminal plasma of men has an inhibitory effect on sperm motility, while removal of semenogelin (Zn binding protein) has resulted in improvement of sperm motility [33]. However, our results observed no effect of Zn nanoparticles on TM and PM. In contrast, in vitro use of an intracellular Zn chelator (diethyldithiocarbamate) has reduced human sperm velocity and inhibited sperm motion [52]. Furthermore, N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) as another Zn chelator has decreased sperm motility in the Japanese eel [48]. These contradictory results suggest that the effect of extracellular Zn on sperm motility may be dependent on both the concentration and the species under investigation, which

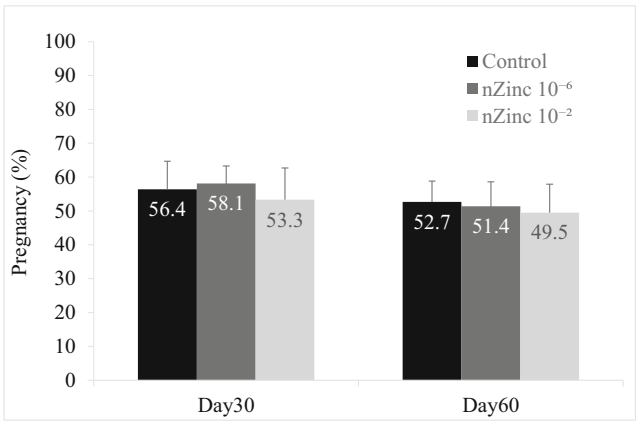

Fig. 7 Effect of semen extenders with different concentrations of Zn nanoparticles on pregnancy rate of inseminated cows 30 and 60 days after AI. No difference ($P \geq 0.05$) was observed among the groups

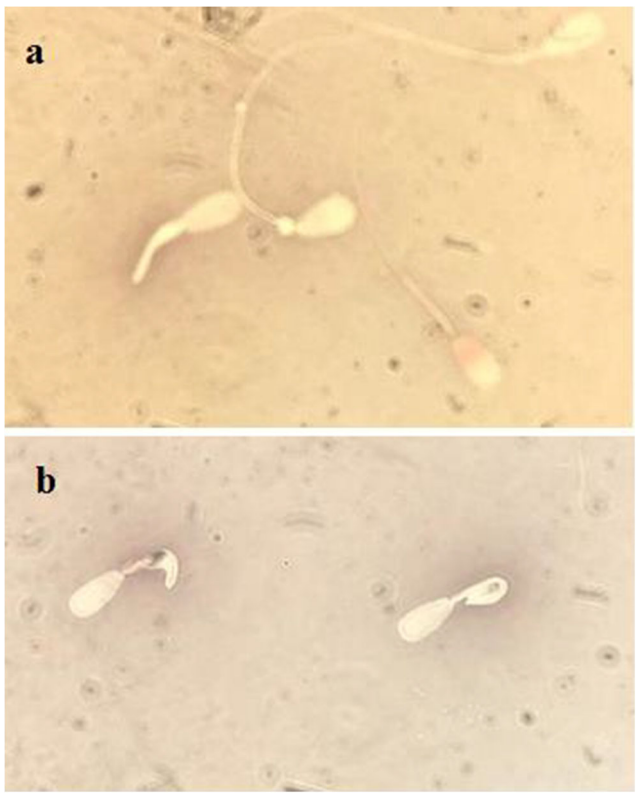

Fig. 8 Image of sperm abnormal morphology after freeze-thawing process, cytoplasmic droplet (a), coiled-tail sperm (b)

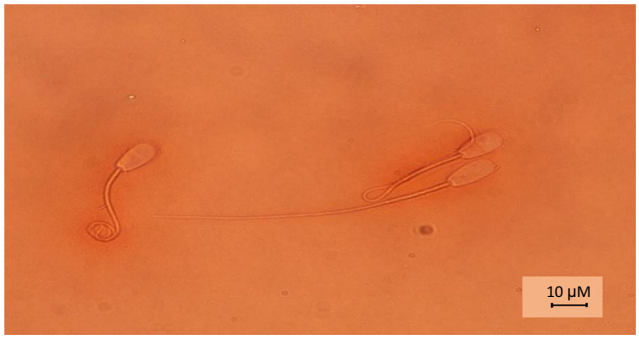

Fig. 9 Image of plasma membrane functionality assessed by hypo-osmotic swelling (HOS) test after freeze-thawing process

suggested a certain level beyond it Zn could have inhibitory effect on sperm motility.

In present study, addition of Zn nanoparticles to semen extender did not influence DNA fragmentation rate, which is in line with data of viability and morphology of sperms. In other study, patients that were treated with antioxidants decreased DNA fragmentation [17]. The form of Zn used in the current study (nano form) may limit some of its biological actions; however, further investigation including different forms of Zn is needed to clarify this idea. The physical characteristics of semen like sperm DNA integrity have a crucial role in transmission of genetic material and preimplantation embryo development [5, 6]. Early embryonic development is crucial for successful implantation and maintenance of pregnancy [53]. Surprisingly, our data revealed that addition of Zn nanoparticles at 10^{-6} M to in vitro maturation media increased embryo development rate compared to control group (Fig. 10 and Table 2). In consistent with our results, blastocyst rate and quality were significantly improved after addition of 1.5 μg/ml Zn during in vitro maturation [53]. This positive effect of Zn supplementation may due to enhancement of SOD activity in cumulus cells, which subsequently decreased DNA damage, and apoptosis in COCs [53]. This result could indicate that Zn nanoparticles could be more effective as antioxidant in oocytes than sperm.

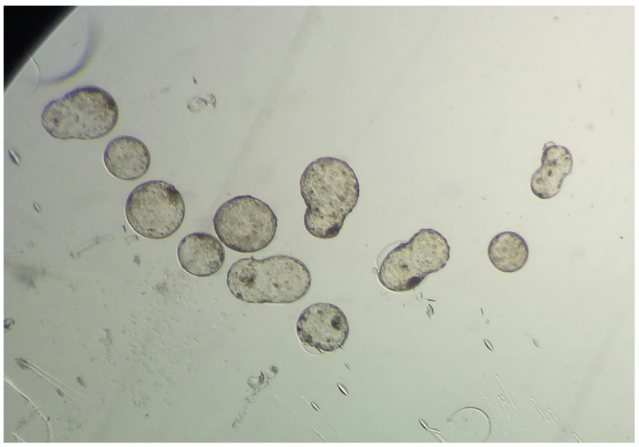

Fig. 10 Image of blastocysts at day 9 after in vitro fertilization of bovine cumulus-oocytes complexes treated with nano Zinc during in vitro maturation

Although Zn nanoparticles have improved some semen quality parameters and embryo development rate during IVM, but there was no effect on embryo development rate and pregnancy outcomes when treated semen was used for in vitro fertilization or A. I in the present report. Consistently, it was also reported [31] that sperm of low-fertility (69% non-return rate) or high-fertility bulls (78% non-return rate) showed similar in vitro fertilization rates (77 and 79%, respectively). Moreover, studies that have focused on the use of micronutrients as antioxidants demonstrated a decrease of sperm oxidative stress and DNA damage in subfertile males, however further research is crucially required to clearly state whether these improvements translate to an improved fertility [54–56]. In addition, the mode of action by which Zn acts as an antioxidant remains partially unknown.

In conclusion, the results of this study have showed partial improvement in bull semen quality after freeze-thawing in presence of Zn nanoparticles which could be beneficial for long time storage. In addition, Zn nanoparticles have a beneficial effect on COCs developmental competence to reach blastocyst stage. However, improved membrane integrity and mitochondrial activity after addition of Zn micronutrients in nanoform did not influence in vitro fertilization, early embryonic development, and pregnancy rate, which require further investigation on large scale of different animal species.

Acknowledgments The authors thank the members of their own laboratories for their helpful discussions.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Karaji RO, Kia HD, Ashrafi I (2014) Effects of in combination antioxidant supplementation on microscopic and oxidative parameters of freeze–thaw bull sperm. Cell Tissue Bank 15:461–470
2. Woelders H, Matthijs A, Engel B (1997) Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. Cryobiology 35:93–105
3. Baumber J, Ball BA, Linfor JJ, Meyers SA (2003) Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl 24:621–628
4. Li MW, Meyers S, Tollner TL, Overstreet JW (2007) Damage to chromosomes and DNA of rhesus monkey sperm following cryopreservation. J Androl 28:493–501
5. Agarwal A, Said TM (2003) Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 9:331–345
6. D'Occhio M, Hengstberger K, Johnston S (2007) Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. Anim Reprod Sci 101:1–17

7. Celeghini E, De Arruda R, De Andrade A, Nascimento J, Raphael C (2007) Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reprod Domest Anim 42:479–488
8. Agarwal A, Virk G, Ong C, du Plessis SS (2014) Effect of oxidative stress on male reproduction. World J Men's Health 32:1–17
9. Lopes S, Jurisicova A, Sun JG, Casper RF (1998) Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod 13:896–900
10. Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI (2010) Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Hum Reprod 25:2415–2426
11. Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC (2000) The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. J Androl 21:895–902
12. Kao SH, Chao H, Chen HW, Hwang TI, Liao TL, Wei YH (2008) Increase of oxidative stress in human sperm with lower motility. Fertil Steril 89:1183–1190
13. Jervis KM, Robaire B (2004) The effects of long term vitamin E treatment on gene expression and oxidative stress damage in the aging Brown Norway rat epididymis. Biol Reprod 71:1088–1095
14. Jin J, Jin N, Zheng H, Ro S, Tafolla D, Sanders KM, Yan W (2007) Catsper3 and Catsper4 are essential for sperm hyperactivated motility and male fertility in the mouse. Biol Reprod 77:37–44
15. Krausz C, Mills C, Rogers S, Tan S, Aitken RJ (1994) Stimulation of oxidant generation by human sperm suspensions using phorbol esters and formyl peptides: relationships with motility and fertilization *in vitro*. Fertil Steril 62:599–605
16. Sanchez-Partida L, Setchell B, Maxwell W (1998) Epididymal compounds and antioxidants in diluents for the frozen storage of ram spermatozoa. Reprod Fertil Dev 9:689–696
17. Ménézo YJ, Hazout A, Panteix G, Robert F, Rollet J, Cohen-Bacrie P, Chapuis F, Clément P, Benkhalifa M (2007) Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. Reprod BioMed Online 14:418–421
18. Malo C, Gil L, Gonzalez N, Martínez F, Cano R, De Blas I, Espinosa E (2010) Anti-oxidant supplementation improves boar sperm characteristics and fertility after cryopreservation: comparison between cysteine and rosemary (Rosmarinus officinalis). Cryobiology 61:142–147
19. Zhang W, Yi K, Chen C, Hou X, Zhou X (2012) Application of antioxidants and centrifugation for cryopreservation of boar spermatozoa. Anim Reprod Sci 132:123–128
20. Atashfaraz E, Farokhi F, Najafi G (2013) Protective effect of ethyl pyruvate on epididymal sperm characteristics, oxidative stress and testosterone level in methotrexate treated mice. J Reprod Infertil 14: 190–196
21. Zanganeh Z, Zhandi M, Zare-Shahneh A, Najafi A, Nabi MM, Mohammadi-Sangcheshmeh A (2013) Does rosemary aqueous extract improve buck semen cryopreservation? Small Rumin Res 114: 120–125
22. Zalata A, Elhanbly S, Abdalla H, Serria M, Aziz A, El-Dakrooy S, El-Bakary AA, Mostafa T (2014) *In vitro* study of cypermethrin on human spermatozoa and the possible protective role of vitamins C and E. Andrologia 46:1141–1147
23. Sarıözkan S, Tuncer P, Büyükleblebici S, Bucak M, Cantürk F, Eken A (2015) Antioxidative effects of cysteamine, hyaluronan and fetuin on post-thaw semen quality, DNA integrity and oxidative stress parameters in the Brown Swiss bull. Andrologia 47:138–147
24. Barkhordari A, Hekmatimoghaddam S, Jebali A, Khalili MA, Talebi A, Noorani M (2013) Effect of zinc oxide nanoparticles on viability of human spermatozoa. Int J Reprod BioMed 11:767–771
25. Kobyliak NM, Falalyeyeva TM, Kuryk OG, Beregova TV, Bodnar PM, Zholobak NM, Shcherbakov OB, Bubnov RV, Spivak MY (2015) Antioxidative effects of cerium dioxide nanoparticles ameliorate age-related male infertility: optimistic results in rats and the review of clinical clues for integrative concept of men health and fertility. EPMA J 6:12
26. Wiwanitkit V, Sereemaspun A, Rojanathanes R (2009) Effect of gold nanoparticles on spermatozoa: the first world report. Fertil Steril 91:e7–e8
27. Ema M, Kobayashi N, Naya M, Hanai S, Nakanishi J (2010) Reproductive and developmental toxicity studies of manufactured nanomaterials. Reprod Toxicol 30:343–352
28. Philbrook NA, Winn LM, Afrooz AN, Saleh NB, Walker VK (2011) The effect of TiO 2 and Ag nanoparticles on reproduction and development of Drosophila melanogaster and CD-1 mice. Toxicol Appl Pharmacol 257:429–436
29. Picco SJ, Anchordoquy JM, de Matos DG, Anchordoquy JP, Seoane A, Mattioli GA, Errecalde AL, Furnus CC (2010) Effect of increasing zinc sulphate concentration during in vitro maturation of bovine oocytes. Theriogenology 74:1141–1148
30. Anchordoquy JM, Picco SJ, Seoane A, Anchordoquy JP, Ponzinibbio MV, Mattioli GA, Peral García P, Furnus CC (2011) Analysis of apoptosis and DNA damage in bovine cumulus cells after exposure in vitro to different zinc concentrations. Cell Biol Int 35:593–597
31. Anchordoquy JM, Anchordoquy JP, Sirini MA, Picco SJ, Peral-García P, Furnus CC (2014) The importance of having zinc during in vitro maturation of cattle cumulus-oocyte complex: role of cumulus cells. Reprod Domest Anim 49:865–874
32. Wooldridge LK, Nardi ME, Ealy AD (2019) Zinc supplementation during in vitro embryo culture increases inner cell mass and total cell numbers in bovine blastocysts. J Anim Sci 97:4946–4950
33. Caldamone AA, Freytag MK, Cockett AT, Cockett T (1979) Seminal zinc and male infertility. Urology 13:280–281
34. Chia SE, Ong CN, Chua LH, Ho LM, Tay SK (2000) Comparison of zinc concentrations in blood and seminal plasma and the various sperm parameters between fertile and infertile men. J Androl 21: 53–57
35. Colagar AH, Marzony ET, Chaichi MJ (2009) Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. Nutr Res 29:82–88
36. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S (2012) The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 10:49
37. Song Y, Leonard SW, Traber MG, Ho E (2009) Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses, and DNA repair in rats. J Nutr 139:1626–1631
38. Zou T, Liu X, Ding S, Xing J (2010) Evaluation of sperm mitochondrial function using rh123/PI dual fluorescent staining in asthenospermia and oligoasthenozoospermia. J Biomed Res 24: 404–410
39. Dodaran HV, Zhandi M, Sharafi M, Nejati-Amiri E, Nejati-Javaremi A, Mohammadi-Sangcheshmeh A, Mohamed Mahmoud Shehab-El-Deen MA, Shakeri M (2015) Effect of ethanol induced mild stress on post-thawed bull sperm quality. Cryobiology 71:12–17
40. Mohammadi-Sangcheshmeh A, Veshkini A, Hajarizadeh A, Jamshidi-Adegani F, Zhandi M, Abazari-kia AH, Cinar MU, Soleimani M, Gastal EL (2014) Association of glucose-6-phosphate dehydrogenase activity with oocyte cytoplasmic lipid content, developmental competence, and expression of candidate genes in a sheep model. J Assist Reprod Genet 31:1089–1098
41. Danscher G, Hammen R, Fjerdingstad E, Rebbe H (1978) Zinc content of human ejaculate and the motility of sperm cells. Int J Androl 1:576–581
42. Fuse H, Kazama T, Ohta S, Fujiuchi Y (1999) Relationship between zinc concentrations in seminal plasma and various sperm parameters. Int Urol Nephrol 31:401–408

43. Mankad M, Sathawara N, Doshi H, Saiyed H, Kumar S (2006) Seminal plasma zinc concentration and α-glucosidase activity with respect to semen quality. Biol Trace Elem Res 110:97–106
44. Ebisch I, Thomas C, Peters W, Braat D, Steegers-Theunissen R (2007) The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 13: 163–174
45. Stanković H, Mikac-Devi D (1976) Zinc and copper in human semen. Clin Chim Acta 70:123–126
46. Stoltenberg M, Sørensen MB, Danscher G, Juhl S, Andreasen A, Ernst E (1997) Autometallographic demonstration of zinc ions in rat sperm cells. Mol Hum Reprod 3:763–767
47. Sørensen MB, Stoltenberg M, Henriksen K, Ernst E, Danscher G, Parvinen M (1998) Histochemical tracing of zinc ions in the rat testis. Mol Hum Reprod 4:423–428
48. Yamaguchi S, Miura C, Kikuchi K, Celino FT, Agusa T, Tanabe S, Miura T (2009) Zinc is an essential trace element for spermatogenesis. Proc Natl Acad Sci 106:10859–10864
49. Elgazar V, Razanov V, Stoltenberg M, Hershfinkel M, Huleihel M, Nitzan YB, Lunenfeld E, Sekler I, Silverman WF (2005) Zinc-regulating proteins, ZnT-1, and metallothionein I/II are present in different cell populations in the mouse testis. J Histochem Cytochem 53:905–912
50. Omu A, Al-Azemi M, Kehinde E, Anim J, Oriowo M, Mathew T (2008) Indications of the mechanisms involved in improved sperm parameters by zinc therapy. Med Princ Pract 17:108–116
51. Contri A, De Amicis I, Molinari A, Faustini M, Gramenzi A, Robbe D, Carluccio A (2011) Effect of dietary antioxidant supplementation on fresh semen quality in stallion. Theriogenology 75:1319–1326
52. Sørensen MB, Stoltenberg M, Danscher G, Ernst E (1999) Chelation of intracellular zinc ions affects human sperm cell motility. Mol Hum Reprod 15:338–341
53. Farin PW, Piedrahita JA, Farin CE (2006) Errors in development of fetuses and placentas from *in vitro*-produced bovine embryos. Theriogenology 65:178–191
54. Eid L, Lorton S, Parrish J (1994) Paternal influence on S-phase in the first cell cycle of the bovine embryo. Biol Reprod 51:1232–1237
55. Ross C, Morriss A, Khairy M, Khalaf Y, Braude P, Coomarasamy A, El-Toukhy T (2010) A systematic review of the effect of oral antioxidants on male infertility. Reprod BioMed Online 20:711–723
56. Gharagozloo P, Aitken RJ (2011) The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Hum Reprod 26:1628–1640