IJAS Iranian Journal of Applied Animal Science

Zinc Oxide Nanoparticles Have Beneficial Effect on Frozen-Thawed Spermatozoa of Holstein Bulls

Research Article

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Received on: 27 Mar 2020
Revised on: 29 May 2020
Accepted on: 15 Jun 2020
Online Published on: Mar 2022

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Online version is available on: www.ijas.ir

ABSTRACT

The aim of this study was to investigate the effect of zinc (Zn) oxide nanoparticles (ZnONPs) on bovine sperm quality after cryopreservation. Semen samples were collected from four healthy, mature Holstein bulls twice a week. The samples with critical quality were then pooled and assigned into four different groups including different concentrations of ZnONPs (0, 0.1, 1.0 and-10 µg/mL of extender). The samples were frozen by using a semi-automatic device and kept until the evaluation. After thawing, total (TM) and progressive -motility (PM), membrane integrity and functionality, acrosome integrity, DNA fragmentation and malondialdehyde (MDA) concentration were assessed. The different concentrations of ZnONPs did not significantly affect TM and PM of bull spermatozoa, when compared to the control ($P \geq 0.05$). The percentage of fast- moving spermatozoa was higher in the treatment supplemented either with 0.1 or 1.0 µg ZnONPs in comparison with the control ($P < 0.05$). Moreover, the results indicated that membrane integrity and functionality and also acrosome integrity of the group treated with 1 µg/mL ZnONP1- was significantly higher than control ($P < 0.05$), while, the percentage of sperm with damaged DNA and MDA concentration was significantly lower in ZnONP1 in comparison with the control ($P < 0.05$). In conclusion, adding 1 µg/mL ZnONP to semen extender, could improve post-thaw bull spermatozoa quality.

KEY WORDS bovine, nanoparticles, reproduction, semen, trace mineral.

INTRODUCTION

Sperm cell membrane is a lipid bilayer enriched with poly-unsaturated fatty acids (PUFAs) which are necessary for membrane fluidity and cell communications, whereas they can also be the major target for peroxidation. Lipid peroxidation triggers a series of actions that increase free radical production which has ability to attack and destroy cell structures including proteins, lipids and nucleic acids (Vishwanath and Shannon, 2000; Medeiros *et al*. 2002). It has been well documented that free radicals are the main reason of oxidative stress during sperm freezing-thawing process. Moreover, semen processing and extending might decrease the concentration of antioxidants and thus antioxidative capacity of sperm cells would be inadequate to prevent lipid peroxidation (Surai *et al*. 1998; Zhandi *et al*. 2020). Antioxidative protection systems are mainly of cytoplasmic origin and spermatozoa lose a large proportion of cytoplasm during the last phase of differentiation which makes them highly sensitive to damages caused by reactive oxygen species (ROS) during the freeze-thawing process (Aitken *et al*. 2016). It has been established that the lower and controlled concentrations of free radicals have regulatory role in specific sperm cell functions especially capacitation and acrosome reaction (Seifi-Jamadi *et al*. 2017; Vafaei *et al*. 2019). Therefore, the equilibrium between

ROS and protective antioxidants have crucial role to prevent spermatozoa from damages which can occur before and during the cryopreservation processes (Hammadeh *et al*. 2009; Mousavi *et al*. 2019). In this scenario, the addition of antioxidants to the semen extender (Seifi-Jamadi *et al*. 2016) or improving sperm intrinsic antioxidant defense system is necessary to decrease lipid peroxidation for protection of sperm cell membrane (Aitken, 2018). Antioxidants can improve semen quality parameters such as motility, viability, acrosome and membrane integrity in bull (Eidan, 2016), goat (Zanganeh *et al*. 2013), horse (Seifi-Jamadi *et al*. 2016) and rooster (Amini *et al*. 2015; Zhandi *et al*. 2019) by reducing detrimental effects of the ROS.

Zn is a trace mineral that plays an important role in cellular functions being a cofactor of DNA and RNA polymerases, which is necessary for normal DNA turnover (Root *et al*. 1979; Prasad, 2009). Moreover, zinc is the cofactor of over 300 different metalo-enzymes involved in protein, lipid and carbohydrate metabolism, DNA transcription and protein synthesis (Cummings and and Kovacic, 2009). Zn is a vital element for reproduction and it has been shown to be essential for sperm formation and testosterone production (Kvist *et al*. 1987; Bray and Bettger, 1990; Colagar *et al*. 2009). Recent studies suggested that inadequate consumption of Zn can negatively affect antioxidative defense barriers and DNA repair mechanism leading to sperm cell oxidative damages (Narasimhaiah *et al*. 2018; Zhandi *et al*. 2019). In addition, it was reported that Zn is important for stabilization of the spermatozoa membrane and chromatin (Prasad, 2009) and is required to maintain mechanical properties and lateral microfilaments, sperm tail morphology and sperm motility (Baccetti *et al*. 1973). Moreover, Bülbül *et al*. (2019) found higher seminal plasma Zn concentration in ram's semen during breeding season. They also suggested that higher concentrations of Zn in ram's semen points out the importance of Zn in spermatogenesis and male fertility.

Zinc oxide is the most common form of Zn used in animals because of its high Zn content and high absorption rates (Dawei *et al*. 2010). Thus, due to its antioxidative properties, addition of Zn to the semen extender can prevent adverse effects of ROS and can effectively reduce DNA damage and lipid peroxidation during semen freezing (Zhandi *et al*. 2019).

Nanoparticles are new form of materials with distinguished biological properties and low toxicity which appear to have high potential passing physiological barriers of the body to reach specific target tissues (Afifi *et al*. 2015). Zinc oxide nanoparticles (ZnONPs) is a Zn derived product with particle diameter between 1-100 nm. These particles have recently gained much attention in animal trials. There are some studies demonstrated the positive effect of nanozinc oxide on reproductive cells and functions in rat (Afifi *et al*. 2015), mouse (Talebi *et al*. 2013) and human (Barkhordari *et al*. 2013). Little known about the effects of Zn nanoparticles on sperm cryopreservation. To the best of our knowledge, this study is the first try to assess the effect of ZnONPs on bull sperm cryopreservation, Hence, this study aimed to evaluate the effect of *in vitro* addition of different concentrations of ZnONPs to semen extender on post-thaw quality of bull spermatozoa.

MATERIALS AND METHODS

Ethical approval

This experiment was approved by the animal care committee and was conducted at the department of animal science, University of Tehran, Iran.

Chemicals

Semen extender was Andromed (Minitube, Germany) and the ZnONPs were obtained from Us-nanomaterial co Inc. (USA). All other chemical reagents including Eosin Y, Nigrosin, Fructose, Sodium citrate, Tris, Acridine orange, NaCl, EDTA, Triton X-100, HCl, Na_2HPO_4, isothiocyanate-conjugated Pisum sativum agglutinin (PSA-FITC), thiobarbituric acid (TBA), trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals and semen collection

Semen samples were collected from four healthy mature Holstein bulls by an artificial vagina. The bulls were housed at a commercial semen freezing center (NDJ Co, Karaj, Iran, 37˚47′ N, 50˚55′ E) and maintained under uniform feeding and management conditions. Ejaculates were collected twice a week for four consecutive weeks. Upon to the collection, ejaculates kept warm in water bath at 37 ˚C until assessment in the laboratory. Ejaculates with 4-6 mL volume, $\geq 1.5 \times 10^9$ sperm/mL sperm concentration (assessed with a normal microtube), ≥ 75% total motility and < 10% sperm abnormalities (subjective assessment), were used for cryopreservation. To eliminate individual differences, semen samples with mentioned criteria, were pooled and then processed for extension.

Semen processing

The pooled ejaculates were divided into four aliquots and diluted (to a final concentration of 20×10^6 spermatozoa/mL) with extenders (Andromed®) containing different concentration of ZnONPs (0, 0.1, 1.0 and 10 µg/mL). The extended semen samples were loaded into 0.5 mL French straws (IMV, France) and sealed with polyvinyl alcohol powder. Then the samples were incubated at 4 ˚C in a normal lab refrigerator for 3-4 h to decrease their temperature.

After equilibration, the straws were frozen at 4 cm above liquid nitrogen, for 9 min. Afterward, the straws were plunged into liquid nitrogen and stored until evaluations. For post-thaw evaluation, the frozen straws were thawed individually at 37 ˚C for 30 s in water bath. Sperm evaluation was performed immediately after thawing (Karimi *et al*. 2017).

Sperm motility

Sperm motility parameters were analyzed using a CASA system (Minitube, Germany). The following variables were recorded: total motility (TM, %), progressive motility (PM, %), percentage of rapid, medium and slow-moving spermatozoa.

Membrane integrity

Membrane integrity was assessed through eosin-nigrosin staining (Evans and Maxwell, 1987; Björndahl *et al*. 2003). The sperm suspension smears were prepared by mixing 10 µL of semen sample with 10 µL eosin-nigrosin stain on a warm slide. The membrane integrity was assessed by counting 200 cells under phase-contrast microscopy (LABOMED, Los Angeles, USA) at 400 magnification. Spermatozoa with white (unstained), light pink head or red appearance restricted to the neck region (Leaky Neck) was considered to be alive, while spermatozoa with stained or partial stained heads were regarded dead (Rahmatzadeh *et al*. 2017).

Membrane functionality

The hypo-osmotic swelling test (HOST) was used to assess the functional integrity of the sperm plasma membrane. This test was performed by incubating 10 µL of semen with 100 µL of a 100 mOsm hypo-osmotic solution (0.9 g fructose and 0.49 g dehydrate sodium citrate dissolved in 100 mL distilled water) at 37 ˚C for 30 min. After incubation, 10 µL of the mixture was placed on a glass slide and covered with a cover slip to evaluate under phase-contrast microscope (×400). A total of 200 spermatozoa were counted in at least 10 different microscopic fields. Then the percentage of spermatozoa with swollen and curled tails was recorded. Swelling is characterized by a coiled tail, indicating that the plasma membrane is intact (Revell and Mrode, 1994; Hedayat-Evrigh *et al.* 2019).

Assessment of DNA damage

Sperm DNA damage was assessed with sperm chromatin structure assay (SCSA). Briefly, frozen–thawed semen was centrifuged (at 500×g for 5 min) and almost all of the supernatant was removed, then the pellets were re-suspended with a buffer (containing 0.15 M NaCl, 1 mM EDTA and 10 mM Tris at pH=7.2). Afterwards, 400 µL of acid/detergent solution (0.1% v/v Triton X-100 in 0.08 M HCl and 0.15 M NaCl) was added to 100 µL re-suspended pellet. After 30 s, 1200 µL of acridine orange solution, containing 6 µg/mL of acridine orange hydrochloride solution (A8097 in 0.15 M NaCl, 1 mM EDTA, 0.2 M Na_2HPO_4 and 0.1 M citric acid at pH=6.0), was added, and the solution was incubated for 30 min at room temperature in dark. Then the DNA status of the spermatozoa was assessed using a flow cytometry. Green fluorescence of double-stranded DNA (intact DNA) and red fluorescence of single-stranded DNA (damaged DNA) were detected with FL (500-530 nm, Becton Dickinson San Jose, CA, USA) (Kadirvel *et al*. 2009; Topraggaleh *et al*. 2014).

Acrosome integrity

Sperm acrosome integrity was assessed with a procedure described by Thys *et al*. (2009). Briefly, 500 mL of thawed sperm samples were centrifuged (600×g, 10 min) and the sperm pellet was dissolved in 100 mL ethanol (96%). After 15 min incubation, the sperm was fixed and smeared on a glass slide to evaporate the remaining ethanol. Then, 30 mL of PSA-FITC (50 µL/mL) was added to the samples and incubated for the next 20 min at room temperature in dark. Subsequently, the prepared slides were dripped for 10 times in distilled water and mounted with glycerol after being dried. At least 200 spermatozoa per slide were assessed by a fluorescence microscope (BX51; Olympus) at 400 magnification. Green head spermatozoa were considered as intact, and the spermatozoa without fluorescence in the head area or with a green fluorescent band at the equatorial level, were considered as damaged or disrupted acrosome, respectively.

Malondialdehyde (MDA) concentration

Malondialdehyde concentration was measured by spectrophotometer (UV-1200, Shimadzu, Japan) using the TBA reaction (Seifi-Jamadi *et al*. 2016). Briefly, 1 mL of diluted sperm was mixed with 1 mL of cold 20% (w/v) TCA to precipitate protein content. The precipitate was pelleted by centrifuging (950×g for 15 min), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (w/v) TBA in a water bath at 100 ˚C for 10 min. After cooling, the absorbance was determined by a spectrophotometer at 532 nm. The result was expressed as nmol/mL.

Statistical analysis

Data were analyzed using general linear models (GLM) procedure of SAS software (SAS, 2003). The dataset was checked for normal distribution by UNIVARIATE procedure and Shapiro–Wilk test and the data without normal distribution were normalized through Arc Sin √x transformation. Results were expressed as LS Means ± standard

error (SE). Tukey test was applied to determine the significant differences. Differences with P-values ≤ 0.05 were considered significant.

$Y_{ijk}= \mu_i + Treat_j + e_{ijk}$

Where:
Y_{ijk}: dependent variable.
μ_i: intercept.
$Treat_j$: fixed effect of the treatments (Different ZnONPs concentration).
e_{ijkl}: residual value.

RESULTS AND DISCUSSION

The effect of ZnONPs on motility parameters of bull spermatozoa are shown in the Table 1. The results indicated that the supplemental ZnONPs did not significantly affect the TM and PM, while the percentage of fast-moving spermatozoa was significantly higher in the treatment supplemented either with 0.1 or 1.0 µg/mL ZnONPs in comparison with the control. The percentage of slow-moving spermatozoa of control and 10 µg/mL ZnONPs was significantly lower than the groups supplemented with 0.1 or 1.0 µg/mL ZnONPs. The effect of ZnONPs on bull sperm membrane integrity and functionality, acrosome integrity, DNA damage and MDA concentration are presented in the Table 2. The membrane integrity and functionality as well as acrosome integrity were statistically higher in the group treated with 1.0 µg/mL ZnONPs compared to the control ($P<0.05$). But treatments with 0.1 or 10 µg/mL ZnONPs didn't showed significant differences with control group ($P>0.05$). Moreover, the production of MDA was significantly lower in the extender containing 1.0 µg/mL ZnONPs than the other groups. The percentage of damaged DNA was significantly lower in the treatments with 0.1 and 1.0 µg/mL ZnONPs than the control ($P<0.05$). There were not significant differences in DNA damage ZnONPs concentration ($P>0.05$). The present study examines the ability of ZnONPs in preventing the cryoinjuries on bull spermatozoa during cryopreservation. The results of this study indicated that the addition of 1.0 µg/mL ZnONPs to the extender, positively affect sperm quality parameters including fast-moving, viability, after thawing. The mammalian seminal plasma has higher concentration of Zn than other tissues (Sørensen *et al*. 1999). While, the seminal plasma has subjected to extending during the cryopreservation, the addition of Zn sources to the extender could be very useful to compensate Zn deficiencies (Hidiroglou *et al*. 1984). The positive role of Zn on the semen quality can be explained by its ability to increasing antioxidant capacity of the spermatozoa to overcome oxidative stress occurs due to ROS overproduction (Afifi *et al*. 2015; Khoobbakht *et al*. 2018; Zhandi *et al*. 2019). In accordance with the results of this study, the addition of Zn sulfate to human semen could cause a higher post-thaw progressive motility, DNA integrity and mitochondrial functionality (Kotdawala *et al*. 2012). Moreover, supplementation of culture medium with 0.6 µmol/mL Zn sulfate, increase human sperm motility after 4 h incubation (Colagar *et al*. 2009).

Moreover, Schäfer and Holzmann, (2000) indicated that the lower dose of Zn sulfate has significant positive effects on the viability of buffalo spermatozoa, while higher dose caused negative effects.

It was reported that some metalloenzymes (i.e. lactate dehydrogenase and sorbitol dehydrogenase) which play an important role in the increasing of sperm motility, may contain Zn in their structure (Dawei *et al*. 2010). In addition, Zn is a part of superoxide dismutase (SOD) structure which is one of the most important antioxidants to neutralize the free radicals (Narasimhaiah *et al*. 2018). This ability makes Zn an important factor to stabilize sperm cell membrane through interaction with sulfhydryl groups and preventing membrane lipids peroxidation (Dawei *et al*. 2010). Furthermore, Zn prevents the accessibility of iron and disrupts the formation of free radicals by replacing iron III with iron II (Zago *et al*. 2001; Talebi *et al*. 2013). Revell and Mrode (1994) demonstrated that there was a remarkable relationship between semen plasma Zn content and the sperm concentration. Moreover, Mohamed and Abdelrahman (2018) showed that ZnONPs supplementation to nicotine exposed rats improved rat semen parameters through decreasing oxidative stress and increasing expression of steroidogenic enzymes. In contrast the addition of ZnONPs to the mouse reproductive cells culture medium negatively affect their viability through increasing ROS level and decreasing glutathione level (Liu *et al*. 2016).

The results of this study showed that the higher dose of ZnONPs might negatively impact bull thawed sperm quality. It has been reported that Zn could increase semen antioxidative capacity and decrease the intensity of oxidative stress (Afifi *et al*. 2015). However, high concentrations of Zn in seminal fluid can decrease oxygen absorption and negatively affect sperm quality parameters (Chohan *et al*. 2004).

Surai *et al*. (1998) indicated that the addition of Zn could significantly decreases sperm lipid peroxidation. While, the higher concentration could disturb sperm cell oxygen consumption by reducing oxygen concentration in the semen fluid. The effect of different levels of nano Zn oxide on rat intestine epithelial cells culture was also investigated and the results showed that SOD and catalase content increased while the MDA concentration decreased in the groups which received nano Zn oxide (Kadirvel *et al*. 2009).

Table 1 The effect of ZnONPs on the motion parameters of frozen-thawed bull spermatozoa

Parameter (%)	Treatment (LS means±SE)[1]				P-value
	ZnONPs (0.1)	ZnONPs (1)	ZnONPs (10)	Control (0)	
Total motility	71.86±1.3	73.44±0.8	70.40±1.6	71.76±1.4	0.46
Progressive motility	65.46±2.1	67.18±2.6	62.36±3.0	63.98±2.2	0.57
Fast moving spermatozoa	47.30±1.6^{a}	46.18±1.2^{a}	45.22±1.7^{ab}	41.26±1.1^{b}	0.04
Moderate moving spermatozoa	18.16±0.6^{bc}	21.00±1.1^{ab}	17.14±1.2^{c}	22.72±1.2^{a}	0.01
Slow moving spermatozoa	6.40±0.2^{b}	6.26±0.2^{b}	8.04±0.4^{a}	7.78±0.4^{a}	0.01

[1] ZnONP (0.1; 1.0 and 10), extender containing 0.1, 1.0 and 10 µg/mL ZnONPs.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SE: standard error.

Table 2 The effect of ZnONPs on bull sperm membrane integrity and functionality, acrosome integrity, DNA damage, and malondialdehyde (MDA) concentration of frozen-thawed bull sperm

Parameter	Treatment (LS means±SE)[1]				P-value
	ZnONPs (0.1)	ZnONPs (1)	ZnONPs (10)	Control (0)	
Membrane integrity (%)	63.0±1.0^{ab}	67.2±1.7^{a}	64.7±1.2^{ab}	61.8±1.3^{b}	0.03
Membrane functionality (%)	74.2±1.1^{ab}	76.4±1.0^{a}	71.8±0.6^{ab}	70.6±0.8^{b}	< 0.01
Acrosome integrity (%)	79.9±1.3^{ab}	81.8±0.7^{a}	79.9±1.4^{ab}	76.8±0.6^{b}	0.04
MDA concentration (nMol/mL)	1.0±0.1^{a}	0.6±0.1^{b}	1.2±0.1^{a}	1.1±0.1^{a}	< 0.01
DNA damage (%)	3.7±0.2^{b}	3.8±0.2^{b}	4.0±0.1^{ab}	4.7±0.1^{a}	0.04

[1] ZnONP (0.1; 1.0 and 10), extender containing 0.1, 1.0 and 10 µg/mL ZnONPs.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SE: standard error.

The results of present study showed that the sperm acrosome status, membrane integrity, membrane functionality and DNA status were enhanced by addition of 1 µg/mL ZnONPs to the extender. In accordance with these results, the membrane protective effect of Zn has been reported in several studies (Gualtieri *et al.* 2014; Afifi *et al.* 2015; Isaac *et al.* 2017).

Therefore, it is assumed that ZnONPs can enhance antioxidant capacity of the semen leading to a decrease in the membrane lipid peroxidation. In agreement, Thys *et al.* (2009) indicated that supplementation of Zn as an antioxidant to the semen extender, caused higher stability of sperm acrosome and plasma membrane after thawing. Moreover, Zn sulfate could increase the percentage of intact acrosomes and DNA integrity in human spermatozoa (Kellokumpu and Rajaniemi, 1981). Similarly, in a recent study we concluded that the addition of Zn sulfate to the extender enhanced rooster semen motility and viability (Zhandi *et al.* 2019), while apoptotic sperm cells were significantly lower in the extenders which supplemented with Zn sulfate. Previously, Omu *et al.* (2008) demonstrated that the oral addition of Zn to men's diet could decrease the apoptotic sperm cells by increasing the anti-apoptotic Bcl-2 and decreasing the Bax protein content. The Bcl-2 plays an important role in the mitochondrial membrane by preventing the cytochrome c which is the main factor in cell apoptosis.

CONCLUSION

In conclusion, the results of current study showed that the addition of 1 µg/mL ZnONP to the bull semen extender had beneficial effects on some sperm quality parameters. The membrane status and acrosome integrity were enhanced, while the DNA damage as well as MDA concentration were decreased by supplemental ZnONP. It can be hypothesized that the enhanced quality parameter is a sign for fertility improvement but further studies may be needed.

ACKNOWLEDGEMENT

The authors acknowledge the financial support provided by the University of Tehran (grant number 7108017/6/23).

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