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Research Article

In vitro Supplementation of Nano Selenium Minimizes Freeze-Thaw Induced Damage to Ram Spermatozoa

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ABSTRACT

Semen cryopreservation induces detrimental effects on the spermatozoa leading to reduction of fertilizing ability in humans and animals. Nano-protectant additives in extenders included in the freezing protocols could improve overall quality of spermatozoa and enhance *in vivo* fertility rates. Selenium nanoparticles (SeNPs) were prepared via sonochemical route and characterized using high resolution transmission electron microscope (HRTEM) and X-ray difractometer (XRD). Thirty-two ejaculates were collected using artificial vagina from four healthy mature Barki rams. Semen was diluted with Tris-based extender containing 0 (control), 0.5, 1 and 2 µg/ml SeNPs and frozen in liquid nitrogen. After thawing, semen parameters as well as oxidative stress were evaluated in semen of each treatment. Extenders containing 0.5 and 1 µg/ml SeNPs improved motility, viability index, membrane integrity and acrosome defects. Moreover, comet assay demonstrated that SeNPs supplemented extenders reduced DNA deterioration. Addition of SeNPs to extender reduced MDA concentrations in ram seminal plasma. It could be concluded that supplementation of SeNPs during cryopreservation enabled ram spermatozoa to bear up freeze-thaw processes competently better than control.

Key words: Semen cryopreservation, Selenium nanoparticles, Lipid peroxidation, DNA integrity and Ram

INTRODUCTION

Preservation of semen of sheep (*Ovis aries*) is one of the essential steps in the artificial insemination process in this species (Allai *et al*., 2018). During freezing process, spermatozoa are exposed to different types of stress which adversely affects their functional competence (Vichas *et al*., 2017). The extreme prone of sperms to low temperatures was related to the high content of polyunsaturated fatty acids (PUFA) in ram sperm plasmatic membrane (Buhr *et al*., 1994) which makes cells sensitive to cold shock and lipid peroxidation in the presence of reactive oxygen species (ROS; Samadian *et al*., 2010). As a result, increases in production of ROS during freezing and thawing change the antioxidant defence status of sperms and seminal plasma and consequently affecting quality of semen as well as fertilizing ability of sperms (Partyka *et al*., 2012). A suitable extender and cryoprotectant is crucial to improve sperm quality during preservation (Allai *et al*., 2018). For this reason, antioxidants supplementation during the preservation process could decrease harmful effects of oxidative stress induced by ROS on ram sperm (Forouzanfar *et al*., 2010). Nanotechnology as an emerging field of biotechnology has potential biomedical applications being confirmed in recent years (Isaak *et al*., 2017). Special properties of materials at nano-scale like greater cellular uptake, surface area, reactivity, binding properties and surface charge as well as antioxidant properties have recently contributed to optimize freezing protocols (Khalil *et al*., 2018). These properties allow the use of a lesser quantity of materials in comparison to conventional materials and new or more effective physical and chemical reactions (Nel *et al*., 2006). Therefore, use of nanotechnology could be efficient to attain bioactive properties of different elements including nano-selenium in reproduction, freezing cells, growth, digestion, and antimicrobial applications (Pelyhe and Mezes, 2013). Selenium nanoparticles (SeNPs) showed lower toxicity than selenite (Zhang *et al*., 2005). However, the available

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data about the role of nano selenium supplementation to preserve ram sperm is very rare. Hence, the current study aimed to explore the effects of modification of cryo-extender by adding three different concentrations of selenium nanoparticles (0.5, 1 and 2 μg/ml) to protect ram spermatozoa from freeze-thaw induced damage.

MATERIALS AND METHODS

Animal management and semen collection

Four sexually and clinically healthy mature Barki rams (2–4 years old) were used to conduct the current study. Thirty-two ejaculates (eight ejaculates for each ram) were collected using artificial vagina twice a week during over a period of three months. Animals were housed in clean semi- open shed stalls belonging to Animal Reproduction Research Institute, Agriculture Research Center, Giza, Egypt.

Synthesis and characterization of selenium nanoparticles

SeNPs were fabricated according to the method of Farghali *et al*. (2018). The morphology and microstructure of synthesized SeNPs were examined by high resolution transmission electron microscope (HRTEM; JEOL-JEM2100, Japan) and XRD patterns were recorded on a PANalytical (Empyrean) X-ray diffraction.

Experimental design

Pooled semen samples were divided into four aliquots and each was diluted with Tris-based extender containing 0 (control), 0.5, 1 and 2 μg/ml SeNPs. Then, samples were plunged into liquid nitrogen and stored until thawing.

Extender preparation

The Tris-based medium was prepared according to the method of Salamon and Maxwell (2000). Briefly, 3.63 g Tris (LobaChemie PVT. Ltd.107,Wodehouse Road, Mumbai 400005, India) was mixed with 0.50 g glucose (ADWIC-El Nasr Pharmaceutical Chemicals Co., Egypt.), 1.99 g citric acid (Alpha Chemika Mumbai-400002-India.), 20 ml egg yolk, 5% glycerol (Fisher Scientific ,United State), 100 ml glass-distilled water to 100 ml and antibiotics (Gentamycin, Tylosine, Lincospecten).

Semen processing

Immediately after collection, ejaculates were transported to the laboratory, and examined for its volume, concentration, sperm motility and morphology. Only ejaculates of good quality (volume: ≥1 ml; motility; ≥70%; sperm concentration $\geq 3 \times 10^9$ sperm/ml) were pooled together. After pooling, semen was divided into four aliquots and each one was diluted with Tris to a final concentration rate, 200×10^6 sperm/ml supplemented with different levels of SeNPs (0, 0.5, 1 and 2 μg/ml). After dilution, all aliquots were gradually cooled to 4°C over 90 min. The concentration of SeNPs was selected based on preliminary study conducted, where semen samples were cryopreserved with different concentration of SeNPs (0.5, 1, 2, 4, 8 and 10 μg/ml). Semen was centrifuged after that at 3000 rpm for 15 min. and seminal plasma was separated and saved for estimating lipid peroxidation. Diluted cooled samples were loaded into 0.25 ml straws (IMV, Laigle, F-61300, France) and thermally sealed then allowed to equilibrate at 4°C for 90 min, before being exposed to liquid nitrogen vapor (4–5 cm above the liquid nitrogen surface) for 15 min. then were put into liquid nitrogen and stored until thawing after one month. For thawing, straws were placed in a water bath at 37°C for 30 seconds and semen parameters (motility, viability index, membrane integrity, acrosome defects) as well as DNA integrity were assessed.

Evaluation of frozen sperm quality

Sperm motility assessment: Motility percentages were assessed under a phase-contrast microscope at 200 x magnification (Hafez and Hafez, 2013).

Viability index calculation: According to a method described by Milovanov (1962), eight straws from each treatment were thawed and incubated in a narrow glass tube at 37ºC. Motility estimations were done at hourly intervals for a period of 3 hours. The viability index was calculated to be equal to half of the post-thaw motility in addition to the summation of recorded motility at 1st, 2nd and 3rd hours post-thawing.

Plasma membrane integrity assessment: Plasma membrane integrity was assessed by means of the hypo-osmotic swelling test as described by Revell and Marode (1994).

Acrosomal membrane integrity assessment: Acrosome integrity was assessed using silver nitrate stain as described by Chinoyc *et al*. (1992).

Assessment of sperm DNA damage using comet assay: Sperm DNA damage was investigated using COMET assay, following the method described by Codrington *et al*., (2004) at neutral conditions.

Determination of lipid peroxidation in ram seminal plasmaLipid peroxidation in seminal plasma was estimated by determination of malondialdehyde (MDA) according to the method of Ohkawa *et al*. (1979).

Statistical analysis

The obtained data was analyzed using SPSS software (version 18 for windows, SPSS Inc., Chicago, IL, USA). Data were analyzed statistically by one-way analysis of variance. Treatment means were compared by the least significance difference (LSD) at 5% level of probability and comparison of means was carried out by Duncan's Multiple Range Test. Semen post thawed motility was analyzed as repeated measures of 1, 2 and 3 h after thawing.Data are presented as the mean ± standard error of mean (SEM).

RESULTS

Characterization of SeNPs

Fig. 1 presents patterns of the X-ray diffraction of sonochemically synthesized SeNPs. The diffraction peaks are at 2θ (degrees) of 29.64°, 23.49° and 43.69° corresponds to (101), (100) and (102) planes of selenium. All the diffraction peaks in the 2θ range are in good agreement with the standard JCPDS data (JCPDS card No. 00-042-1425). The microscopic analysis using HR-TEM results presented in Fig. 2 showed that selenium nano-particles were spherical in shape with size about 32 nm.

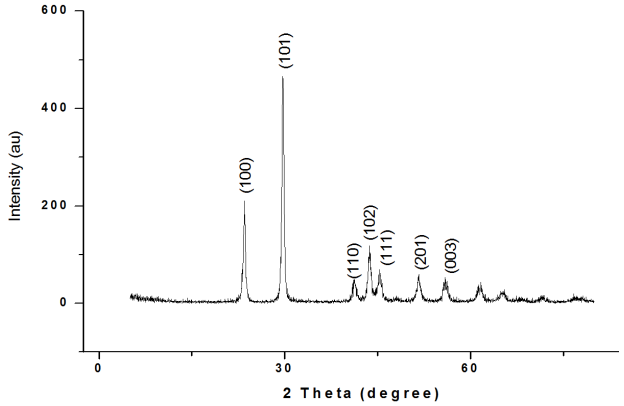

Fig. 1: X-ray diffraction of sonochemically synthesized SeNPs.

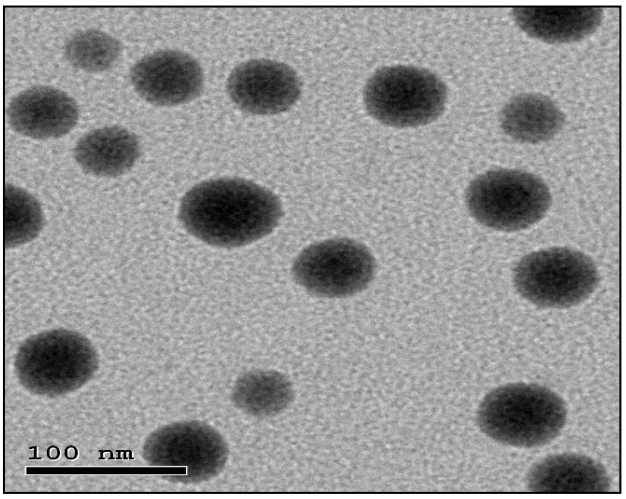

Fig 2: HR-TEM micrograph of sonochemically synthesized SeNPs.

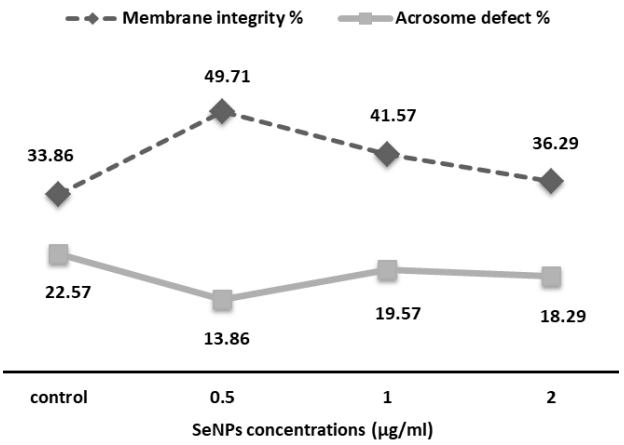

Fig. 3: Effect of Nano-selenium different concentration on plasma membrane integrity % and acrosome defect % of frozen-thawed ram spermatozoa.

Effect of Nano-selenium different concentrations on sperm motility % and viability index of frozen-thawed ram spermatozoa

Means for motility % of sperms subjected to extender with different concentrations of SeNPs are presented in Table 1. Post thaw motility % was improved significantly ($P<0.05$) with selenium nanoparticles conc. 0.5 µg/ml and conc. 1 µg/ml (52.14±2.64 and 47.86±2.40, respectively) in comparison with control and selenium nanoparticles conc. 2 µg/ml (35.71±2.02 and 41.43±1.43, respectively). Also, nano-Se 0.5 µg/ml in extender significantly ($P<0.05$) increased viability index (146.07±6.50) when compared to the control group (77.86±8.34).

Effect of Nano-selenium different concentrations on plasma membrane integrity and acrosome defect% of frozen-thawed ram spermatozoa

Data presented in Fig. 3 shows that supplementation of semen extender with SeNPs (0.5 and 1, µg/ml) attained improvement of post thawing plasma membrane integrity (49.71±2.56 and 41.57±2.60, respectively) when compared to the control and semen extender with SeNPs 2 µg/ml (33.86±1.56 and 36.29±1.84, respectively). Likewise, nano selenium 0.5 µg/ml exhibited a protective effect and significantly ($P<0.05$) decreased acrosome defect % (13.86±0.74) followed by both conc. 1 and 2 µg/ml SeNPs (19.57±0.65 and 18.29±0.61, respectively) in comparison with control group (22.57±0.65).

Effect of Nano-selenium different concentrations on sperm DNA integrity of frozen-thawed ram spermatozoa

It is evident from values shown in Table 2 thatnano selenium 0.5 µg/ml offered the maximum protection to sperm DNA and significantly ($P<0.05$) increased head DNA% and decreased the tail DNA%, tail moment & olive moment (96.38±0.49, 3.62±0.49, 0.22±0.09, 1.41±0.24, respectively) when compared to SeNPs extender free (85.58±0.99, 14.42±0.99, 2.56±0.41 and 6.39±0.63, respectively). As shown in Fig. 4, spermatozoa with non-fragmented DNA do not have a comet (without tail) and spermatozoa with fragmented DNA exhibit the characteristic formation of comet (with tail).

Effect of Nano-selenium different concentrations on lipid peroxidation levels of frozen-thawed ram spermatozoa

It is evident from data presented in Fig. 5 that seminal plasma of post thaw ram semen of nano Se 0.5 µg/ml group significantly ($P<0.05$) decreased concentrations of MDA (10.25±1.25) as compared to control group (12.20±1.99) as well as nano Se 1 µg/ml and nano Se 2 µg/ml groups (13.17±1.06 and 12.32±1.41), respectively.

Table 1: Effect of Nano-selenium different concentrations on sperm motility % and viability index of frozen-thawed ram spermatozoa:

Sperm Parameters	SeNPs (µg/ml)			
	Control	0.5	1.0	2.0
Post-thaw motility %	35.71±2.02^{b}	52.14±2.64^{a}	47.86±2.40^{a}	41.43±1.43^{b}
Viability index	77.86±8.34^{c}	146.07±6.50^{a}	123.21±8.76^{b}	102.14±4.80^{b}

Data indicates Mean ±SEM; Means with different superscripts (a, b, c) within each row are significantly different ($P<0.05$).

Table 2: Effect of Nano-selenium different concentrations on sperm DNA integrity of frozen-thawed ram spermatozoa.

DNA Integrity Parameters	SeNPs (µg/ml)			
	Control	0.5	1.0	2.0
Head DNA%	85.58±0.99[d]	96.38±0.49[a]	92.23±0.63[b]	89.39±1.08[c]
Tail DNA%	14.42 ± 0.99[a]	3.62±0.49[d]	7.77±0.6[c]	10.61±1.08[b]
Tail moment	2.56±0.41[a]	0.22±0.09[b]	1.07±0.37[b]	1.08±0.35[b]
Olive moment	6.39 ±0.63[a]	1.41±0.24[c]	3.73±0.82[b]	4.13±0.65[b]

Data indicates Mean ±SEM; Means with different superscripts (a, b, c) within each row are significantly different (P<0.05).

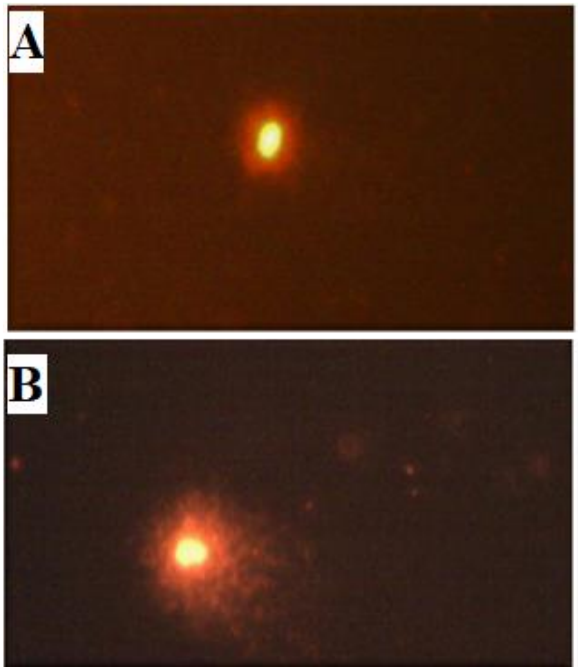

Fig. 4: DNA fragmentation analyzed by the comet assay A) Ram sperm showing intact DNA (no fragmentation) B) Ram sperm showing DNA fragmentation.

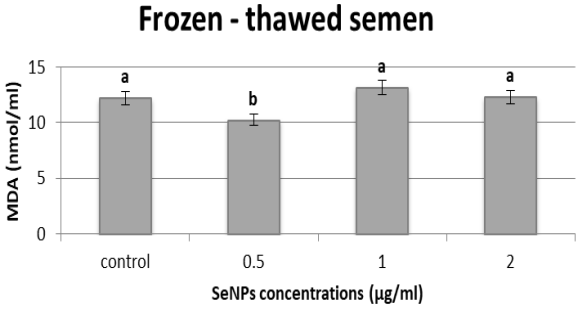

Fig. 5: Effect of Nano-selenium different concentrations on lipid peroxidation levels of frozen-thawed ram spermatozoa.

DISCUSSION

Applications of nanoparticles based on their antioxidative properties can be particularly valuable for sperm functions and male fertility (Khalil *et al*., 2018). HRTEM data reveals that the average size of SeNPs used in the present work was about 32 nm at which size; SeNPs in extender of frozen ram semen (0.5, 1 and 2 µg/ml) enhanced motility of spermatozoa and increased the viability index. Furthermore, negative changes in motility and percentage of viable spermatozoa were significantly higher in extender free selenium than SeNPs supplemented extenders. These results are in agreement to that recently observed in bulls by Khalil *et al*. (2019) who reported that supplementation of SeNPs up to 1.0 mg/ml to Tris-yolk fructose extender significantly improved motility and viable sperm percentage and decreased apoptotic sperm percentage in frozen/thawed bull semen. The detrimental effects associated with cryopreservation may be as a result of ROS production causing a series of events that reduce phosphorylation of axonemal protein and result in sperm immobilization (De Lamirande and Gagnon, 1995). SeNPs positive effects in respect to motility could be attributed to the role of selenium in reducing lipid peroxidation. Additionally, enhancements in sperm motility may be attributed to enhanced antioxidant enzymes including glutathione peroxidase activity suggesting that supplementation of SeNPs could improve the ability of seminal plasma to diminish oxidative stress (Khalil *et al*., 2019). In addition, Kantola *et al*. (1988) reported positive correlation between sperm quality and selenium concentration in seminal plasma. Cryopreservation exposes spermatozoa to severe osmotic stress (Sieme *et al*., 2015) and induces damage of membranes and acrosomes affecting the fertilization capacity of spermatozoa (Vichas *et al*., 2017). The obtained results of the present study revealed that modification of extender of frozen ram semen with SeNPs improved plasma membrane integrity and decreased acrosome defects in ram semen. Effect of nanoscale selenium on the reduction of oxidative stress is documented in literature (Kojouri and Sharifi, 2013) and Gao *et al*. (2002) confirmed the antioxidant properties of hollow spherical SeNPs. This might be attributed to much smaller size of SeNPs which allow more surface area to react with free radicals and offers plenty of space to absorb oxygen (Safa *et al*., 2016). It has been reported that acrosomal integrity of ram sperm greatly affected by the cryopreservation process specially freezing thawing steps (Alcay *et al*., 2016). Our results are in agreement with Rezaeian *et al*. (2016) in their earlier study who observed that 5 mg/ml selenium in its bulk form (sodium selenite) offered protection to human sperm parameters and improved sperm vitality after freezing/thawing process. Also, Dorostkar *et al*. (2012) reported that supplementation of sodium selenite 2 mg/ml to extender could improve semen parameters and decreased abnormality in frozen buffalo semen.

Efforts to improve the outcome of semen cryopreservation concentrates on protecting DNA from freeze-thaw-induced stress (Isaac *et al*., 2017). In the present study, different concentrations 0.5, 1 and 2 µg/ml of SeNPs showed positive effects on DNA integrity of cryopreserved ram semen. Whereas nano selenium free extender (control) was associated with increased DNA deterioration in frozen-thawed sperm, extender containing 0.5 µg/ml SeNPs provided the best results in terms of DNA integrity. Damage occurring during the freeze-thaw

process has been attributed to cold shock, ice crystal formation, oxidative stress, osmotic changes and lipid–protein reorganizations within the cell membranes (Barkalina *et al*., 2015). Although loss of motility and poor mitochondrial function as well as ultrastructural changes in the membrane (Ozkavukcu *et al*., 2008) are common consequences of freeze-thaw process, damage of DNA is considered to be most serious problem due to its close association with abnormal post-fertilization developmental events (Simões *et al*., 2013). The importance of selenium is pronounced from the fact that supplementation of selenium provides better storage along the lessrelease of lipids from the sperm cell during long time storage (Dimitrove *et al*., 2007). The positive effect of SeNPs on the sperm cryopreservation in our study may be attributed to its ability to protect sperm against oxidative damage and reduction of oxidative stress-induced DNA oxidation and DNA fragmentation. Nano-selenium has been used in several studies as a scavenger of ROS to protect against oxidative damage in sperm cells. Supplementation of semen extender of rooster semen with SeNPs successfully enhanced the post-thawing quality as well as oxidative biomarkers (Safa *et al*., 2016). Also, oral supplementation of SeNPs protected the quality of spermatozoa (motility, DNA integrity) and spermatogenesis against oxidative damage induced by Cisplatin, an anticancer agent with male reproductive toxicant properties (Rezvanfar *et al*., 2013).

Small ruminants' sperm is sensitive to ROS damage due to the relatively high content of unsaturated fatty acids in the phospholipids of the sperm membrane (Ashrafi *et al*., 2011). Measurement of MDA is widely used as lipid peroxidation indicator in different cell types, including spermatozoa (Sikka, 1996). The present results showed that supplementation of the extender with SeNPs 0.5 μg/ml resulted in reduction of MDA level in frozen-thawed spermatozoa when compared to control group. On the other hand, adding SeNPs in levels 1 and 2 μg/ml increased levels of lipid peroxidation in frozen ram semen. These results prove that one of the most advantageous effects of supplementing antioxidants during cryopreservation is reduction of the membrane lipid peroxidation, throughout reduction of lipid peroxidation. Biological membranes are characterized by the asymmetrical arrangement of lipids within the bilayer. However, the composition of lipid in sperm cell plasma membrane differs markedly from that of somatic cells. The higher content of PUFA in sperm cells makes sperm membranes more prone to oxidative damage. Consequently, it was hypothesized that sperm membrane is a primary target of freezing shock damage (Alvarez *et al*., 1995). The fact of high concentrations of PUFA within the lipid structure of cell membranes requires efficient antioxidant systems to defend against oxidative damage and associated sperm dysfunction (Aitken *et al*., 1994). The decline in levels of lipid peroxidation SeNPs supplemented extenders in the present study was associated with higher DNA integrity indicating that deterioration occurs as a result of the harmful effect of cryopreservation on mammalian cells may be attributed to the oxidative stress which is detrimental to sperm DNA integrity (Fraser and Strzeźek, 2005).

Conclusions

Supplementation of selenium nanoparticles as nano-protectant additives in ram semen extender during freezing process potentially protected spermatozoa from lipid peroxidation and maintained motility, sperm membrane integrity. It is important to note that ram semen quality was the best in extender containing 0.5 followed by 1 μg/ml SeNPs. Moreover, nano selenium preserved DNA integrity of ram spermatozoa after freezing in concentration dependent manner.

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Authors contributions

All authors participated and did research work as study protocol.

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