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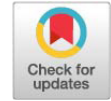
Detrimental Effects of Selenium Nanoparticles on Growth and Development of Mice Embryos: An *In-Vitro* Study



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Key words
Nanotechnology, Selenium nanoparticles, Mice embryos, *In vitro* fertilization, Embryo development

ABSTRACT

Nanotechnology has numerous applications in medicine, diagnostics, and science. Over the last ten years, nanotechnology has significantly influenced reproductive techniques such as *in vitro* maturation, fertilization, and follicular oocyte culture. Selenium nanoparticles have substantial applications in numerous fields including disease prevention and treatment, mammary glands' development, reproduction, and immune systems. In present study, *in vitro* trial was performed to examine the effect of selenium nanoparticles (SeNPs) on mice embryo growth and development. For this purposes, sixty (n= 60) female mice were divided randomly into three equal groups (n= 20) including control and two treatment groups. The treatment groups received SeNPs at doses of 5 and 25 µg/ml, respectively. During the experiment period, mice embryo growth and development, cleavage rate, and blastocyst rate were analyzed. After the collection of oocytes from female mice, insemination and *in vitro* fertilization were done to culture embryo through *in vitro* study. It was found that during the embryonic stages, the treated group showed no significant (P>0.05) differences compared to control group. Moreover, the blastocyst rate exhibited no notable differences. Whereas, the cleavage rate was significantly (P<0.05) decreased compare with control group. Altogether, the current study showed that exposing mice embryos to SeNPs had detrimental effects on the cleavage rate in *in vitro* trial.

INTRODUCTION

Nanotechnology has numerous applications in medicine, diagnostics, and science (Ray *et al*., 2009; El-Dawy *et al*., 2023; Elbehary *et al*., 2023). Over the last ten years, nanotechnology has significantly influenced reproductive techniques such as *in vitro* maturation, fertilization, and follicular oocyte culture (Albanese *et al*., 2012; Arguelles, 2022). The field of nanotechnology is concerned with particles with a dimension of less than 100 nanometers. Nanomaterials are also referred to as zero-dimensional nanomaterials. They are classified according to their properties, shapes, and sizes (Joseph *et al*., 2023; Elbehary *et al*., 2023), and are made up of a variety of chemical substances including nanocrystals of semiconductor, dendrimers made from organic materials, and fullerenes of carbon (Jeng and Swanson, 2006). Scientists are developing nanomaterials for biomedical applications such as cell labeling, drug delivery, gene therapy, biosensors, and hyperthermia therapy (Jeng and Swanson, 2006).

Magnetic nanoparticle can be used to detect metastatic lesions in lymph nodes because they can exit the systemic circulation through the permeable vascular epithelium (Jeng and Swanson, 2006). Many new doors in disease pathophysiology and treatment options have been opened with nanotechnology development of over the last three decades. Despite their profound use, nanoparticles could harm the living things in three ways: they directly interact with biological membranes, disintegrate and release harmful ions, and they increase oxidative stress (Aktas *et al*., 2023). Humans are generally exposed to nanoparticles by inhalation, ingestion, skin contact and injection (Ajdary *et al*., 2018). The size of nanoscale constructs is similar to that of biological macromolecules such as enzymes, receptors, and hemoglobin, but it is smaller than human cells and organelles. Blood vessel walls can be penetrated by nanoparticles smaller than 20 nanometers.

A variety of nanostructures of metals have been used

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as nanoparticles (Khurana *et al.*, 2019), but selenium nanoparticles (SeNPs) are attracting much attention (Bhattacharjee *et al.*, 2014). SeNPs can be produced by physical, chemical and biological techniques (Tan *et al.*, 2018). Most of selenium compounds play a biological role by reducing oxidative stress in living cells (Jeng and Swanson, 2006). The antioxidant activity and redox regulation of selenium are carried out through selenoproteins (Chandramohan *et al.*, 2018). In the body, biologically synthesized selenoproteins can exert reductive and oxidative effects on immune, reproductive, cardiovascular, and endocrine systems. Human bodies contain the greatest concentrations of selenium in the thyroid (Tan *et al.*, 2018).

Selenium is a cancer-preventive chemotherapeutic agent. It also lowers the risk of heart disease and other cardiovascular conditions, slows the aging process, and prevents virus growth (He *et al.*, 2014; Zafar *et al.*, 2021; Nossier *et al.*, 2022). Selenium, as an antioxidant, protects cells and tissues from damage by influencing the activity of seleno-enzymes such as glutathione peroxidase (Skalickova *et al.*, 2017). Current research on the possible negative effects of SeNPs is largely debatable. According to some studies, SeNPs have strong antioxidant properties that inhibit oxidative stress and DNA damage (Bhattacharjee *et al.*, 2014; Karami *et al.*, 2018). SeNPs have also been shown to act as a chemo-preventive agent, reducing toxicity (Ray *et al.*, 2009). Several studies, on the other hand, have revealed the genotoxic and cytotoxic effects and abnormal effects on embryonic development following SeNPs exposure (Ray *et al.*, 2009). In female mice, maternal dietary exposure to SeNPs can result in offspring malformations (Hou and Zhu, 2017). Another study found that after exposing zebrafish oocytes to SeNPs only 50% of the embryos were viable (Shi *et al.*, 2018).

In contrast, several studies have shown that SeNPs have beneficial effects on embryos. For instance, a recent study found that in buffalo SeNPs improve oocytes' nuclear maturation, regulate antioxidant defense genes, and reduced apoptosis (Hassan *et al.*, 2023). In another study, it reduced the oxidative stress and DNA damage caused by Cyclophosphamide (CP) in mice's peripheral blood and bone marrow cells (Bhattacharjee *et al.*, 2014). Furthermore, pretreatment with SeNPs was reported to alleviate the negative effects of gamma radiation that induces nephropathy in mice (Karami *et al.*, 2018). The aim of this study was to determine the effects of SeNPs exposure on the *in vitro* mice embryos development, cleavage rate, and blastocyst rate.

MATERIALS AND METHODS

Chemicals and reagents

Most of the chemicals were purchased from Merck (St. Louis, MO, USA). A dark powder SeNP, with a size of < 80 nm and 99.9% purity (CAS No. 7782-49-2) was obtained from Nanoshel company (West Valley City, Utah, USA).

Solutions and media preparation

Human tubular fluid (HTF) medium used for gametes collection, and fertilization was prepared freshly through two steps; the stock solution and the working solution. The stock solution was prepared by dissolving 594 mg NaCl, 35 mg KCl, 5 mg $MgSO_4 \cdot 7H_2O$, 210 mg $NaHCO_3$, 5 mg KH_2PO_4, and 60 mg $CaCl_2.2H_2O$ in 100 mL of Milli-Q water. The working medium was prepared a day before the experiment by adding 370 µL D-glucose anhydrous, 68.4 µL Na-Lactate, 68 µL Na-pyruvate, 20 µl penicillin, 20 µL streptomycin, and 80 mg bovine serum albumin to 20 mL of the stock solution. The final step was to optimize the pH for the media at 7-7.5 then the medium was filtered by 0.22 µm Syringe filter into a 15 ml sterile centrifuge tube and the media was stored for two weeks at 4 °C and used only in this period of time (Arroyo-Salvo *et al.*, 2019). KSOM medium used for embryos culture was freshly prepared. The stock solution was prepared by dissolving 560 mg NaCl, 19 mg KCl, 5 mg KH_2PO_4, 5 mg $MgSO_4.7H_2O$, 210 mg $NaHCO_3$, and 25 mg $CaCl_2.2H_2O$) in 100 mL of Milli-Q water. The working medium was prepared by adding 20 µl Na-pyruvate, 10 µL L-glutamine, 10 µl EDTA, 100 µl Ess. AA50X, 50 µL NonEss.AA100X, 8.4 µl penicillin, 10 µL streptomycin, 10 mg bovine serum albumin, 17.4 µl Na-lactate, and 13.3 µL D-glucose anhydrous to 10 ml of the stock solution (ALRashd *et al.*, 2023).

Characterization and preparation of selenium nanoparticles

To prepare the aqueous solution of nanocrystalline selenium, the nano-powder was dissolved in Milli Q water to get a stock concentration of 1 mg/ml. To avoid nanoparticle agglomeration, the suspension was sonicated using an ultrasonic for 15 min at 40 W before exposure to the gamete cells. After the sonication process, a suitable volume of the stock aqueous nanocrystalline selenium was added to the sperm, *in vitro* fertilization (IVF), and *in vitro* culture (IVC) dishes (El-Naby *et al.*, 2020). Nanoparticle was described and characterized by using various microscopic and spectroscopic methods (KSU, Saudi Arabia). The particle size, morphology and structure were determined by using X-Ray diffraction (Bruker, Karlsruhe, Germany) and Scanning Electron Microscopy (JEOL, Tokyo, Japan)

in conjunction with Energy Dispersive X-ray spectroscopy (EDX) according to previous study (Mourdikoudis *et al*., 2018; Samy *et al*., 2022; Liaqat *et al*., 2023).

Experimental design

In this experimental study, 60 female Balb/c mice weighing approximately 25-30 g and 4–8 weeks old were used. All mice were housed under standard animal conditions (24 ± 2°C; 12-h light/dark cycles) with free access to food pellets and water. The mice were distributed randomly into three groups (n = 20/each) including Group I (control without SeNPs), Group II (received SeNPs @ 5 µg/ml), and Group III received SeNPs @ 25 µg/ml SeNPs) respectively (Chandramohan *et al*., 2018; Takeo and Nakagata, 2018).

Oocyte recovery

Sixty adult female Balb/c mice (4-8 weeks old) were used to obtain a sufficient number of oocytes for IVF. Superovulation was induced by injecting female mice intraperitoneally with 5 IU of equine chorionic gonadotropin (eCG), followed by 5 IU of human chorionic gonadotropin (hCG) 48 h later in order to obtain mature oocytes from the oviducts. Next, 15-17 h after the hCG injection, the mice were euthanized by exposing to 80% CO_2/ 20% O^2 for 120 s in a sealed chamber, and the oviductal ampullae of each mouse were collected and placed in a petri dish containing drops of HTF medium covered by paraffin oil previously adjusted in an incubator with 5% CO_2 at 37°C. Then, the ampulla was opened and the cumulu oocytes complexes (COCs) were dragged into the HTF medium drops, which contained an appropriate volume of SeNPs stock in the two treated groups (Takeo and Nakagata, 2018).

Collection of spermatozoa

Fertile male mice (3–6 months old) were used as sperm donors in general. The mice were euthanized by exposing to 80% CO_2, 20% O^2 for 120 s in a sealed chamber. In order to obtain sperm, each animal's caudal epididymis was severed and transferred to the sperm collection dish, which contained drops of HTF covered by paraffin oil, and previously adjusted in an incubator with 5% CO_2 at 37°C. Sperm cell concentration was adjusted to the appropriate volume and final concentration of ten million sperm /ml. Then a suitable volume of the SeNPs stock solution was added to the IVF medium to adjust the desired concentrations. After that, it was incubated for 60 min at 37°C under 5% CO_2 to induce sperm capacitation.

Insemination and in vitro *fertilization*

The fertilization dish was incubated at 37°C, 5% CO_2 for overnight to allow fertilization medium equilibration. Before insemination, the sperm cell concentration was determined by hemacytometer and sperms were further incubated for 60 min at 37°C under 5% CO_2 to induce sperm capacitation. Finally, the appropriate number of sperms was transferred to the COCs drop on the fertilization dish to a final sperm cell concentration one million sperm/ml. After insemination, the fertilization dish was incubated at 37°C, 5% CO_2 in air for 6 h (Takeo and Nakagata, 2018). Finally, the fertilization process was evaluated under an inverted microscope after 4-6 h by observing the second polar body and two pronuclei.

Embryo culture

After insemination, all zygotes were transferred to the washing dish containing HTF supplied with 300 µg/ml of hyaluronidase enzyme to remove cumulus cells. After that, the zygotes were washed twice by HTF to remove excess enzyme. All the oocytes were transferred from the washing dish into the IVC dish containing KSOM media and then placed in 5% CO_2 incubator at 37°C.

Examination of the growth and development of mice embryos

The growth of the embryos was monitored during the 5th day of culturing and the cleavage rate and blastocyst rate calculated. Then, the embryos were collected and stored in 5 µl of 0.1% PVA-PBS at −80 °C.

Statistical analysis

Statistical analysis of data from at least four replicates for each treatment was carried out by using SPSS. Data normality was first evaluated using the Shapiro-Wilk test. The means of cleavage rates, different stages of *in vitro* development, and blastocyst rates in all groups were compared by ANOVA. All the values are presented as Mean ± SEM, while $P \leq 0.05$ is for significance.

RESULTS

Analysis of selenium nanoparticles using XRD, SEM, and EDX

XRD, EDX, and SEM were used to investigate the size, structure, and purity of selenium nanoparticles. The SeNPs XRD analysis was carried out in the 2 range of the spectrum, and the diffraction peaks correspond to the standard JCPDS data (JCPDS card No. 01-086-2246) (Fig. 1A). The average calculated crystalline size of SeNPs was found to be 58 nm approximately (Fig. 1B). The presence of Se with ultra-high purity was confirmed through EDX analysis (Fig. 2A). SEM image indicated the presence of only one phase, implying the purity of the Se nanoparticles (Fig. 2B).

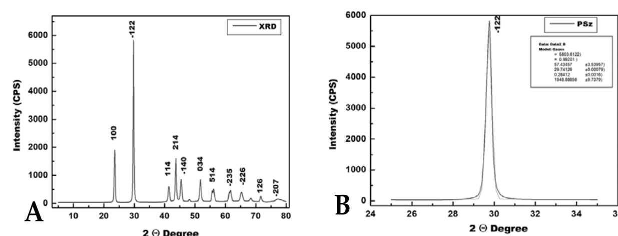

Fig. 1. Characterization of synthesized SeNPs. (A) The XRD spectra of pure SeNPs. (B) Particle size calculations of pure SeNPs.

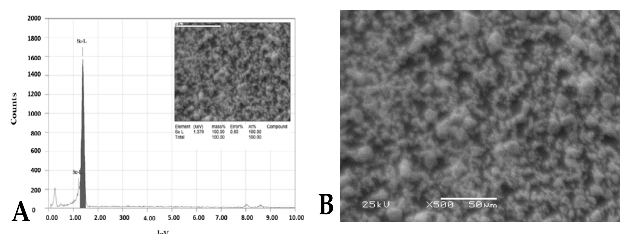

Fig. 2. Purity of synthesized SeNPs. (A) EDX profile for pure SeNPs. (B) SEM analysis of the SeNPs.

Table I. Effect of SeNPs on the number, percentages, and mean of embryos in different development stages related to different treated groups.

Groups	Control (0µg/ml)	Treatment 1 (5µg/ml)	Treatment 2 (25µg/ml)
No. of cultured oocytes	272	226	199
Degenerated oocytes	26(9.56%) (6±2.27) [a]	20(8.85%) (8.5±2.4) [a]	20(10.05%) (12±2.45) [a]
1-Cell stage	19(6.99%) (0.5±0.5) [a]	30(13.27%) (0.5±0.29) [a]	12(6.03%) (0.25±0.25) [a]
2-Cell stage	31(11.40%) (2.5±0.87) [a]	24(10.62%) (3.25±1.97) [a]	24(12.06%) (2.75±0.85) [a]
4-Cell stage	10(3.68%) (7.75±2.32) [a]	13(5.75%) (6±2.04) [a]	11(5.53%) (6±1.16) [a]
8-Cell stage	2(0.74%) (4.57±1.44) [a]	2(0.88%) (7.5±3.069) [a]	1(0.5%) (3±0.913) [a]
Morula stage	24(8.82%) (6.5±2.7) [a]	34(15.04%) (5±1.47) [a]	48(24.12%) (5±1.47) [a]
Blastocyst stage	131(48.16%) (68±16.72) [a]	87(38.50%) (56.5±16.3) [a]	73(36.68%) (49.8±3.92) [a]
Fragmented embryos	29(10.66%) (7.25±2.32) [a]	16(7.08%) (4±1.472) [a]	10(5.03%) (2.5±1.041) [a]

Data between parenthesis indicated percentage (%) and the mean number of embryos replicates ± SEM. Superscripts that were similar indicated that the difference was not statistically significant. Different superscripts mean significant differences as indicated by ($P \leq 0.05$).

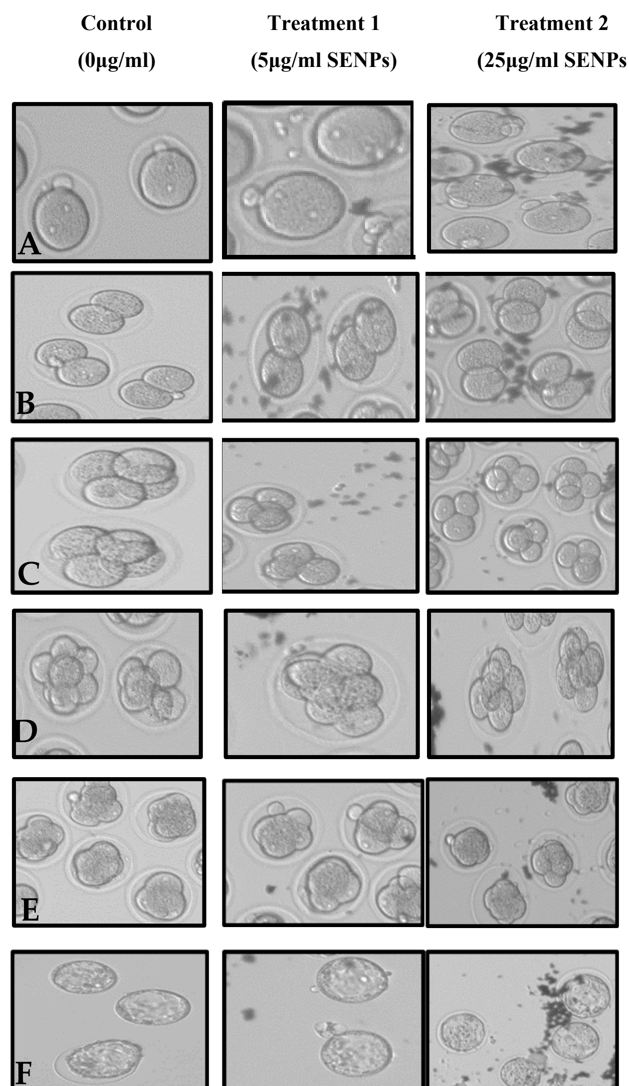

Fig. 3. Different stages of embryonic development of mice embryos produced *in vitro*. (A) Fertilized oocyte; (B) two-cell stage; (C) four-cell stage; (D) eight-cell stage; (E) Morula; (f) Blastocyst.

In vitro *fertilization and culture*

To determine the toxicity of SeNPs, mice embryos were exposed to 58 nm SeNPs at two different concentrations (5 and 25 µg/ml). Data regarding the effect of selenium nanoparticles on the embryonic development of mice embryos was presented in Tables I, II and Figure 3. The experiment recorded neither significant differences in blastocyst rate nor means of embryonic development stages of mouse oocytes across groups. As shown in Table I and Figure 4A-D, the results indicated that the embryonic stages include both the early stages and the late stages exhibited no significant differences

Table II. Effect of SeNPs on the number, percentages, and mean of embryos in cleavage rate and blastocyst rate related to different treated groups.

Groups	Control (0µg/ml)	Treatment 1 (5µg/ml)	Treatment 2 (25µg/ml)
No. of cultured oocytes	272	226	199
No. of cleaved oocytes	198	160	157
No. of blastocysts	131	87	73
Cleavage rate (%)	198/272 (72.79%) (0.655±0.041) [a]	160/226 (70.8%) (0.525±0.024) [b]	157/199 (78.89%) (0.45±0.044) [b]
Blastocyst rate (%)	131/198 (66.16%) (0.72±0.067) [a]	87/160 (54.38%) (0.72±0.037) [a]	73/157 (46.50%) (0.78±0.036) [a]

Data between parenthesis indicated percentage (%) and the mean number of embryos replicates ±SEM. Superscripts that were similar indicated that the difference was not statistically significant. Different superscripts mean significant differences as indicated by ($P \leq 0.05$).

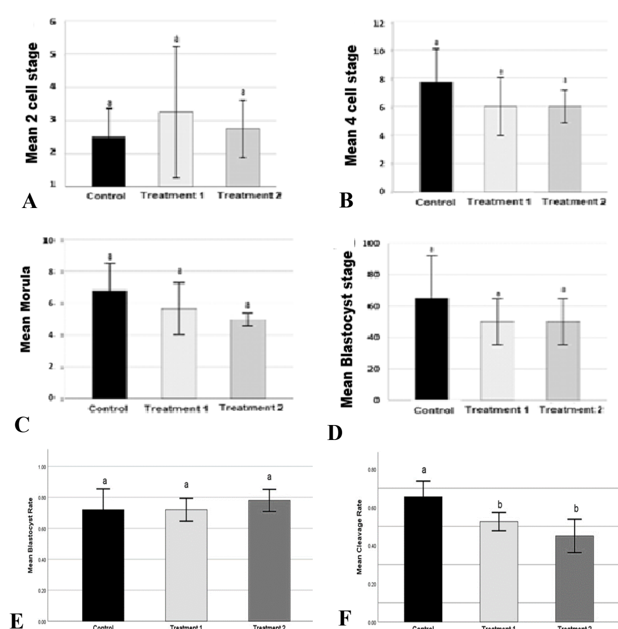

Fig. 4. Effect of selenium nanoparticle on the number of embryos in two-cell stage (A) four-cell stage (B) morula stage (C) blastocyst stage (D) blastocyst rate (E) and cleavage rate (F) development in various experimental groups. Similar letters indicated that the difference was not statistically significant. Different letters mean significant differences ($P \leq 0.05$).

between treated groups with concentrations of 5 µg /ml and 25 µg /ml compared with control group. As shown in Table II and Figure 4E, the blastocyst rate did not differ significantly in 5 µg /ml (0.72 ± 0.037) and 25 µg /ml (0.78 ± 0.036) concentrations in comparison to the control group (0.72 ± 0.067). Our results also showed that there was a significant difference ($P<0.05$) in cleavage rate between treatment groups and the control group. In SeNPs treated groups, the cleavage rates were lower than in the control group. Table II, and Figure 4F revealed that the cleavage rate was significantly ($P<0.05$) reduced in the groups that were exposed to 5 µg /ml (0.525 ± 0.024) and the 25 µg/ml group (0.45 ± 0.044) compared with the control group (0.655 ± 0.041).

DISCUSSION

In the past few years, the need for *in vitro* fertilization has been considerably increased. Despite their beneficial applications, there are some factors that limit the success of *in vitro* fertilization. The use of selenium as nanoparticles instead of selenium is due to its physical and chemical properties, which is distinguished from material properties in its large size. As a result, it has played a significant role in a variety of fields that benefit humans. Therefore, there is a noticeable increase in the use of this technique in the field of research. It has been shown that inflammation, necrosis, ROS, and apoptosis play key roles in NPs' toxicity mechanisms. The dose of NPs through intravenously injection are more toxic than those administered to the skin (Bhattacharjee *et al*., 2014; Ullah *et al*., 2022, 2023). SeNPs provide improved biological availability with an additional feature of low toxicity (Khurana *et al*., 2019). This study was motivated by the need for comprehensive scientific data on the role of selenium nanoparticles on the growth and development of mice embryos *in vitro.*

The SeNPs were thoroughly characterized to validate their purity and structure using different analytical techniques, including XRD, EDX, and SEM. The characterization results indicated the SeNPs' ultra-high purity. In addition, the size of SeNPs was found to be 58 nm, which is consistent with the previous study on SeNPs with sizes around 20–80 nm (Alagesan and Venugopal, 2019). For the first time, the current study evaluated

the putative effect of adding the SeNPs in the culture media of mouse embryos *in vitro*. The experiment did not record any significant changes in blastocyst rate and means of embryonic development stages of mice oocytes in different groups. Our findings also demonstrated that a significant difference was observed in cleavage rate between treatment groups compared with the control group, where the cleavage rate was lower in the SeNPs treated groups than the control group, which indicated that the 58 nm selenium nanoparticles have a cytotoxic effect in the culture media of mice embryos. Our data is in line with previous studies that clearly indicated that when fish were exposed to SeNPs (5–25 mg/mL) at size 100–200nm and sodium selenite (5–25 mg/mL) before 48 h post-fertilization, SeNPs seemed more toxic than sodium selenite (Bano *et al*., 2022). The most common malformations observed were pericardial edema and tail malformations when embryos were incubated with SeNPs at 5–25 mg/mL. It was found that only half of the zebrafish embryos were viable after treatment with 25 mg/ml of SeNPs (Kalishwaralal *et al*., 2016). The results of the present work are also consistent with previous findings (He *et al*., 2014), who showed that several abnormalities were observed in zebrafish embryos after treatment with Hollow selenium nanoparticles (hSeNPs) of various concentrations ranging from 10-50 µg/ml, including abnormal heartbeat, oedema of the embryo sac, oedema of the eye, and swelling of the head. The results agree with previous studies on SeNP antioxidant activity, high concentrations of hSeNPs showed high antioxidant activity (Torres *et al*., 2012). In previous study (Shi *et al*., 2018), a comparison was made between elemental selenium nanoparticles (SeNPs), selenite, and selenomethionine (Se-Met) and the results showed that maternal dietary exposure to SeNP was associated with significant malformations in offspring along with other common Se species. These findings further support the idea of that NPs can enter the fetus via passive diffusion and cause detrimental effect in fetus (Hou and Zhu, 2017). Furthermore, treatment of embryos with 50 M nanoAg *in vitro* led to increased resorption and decreased fetal weight (Grumezescu, 2018). Other nanoparticles were studied on mice like ZnO-NP which caused cytotoxic on mouse ovarian germ cells based on concentration and time. Increasing ROS levels were observed when cells were exposed to ZnO-NPs, and premeiotic germ cell markers were significantly increased but meiotic and post-meiotic markers were decreased compared to untreated cells (Farroh *et al*., 2020).

In other study CeO_2 nanoparticles affected the testis tissue, sperm parameters in mice, (Hosseinalipour *et al*., 2021). On the other hand, our results differ from some published studies (Ray *et al*., 2009), they studied the effects of SeNPs on Swiss albino mice *in vitro*, and the results showed that the activity of antioxidant enzymes increased, causing less bone marrow cell death and prevented DNA damage, it might act as a potential chemo-preventive. A study has shown that the SeNPs at supra-nutritional levels were not toxic to rats and could potentially be used as cancer chemo-preventive agents (He *et al*., 2014), although doses greater than 2.0 mg Se/kg b.wt. caused chronic toxicity. Previous study also evaluated the effect of SeNPs and bulk selenium on buffalo oocytes maturation *in vitro* (El-Naby *et al*., 2020). This finding has been shown that Se and SeNPs (40 nm) had a positive effect on oocytes nuclear maturation rates via regulation the expression of development competence and antioxidant defense gene. It is proved that the SeNPs have been marketed as anti-apoptotic effecting human lymphocytes that exposed to UVB radiation *in vitro* experiments, selenium nanoparticles in this case may be helpful in reducing reactive oxygen species (Prasad *et al*., 2013). Kalishwaralal *et al*. (2016) identified optimal concentrations of SeNP for potential therapeutic applications. Researchers found that SeNPs at 5–10 µg/ml may provide an economical method of treating cardiovascular diseases.

CONCLUSION

In the current study, we studied the putative impact of selenium nanoparticles on the growth and development of mice embryos *in vitro*. Based on the results of the present study we can say that mice embryos that supplemented by 5 µg/ml or 25 µg/ml SeNPs in size of 58 nm at concentration exhibited a reduced cleavage rate and consequently embryonic development. It led to minor toxic effects and abnormal embryo development. Eventually, SeNPs have a very narrow margin between beneficial and drawbacks effects. Moreover, studies based on both *in vitro* and *in vitro* models are needed to identify the mechanisms of nanoparticle toxicity.

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IRB statement and ethical approval

This research work belongs to master student which was approved by supervisory committee. Furthermore, all animal experiments took place following the ethical approval number KSU.SE-21-37 dated in 27-05-2021 from the King Saud University (KSU), Riyadh, Saudi Arabia.

Statement of conflict of interest

The authors have declared no conflict of interest.

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