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Screening of genotoxicity and oxidative stress effect of selenium nanoparticles on ram spermatozoa

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ABSTRACT

The widespread use of different nanoparticles raised great concerns about their biological safety. To examine reproductive toxicity of nanoscale selenium, ram spermatozoa were challenged with selenium nanoparticles (SeNPs) and DNA damage of spermatozoa was evaluated using comet assay. Sperm motility, membrane integrity and acrosome defects were also measured. To determine oxidative stress role of SeNPs spermatotoxicity, malondialdehyde (MDA) level as a biomarker of lipid peroxidation was measured in seminal plasma. Spermatozoa were gradually incubated to 4°C over 90 min in Tris-based extender containing 0 (control), 0.5, 1 and 2 µg/ml SeNPs and cooled at 4°C for 120 min. The sperm DNA damage was not significantly different regardless of SeNPs concentrations ($P < 0.05$). Sperm motility increased with increasing SeNPs concentrations up to 2 µg/ml. In addition, sperm membrane integrity remained unimpaired at all concentrations. MDA decreased with the concentration of 0.5 µg/ml SeNPs along with a non-significant ($P < 0.05$) increase with concentrations 1 and 2 µg/ml SeNPs. Adding 0.5 and 1 µg/ml SeNPs to extender produced significant positive effects on motility, acrosome protection and preservation of sperm membrane integrity compared to SeNPs free group. In conclusion, this study demonstrated that low concentrations of SeNPs had no toxic effect on ram spermatozoa indicating their potential application in sperm cooling processing.

Keywords: Ram spermatozoa, Selenium nanoparticles, Genotoxicity, Lipid peroxidation.

Introduction

Nanotechnology as a growing field for research in animal reproduction offers outstanding opportunities to improve reproductive functions either *in vitro* or *in vivo* (Silva, 2014 and Barkalina *et al.,* 2014). On the other hand, there are concerns about environmental impact of the expanding use of nanotechnologies on reproduction and detrimental effects of nanoparticles on sperm cells (Falchi *et al.,* 2018). Nanoparticles are substances the diameter of which does not exceed 100 nm and this size provides them with different physical and chemical properties from their bulk materials (ISO 2004 and Laurent *et al.,* 2010). Many recent studies reported *in vitro* and *in vivo* effects of different types of nanoparticles, either beneficial or detrimental, on male fertility and sperm cell function in farm animals (Falchi *et al.,* 2018).

In vitro toxicity assessment imposed by nanoparticles could be evaluated by several assays including DNA damage and oxidative stress assays (Kumar *et al.,* 2017). Comet assay or single cell gel electrophoresis assay is a sensitive method for assessment of genetic damage and apoptosis (Fraser and Strzeżek 2005 and Jin *et al.,* 2007). Besides, it is used to detect *in vitro* and *in vivo* DNA breaks in individual cells (Bajpayee *et al.,* 2013). It is also used to quantify oxidative DNA damage (Kumar *et al.,* 2013). The comet assay is based on the principle that fragments of damaged DNA will migrate out of the cell when an electric current is applied, while the undamaged DNA will remain in the cell nucleus (Fairbairn *et al.,* 1995). The extent of DNA damage is correlated with the size and shape of the tail and the distribution of DNA within the comet (Malyapa *et al.,* 1998 and Lemay and Wood, 1999). According to Li and Osborne (2008), excessive generation of free radicals is considered

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the cause of apoptosis and DNA damage. In this context, evidence suggested that DNA damage can be caused by oxidative stress in cell culture systems (Ryter *et al.,* 2007).

Exposure to nanoparticles leads to the production of reactive oxygen species (ROS; Magder, 2006). In addition, nonspecific oxidative stress is a major mechanism of nanoparticle-induced toxicity (Nel *et al.,* 2006). Oxidative stress can be assessed by measuring lipid peroxidation using thiobarbituric acid (TBA) assay for MDA (Fantel, 1996). Selenium is an essential micronutrient which generally acts as a co-factor and is present in some enzymatic structures called selenoproteins *in vivo* (Sarkar *et al.*, 2015; Zeliha *et al.*, 2015). However, there is a very narrow margin between activity and toxicity of selenium (Lanctot *et al.,* 2017).

Among the biological nanoparticles, SeNPs have attracted intense interest. Moreover, applications of nanoparticles based on their antioxidative properties can be particularly valuable for sperm functions and male fertility (Falchi *et al.,* 2018). The available data about the effects of selenium at nanoscale either beneficial or detrimental on ram sperm is very rare. Therefore, the aim of this study was to examine toxicity of different doses of SeNPs (0.5, 1 and 2 µg/ml) on sperm DNA damage, oxidative stress levels and some sperm characteristics (sperm motility, membrane integrity and acrosome defects).

Materials and Methods

Nanoparticles and Chemicals:

Black powder selenium nanoparticles (CAS. No. 7782 – 49 - 2) were purchased from LobaChemie (PVT. LTD., India) with 40 nm average particle size. Tris was obtained from LobaChemie (PVT. Ltd.107, Wodehouse Road, Mumbai 400005, India). Glucose were purchased from ADWIC (El Nasr Pharmaceutical Chemicals Co., Egypt.), citric acid was obtained from Alpha Chemika (Mumbai-400002-India and glycerol from Fisher Scientific (United State).

Semen collection, processing and exposure to nanoparticles

Thirty-Five ejaculates (seven ejaculates for each ram) were collected from five sexually mature Barki rams (2–4 years old) using artificial vagina. The animals were housed in the farm belonging to Animal Reproduction Research Institute, Giza governorate. Semen was processed according to the procedure of Salamon and Maxwell (2000). Pooled semen was diluted with Tris to a final concentration rate 200 × 106 sperm/ml and divided into aliquots that were treated with 0.5 µg/ml, 1 µg/ml and 2 µg/ml SeNPs. Sperm suspensions were cooled gradually to 4 °C over 90 min then diluted cooled samples were kept in refrigerator at 4 °C for 2 h. The control samples were treated with the same procedure but without SeNPs.

Evaluation of sperm characteristics after exposure to SeNPs

Sperm motility was assessed by placing a drop of semen on a pre-warmed slide and covered with a cover slip. Motility percentages were assessed under a phase-contrast microscope at x 200 magnification (Hafez and Hafez, 2013).

Plasma membrane integrity was assessed by means of the hypo-osmotic swelling (HOS) test as described by Revell and Mrode (1994) with some modifications. Briefly, 10 µl of semen sample were mixed with 100 µl of a hypoosmotic solution [9 g fructose, 4.9 g sodium citrate in 1000 ml distilled water (osmolarity: 100 mOsm/kg)] and incubated at 37°C for 30 min. After incubation, smear was prepared and 300 spermatozoa were counted by phase-contrast microscope (× 400 magnification) under the phase-contrast microscope. The percentages of sperm with swollen and curled tails were then recorded. Acrosome integrity was assessed using silver nitrate stain as described by Chinoy *et al.* (1992).

Evaluation of sperm DNA damage after exposure to SeNPs

The potential genotoxicity of SeNPs was assessed using comet assay, following the method described by Codrington *et al.* (2004) at neutral conditions. Slides were coated with 300 µl 1% normal melting gel (NMG) then dried overnight. After that, 20000 sperm cells in10 µl phosphate buffer saline were mixed with 90µl 1% low melting gel (LMG), layered on the first dried agarose layer, covered with coverslip and left to solidify at 4˚C for 30 min. Slides were immersed in a vertical coupling jar

filled with lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl [pH10], 1X triton, 40 mM Dithiotheritol (DTT) for 1 hour at room temperature then 10 µg/ml proteinase were added and the slides were incubated overnight at 37°C. After lysis, slides were washed with distilled water, then were loaded with unwinding alkaline solution (1ml EDTA, 0.05 M NaOH, pH 12.1) for 15 min. Slides were washed with 1 x Tris – base borate EDTA (TBE) pH 7.4 twice for 5 min each and placed onto horizontal electrophoresis tank. Following the electrophoresis, the slides were drained and flooded with absolute ethanol for 10 min. and each gel was stained with 50 µl of 20 µg/ml ethedium bromide. Slides were then visualized using 40X objective of Leica epifluorescent microscope (Green filter: N2.1 with Exitation filter: BP 515-560, Dichromatic Mirror: 580, Suppression filter: LP 590) and the images for the sperm nuclei were digitalized with true chrome retina screen camera version 4.2 build 5001 (copyright Tucsen photonics co. ltd). As a minimum 100 sperm nuclei were randomly selected for each group and measured for DNA% in tail, tail moment and Olive moment using COMETSCORE software.

Evaluation of lipid peroxidation after exposure to SeNPs

Lipid peroxidation in seminal plasma was estimated by determination of malondialdehyde (MDA) according to the method of Ohkawa et al. (1979) with minor modifications. Concisely, five volumes from the stock solution containing equal volumes of chromogen and 2-thiobarbituric acid (25 mmol/L) was mixed with one volume of seminal plasma in test tubes covered with glass bead, vortexed and heated for 30 min in a boiling water bath. After cooling, absorbance of the resultant pink product was measured at 534 nm against blank (containing all the reagents except test sample).

Statistical analysis:

The obtained data were analyzed by one-way analysis of variance (ANOVA) using SPSS software (version 18 for windows, SPSS Inc., Chicago, IL, USA). Comparison of means was carried out by Duncan's Multiple Range Test. Data were presented as the mean ± standard error of mean (SEM).

Results

Sperm characteristics after exposure of ram spermatozoa to different concentrations of SeNPs:

Figure (1) shows that sperm motility percentage was reduced significantly *($P < 0.05$)* by increasing SeNPs levels 0.5, 1 and 2 µg/ml (85.00 %, 80.71 % and 77.86 %, respectively). However, sperm motility % at concentrations 0.5 µg/ml and 1 µg/ml SeNPs were significantly *($P < 0.05$)* higher than control group. It is also clear from figure (1) that SeNPs at concentrations 1 and 2 µg/ml had no significant *($P < 0.05$)* effect on HOS response in comparison with control while, 0.5 µg/ml SeNPs significantly *($P < 0.05$)* improved HOS response. In addition, acrosome defect % decreased significantly *($P < 0.05$)* with concentrations 0.5 and 1 µg/ml SeNPs (Figure 1).

Sperm DNA damage after exposure of ram spermatozoa to different concentrations of SeNPs:

Figure (2A) shows typical nuclei of undamaged cells of control group while figure (2B) represents SeNPs treated spermatozoa that show normal nuclei of undamaged sperm cells. The results in figure (3) show a non-significant decrease in tail DNA% in 0.5 µg/ml and 1 µg/ml SeNPs treated groups vs. the control one (3.45 ±1.28, 3.47 ±0.62 and 4.6 ± 0.96, respectively). Tail moment of 0.5 µg/ml SeNPs treated group (0.53 ± 0.31) was non-significantly *($P < 0.05$)* higher than control group as well as 1 µg/ml and 2 µg/ml SeNPs treated groups (0.38 ± 0.25, 0.34 ± 0.13 and 0.40 ± 0.24, respectively). Also, olive moment did not differ significantly *($P < 0.05$)* in all SeNPs treated groups vs. the control one.

Lipid peroxidation levels in seminal plasma of ram spermatozoa after exposure to different concentrations of SeNPs:

As shown in figure (4), MDA concentrations in seminal plasma of 0.5 µg/ml SeNPs group (11.85±0.21) were significantly ($P < 0.05$) lower than control group (15.19±2.33). Moreover, there were non-significant ($P < 0.05$) increases in MDA levels in both nano selenium 1and 2 µg/ml groups (16.07 ±1.71 and 18.02 ±1.82, respectively) in comparison with the control group.

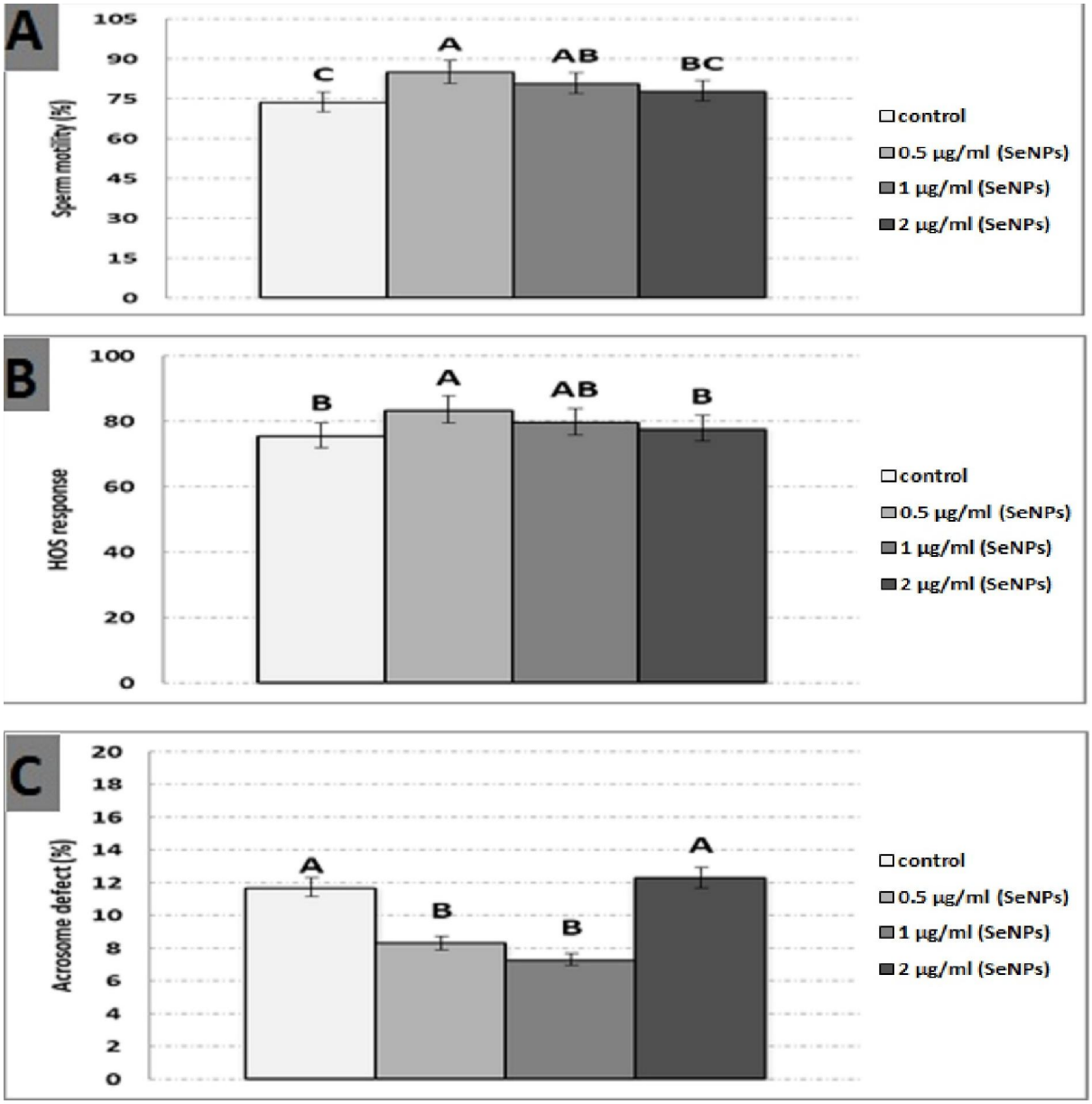

Fig. 1: Sperm characteristics after exposure of ram spermatozoa to different concentrations of SeNPs; (A) sperm motility (%); (B) HOST response; (C) Acrosome defect (%). Different letters indicates significant effect at *(P<0.05)*.

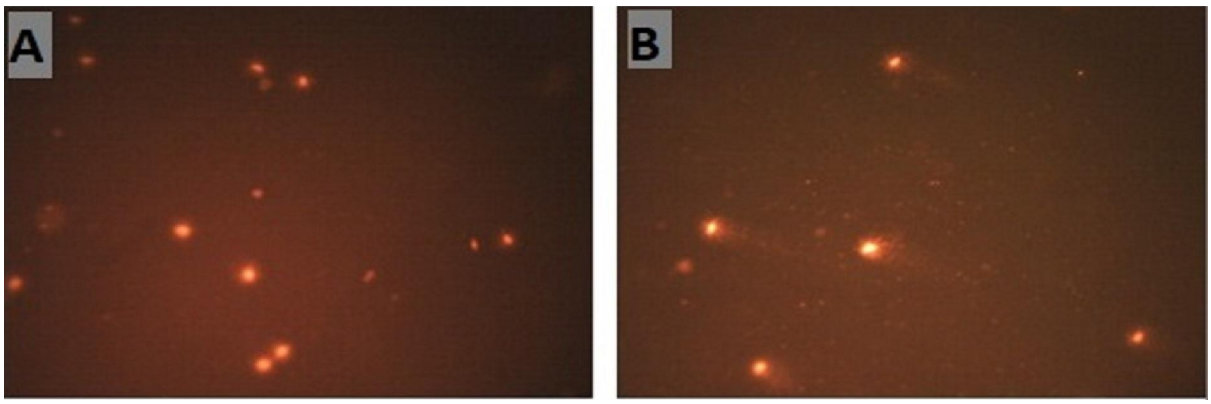

Fig. 2: Comet assay micrographs; (A) typical nuclei of undamaged sperm cells of control group; (B) normal nuclei of undamaged sperm cells after exposure of ram sperms to SeNPs.

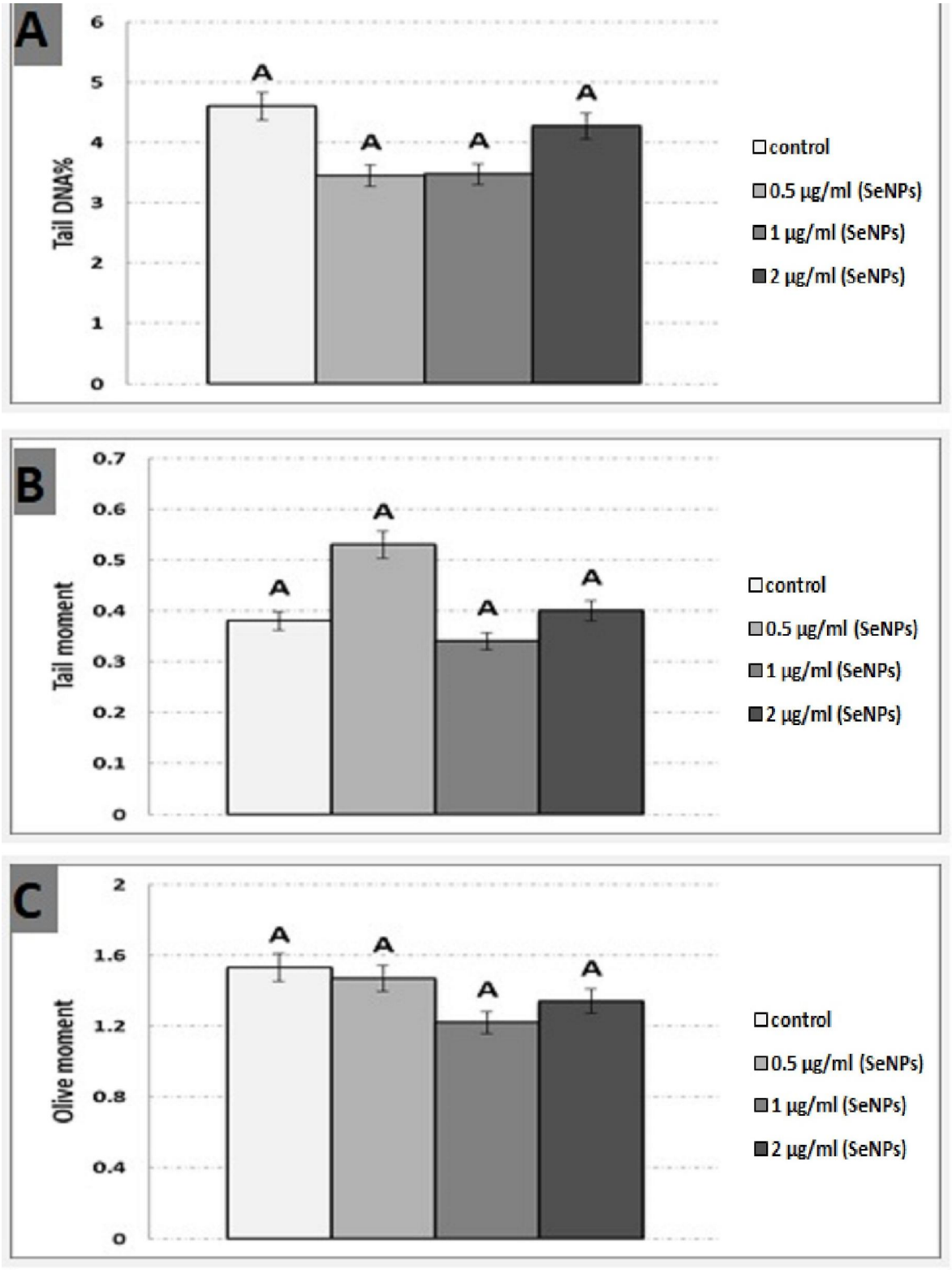

Fig. 3: Sperm DNA damage after exposure of ram spermatozoa to different concentrations of SeNPs; (A) Tail DNA (%); (B) Tail moment; (C) Olive moment. Different letters indicates significant effect at *(P<0.05)*.

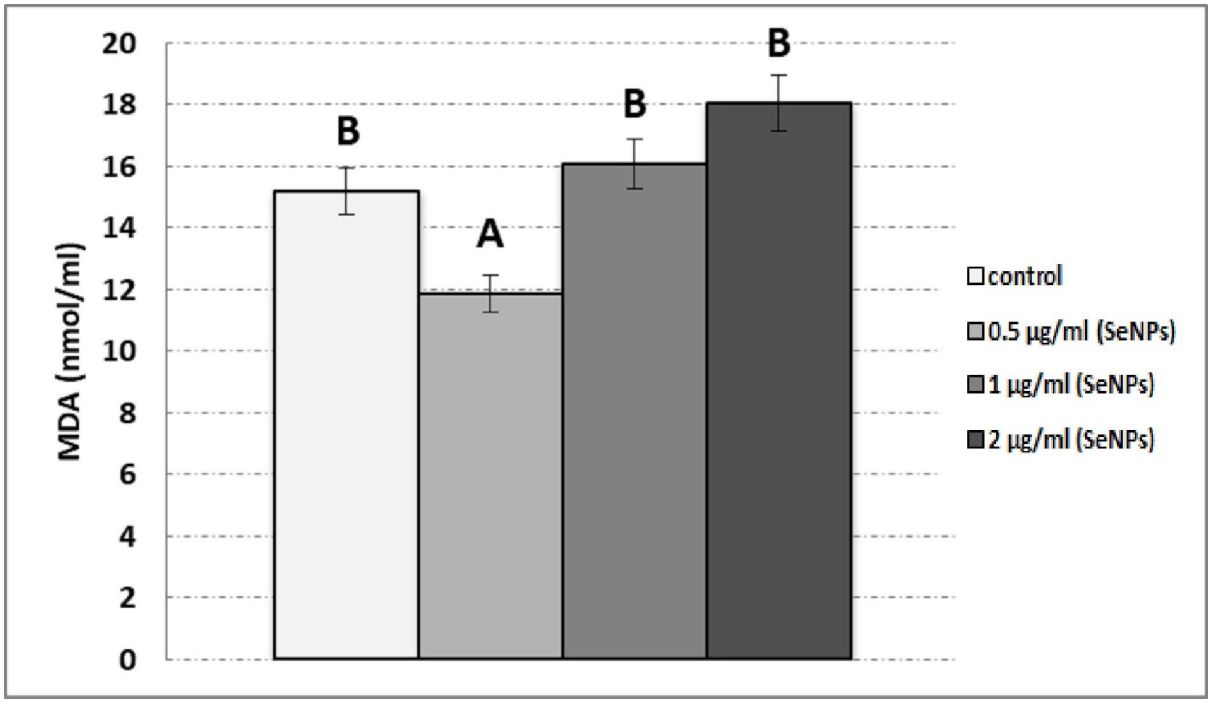

Fig. 4: Lipid peroxidation in seminal plasma of ram spermatozoa after exposure to different concentrations of SeNPs. Different letters indicates significant effect at *(P<0.05)*.

Discussion

The present study was designed to evaluate the toxicity of SeNPs at concentrations of 0.5, 1 and 2 µg/ml on ram spermatozoa. Our results showed that although sperm motility % and HOS response decreased with increasing levels of SeNPs, sperm cells seem to have passed undamaged, with no adverse effects on motility as well as plasma and acrosome membrane integrity. Furthermore, SeNPs at concentrations of 0.5 and 1 µg/mL improved motility and membrane integrity in comparison with the control. Motility of sperm is an indicator of membrane integrity and active metabolism (Johnson *et al.,* 2000). Moreover, it is crucial for sperms to have intact acrosome and plasma membranes to fertilize an oocyte (Yanagimachi, 1994). SeNPs administration have improved semen quality in goat bucks (Shi *et al.,* 2010), rats (Ghazanfarpoor *et al.,* 2014) and *in vitro* in roosters (Safa *et al.,* 2016). Scanning germ cells for damage of DNA is essential to study toxic effect of materials on reproductive system (Baumgartner *et al.,* 2009). A sensitive, quick and consistent tool to evaluate genome integrity inside single cells and to detect damage of DNA is that of the comet assay (Baumgartner et al., 2009). In the present research, the percentage of DNA in tail of spermatozoa as well as tail and olive moments detected by comet did not differ significantly (P<0.05) in all SeNPs groups vs. the control one. Intact DNA look like the head in comet assay whereas, damaged DNA resembles the tail (Fairbairn *et al.,* 1995). According to Lee *et al.* (2004), tail moment delivers the most stable evaluations for DNA damage and to be considered the favored comet parameter to describe the observed DNA damage because it has a large degree of uniformity in quantile dispersions. Oral supplementation of SeNPs protected motility of spermatozoa and integrity of DNA against oxidative damage induced by the anticancer agent Cisplatin which has male reproductive toxicant properties (Rezvanfar *et al*., 2013). However, it was reported that some materials at the nano scale like silica nanoparticles affected the maturation process of spermatozoa in the epididymis (Xu *et al*., 2014). ROS generated by endogenous and exogenous sources cause significant damage to macromolecules including DNA (Salmon *et al*., 2004). Oxidative stress elicited at the membrane level could be related to MDA level which is frequently used as biomarker for membrane lipid peroxidation of polyunsaturated fatty acids as it can readily react with TBA to give colored fluorescent red adduct (Esterbauer and Cheeseman, 1990). In this study, levels of MDA in seminal plasma as indicator of lipid peroxidation were directly proportional with increasing concentration of SeNPs. Though, increases in MDA levels in both nano selenium 1 µg/ml and 2 µg/ml groups were non-significant in

comparison with control group. Moreover, supplementation of semen extender with 0.5 µg/ml SeNPs had beneficial effect and significantly lowered MDA concentrations. Spermatozoa are susceptible to injury by oxidative stress because of the high levels of plasma membrane unsaturated fatty acids (Aitken *et al*. 1989). Additionally, lack of effective system for scavenging free radicals due to negligible quantity of cytoplasm makes the mature spermatozoa extremely prone for oxidative damage. The significantly lower level of MDA observed in the 0.5 µg/ml SeNPs group in the present study suggests that SeNPs at low concentration efficiently scavenge free radicals. The free radical scavenging function of SeNPs was reported in earlier studies by Gao *et al*. (2002) and Kojouri and Sharifi (2013). Our findings agree with a recent study by Khalil *et al*. (2018) who have demonstrated that selenium nanoparticles have membrane protective function in bulls. These results attest the hypothesis that one of the most beneficial effects of antioxidants is the reduction of membrane lipid peroxidation. Proper dose of antioxidant should be selected to keep the natural balance that exists between ROS generation and scavenging activities (Badr *et al*., 2009). Our results explained that lipid peroxidation began to increase with increasing levels of nano selenium in ram semen extender. These results are in accordance with Aitken *et al*. (1998) who indicated that high levels of antioxidants are connected to reduce sperm function and this could be attributed to increased susceptibility of spermatozoa to H_2O_2 cytotoxic effect or O_2 removal which is an important mediator of function of normal sperms. The noteworthy positive effects of nano selenium exerted in this study with regard to acrosome protection may be attributed to the role of selenium in decreasing lipid peroxidation. Also, the non-toxic effect of SeNPs on spermatozoa was supported by the motility data in the present study.

Conclusion

Based on DNA damage and lipid peroxidation, it could be concluded that adding SeNPs to semen extender at concentrations (0.5, 1 and 2 µg/ml) did not induce any toxicity on ram spermatozoa. In addition, exposure of spermatozoa to these concentrations of SeNPs enhanced sperm motility and improved membrane integrity which shows that antioxidant properties of SeNPs have beneficial effects on ram spermatozoa and further confirms that SeNPs may not exert any toxic effect on spermatozoa at low concentrations. Further studies are essential to confirm that SeNPs are safe in freezing protocols and to test their effects on fertilization and early embryonic developmental process.

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