Study of the Effects of Silver Nanoparticles Exposure on the Ovary of Rats

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Abstract: Purpose: The aim of the work is to investigate the effects of silver nanoparticles exposure on the ovary of adult rats. **Material and methods:** 36 adult Wister albino rats were used in this experiment. They were divided into six groups of (2 control and 4 experimental), 6 rats each. The first and second group were the control groups where physiological saline was given for 2 and 4 weeks, respectively. The third and fourth groups received low (30 mg/kg) and high dose (300 mg/kg) of silver nanoparticles orally once per day for two weeks, respectively. The fifth and sixth groups received low (30 mg/kg) and high dose (300 mg/kg) of silver nanoparticles orally once per day for four weeks, respectively. The samples were obtained after sacrificing of the rats and were prepared to be examined by light microscopy (after staining with Haematoxylin and Eosin stain, Masson's Trichrome stain and Immunohistochemistry for Caspase 3)**. Results:** All experimental groups showed congestion and haemorrhage that increase as dose and duration increase. Mononuclear cell infiltrations were observed indicating the presence of inflammation. Also, there were excess of collagen deposition in all groups. Immunohistochemical studies showed that there were increased immunorespose to Caspase 3 with the increase of the dose and duration which revealed the increase of cell apoptosis in the specimens. **Conclusion:** Silver nanoparticles showed evidences of congestion, bleeding, fibrosis and apoptosis in cells of the ovary in all examined doses and durations.
[M. Amr El-Nouri; Osama Mahmoud Azmy; Awatif Omar Ibraheim Elshal; Ayah Mohamed Hassan Ragab and El-Sayed Abdel-Majied Elsherbini. **Study of the Effects of Silver Nanoparticles Exposure on the Ovary of Rats.** *Life Sci J* 2013;10(2):1887-1894]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 265

Keywords: Silver nanoparticles, ovary, apoptosis.

1. Introduction

Nanoparticles are clusters of atoms in the size range of 1–100 nm where the prefix 'nano' indicates one billionth or 10^9 units **(Kholoud *et al.*, 2010).** At the nanoscale, metallic nanoparticles exhibit different properties than they would at larger bulk size. These different properties are attributed to their small size and larger surface area to volume ratio**(Lanone & Boczkowski, 2006; Yu *et al.*, 2007; Jennings &Strouse, 2007).**The nanoparticles with size smaller than 50nm are capable of entering the cells and they can even move out of the blood vessels if their size is less than 20 nm **(Yih & Wei, 2005).** These properties allow their incorporation in many applications ranging from catalysts and sensing to optics, antibacterial activity and data storage **(Hutter and Fendler, 2004; Sudrik *et al.*, 2006; Choi *et al.*, 2007; Yoosaf *et al.*, 2007).**

The widespread inclusion of silver nanoparticles within a number of diverse products (including clothes and wound dressings) arises primarily from its antibacterial behaviour **(Johnston *et al.*, 2010).** This antibacterial behaviour has resulted also in the widespread use of silver nanoparticles in washing machines, water purification, toothpaste, shampoo and rinse, nipples and nursing bottles, fabrics, deodorants, filters, kitchen utensils, toys, and humidifiers where the main body or inner surface of the product is mixed or coated with germ-resistant nano-silver to prevent the growth of fungi and bacteria **(Maynard, 2006; KISTI, 2006).**

Moreover, silver nanoparticles are considered as a potential additive to animal feed to replace antibiotics **(Ahmadi, 2009; Fondevila *et al.*, 2009).** Therefore, oral intake of silver nanoparticles is a relevant route of exposure for the consumer. However animal studies dealing with oral exposure to silver are nevertheless scarce **(Loeschner *et al.*, 2011).**

Also, the majority of the scientific reports that investigate the cellular impact of nanomaterials were *in vitro*, while fewer studies were conducted to investigate the impact *in vivo* **(Fischer & Chan, 2007)**. Recently there has been a concern of the wide use of nanoparticles and its potential hazards. But regulatory control over the use or disposal of such products is lagging due to insufficient assessment on the toxicology of silver nanoparticles **(Stensberg *et***

***al.*, 2011).** Accordingly, the effects of daily oral exposure of silver nanoparticles on ovary of the adult rats were examined in this study.

2. Material and methods

Preparation of silver nanoparticles

Preparation of $5x10^{-3}$ mol/l of Ag nanospheres (puress, Fluka) was done by citrate-reduction route **(Turkevich *et al.*, 1951)** according to the following: 0.0850g of (AgNO3) (puress, Fluka) were added to 100 ml of double distilled water. Then, 25 ml of the stock solution was added to 100 ml of double distilled water. The solution was heated until it begins to boil where 5ml 0f 1 % of sodium citrate was added with vigorous magnetic stirring. Heating was continued until the colour of the solution gradually changed to yellow. Then, it was continued for another 15 minutes after that the solution was removed from the heater and stirred for a further 15 minutes. The silver nanoparticles solution were completed to 125 ml by double distilled water and stored at 4°C.

Transmission electron microscopy [TEM, Hitachi HU-11B] at a voltage of 80 kV was used to study the particles size, and morphology of the Ag NPs where the aqueous dispersion of the NPs was drop cast onto a carbon coated copper grid, and the grid was air dried at room temperature before viewing under the microscope, and the diameter was determined from the micrographs. The electronic UV-Visible absorption spectra were recorded on a *Perkin Elmer lambda-17* spectrophotometer, using a quartz cell with a path length of 1 cm.

Animals

Thirty six female Wister albino rats, weighing 140-160g and their ages range between 12 and 14 weeks (in animal house- National Research Centre) were used in the present experiment. The animals were kept under optimum conditions of temperature, humidity and maintained on standard pellet diet with adequate water. They were divided into six groups of (2 control and 4 experimental), 6 rats each. The first and second group were the control groups where physiological saline was given for 2 and 4 weeks, respectively. The third and fourth groups received low (30 mg/kg) and high dose (300 mg/kg) of silver nanoparticles orally once per day for two weeks, respectively. The fifth and sixth groups received low (30 mg/kg) and high dose (300 mg/kg) of silver nanoparticles orally once per day for four weeks, respectively. Rats were sacrificed by cervical dislocation on the next day at the end of the dosing period, and then the ovaries were removed. Samples for light microscopy were fixed in 10% neutral buffered formalin. The research was approved by the ethical committee for medical research of the National Research Centre.

Histological studies

1. Light microscopic study

Tissues fixed with 10% formalin were embedded in paraffin blocks, and then sliced into 6 micron in thickness **(Detafield, 1989).** Haematoxylin and Eosin (H&E) staining was performed **(Horobin& Bancroft, 1998)** to study the general histology features and Masson's Trichrome staining was performed **(Masson, 1929)** to investigate collagen deposition**.** Both were examined by light microscope (Olympus optical lamp housing, BX50 F4) equipped with a camera (Olympus imaging Corp, digital camera E420, China).

2. The Immunohistochemistry

Caspase 3 protein is a member of the cysteine aspartic acid protease (Caspase) family. Sequential activation of Caspase plays a central role in cell apoptosis. Caspase 3 was immunohistochemically detected in paraffin embedded ovarian sections. Sections were deparaffinised and rehydrated using alcohol series. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide-methanol (1:1 v/v) solution at room temperature for 20 minutes. For antigen retrieval, the slides were placed in 10mM citrate buffer solution (pH 6.0), blocked with 5% human serum albumin for 30 minutes, and incubated overnight at 4º C with primary antibodies (anti-CASP3 polyclonal antibody, dilution 1:100, obtained from Lab Vision Company). Slides were incubated with Biotinylated Goat Anti-polyvalent for 10 minutes at room temperature. Immunoreactions complexes were detected using the avidin biotin peroxidase complex technique (Sigma Chemical Company) **(Buchwalow&Bocker, 2010)** and visualized using 0.05% diaminobenzidine (DAB) as a chromogen. Slides were counterstained with Haematoxylin and examined under a light microscope.

Statistical analysis

Data were statistically described in terms of mean ± standard deviation (± SD), median and range when appropriate. Comparison of numerical variables between the study groups was done using Student t test for independent samples. *P* values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

3.Results

Silver nanoparticles show a strong light absorption in the visible region at λ max = 405 nm, shown in Figure 1. This strong absorption results from nanoparticles' coherent oscillation of the free electrons on the particle surface (Surface Plasmon

Resonance). The prepared silver nanoparticles were spherical in shape and about 15nm in size.

Body weight

There was a decrease in the mean body weight gain in groups 3 & 4 (low and high dose of silver for two weeks, respectively) which, when compared with mean body weight gain in group one (control for 2 weeks), was highly significant (Table 1). Also, the decrease in the mean body weight gain in groups 5 & 6 (low and high dose for 4 weeks, respectively), when compared with mean body weight gain in group2, was highly significant too (Table 1). Only one rat died from group 3 (low dose, 2weeks) on the eighth day of the study.

Light Microscope study:

When compared to control groups (Fig 2), all groups of silver showed congested blood vessels and areas of congestion in the stroma with extravasations of blood with mononuclear cell infiltration that increase with the increased dose and duration as seen in (Figs 3,4). Also there was a relative increase in atretic and degenerated follicles.

Also when the control groups compared with the experimental groups (stained with Masson trichrome), excess deposition of collagen fibres were recognised which vary from mild to severe depending on dose and duration of exposure to silver nanoparticles (Figs 5 & 6). The control groups (Fig 7 & 8) showed negative reaction to Caspase 3 while there was positive reaction for caspase3 in ovarian tissue of the experimental groups, which was moderate in group 3 and varied between moderate to strong in groups 4, 5 and 6 as shown in Fig(9).

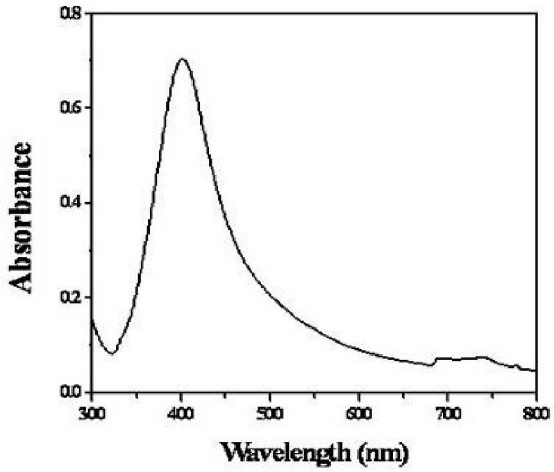

Figure 1 The absorption spectra of silver nanoparticles

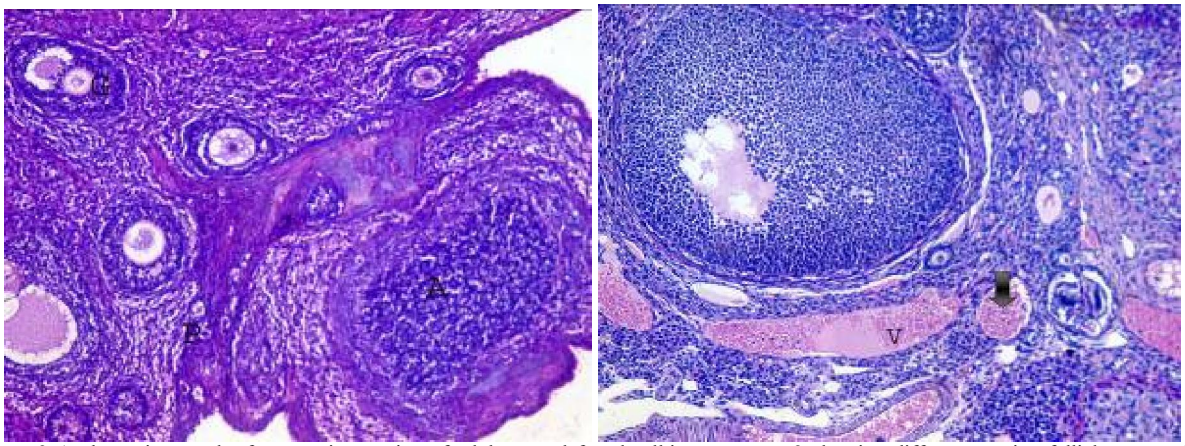

Figure 2 A photomicrograph of an ovarian section of adult control female albino rat group 2 showing different ovarian follicles, secondary growing follicles(G), atretic follicles (A) ,primordial follicles(P) (H&E x 200)

Figure 3 A photomicrograph of an ovarian section of adult female albino rat ,group4, after 2 weeks of high dose of silver nanoparticles administration showing multiple areas of congestion in the stroma, dilated congested blood vessel (V) with mononuclear cell infiltration (Arrow) (H&E x 200)

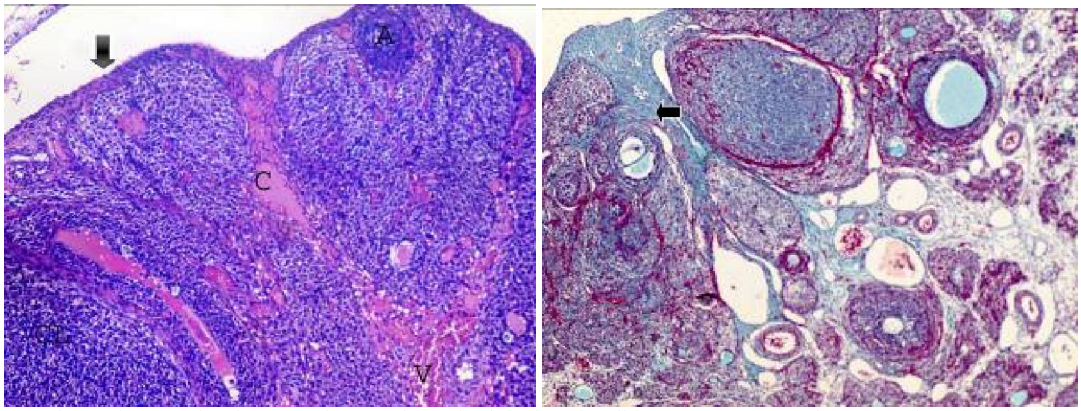

Figure 4 A photomicrograph of an ovarian section of adult female albino rat ,group6, after 4 weeks of high dose of silver nanoparticles administration showing different ovarian follicles, corpora lutea(CL), atretic follicles(A) ,multiple areas of congestion in the stroma(C) and dilated congested blood vessel(V) in the stroma and covering ovarian epithelium (Arrow) (H&E x 200)

Figure 5 A photomicrograph of an ovarian section of adult female albino rat ,group4, after 2 weeks of high dose of silver nanoparticles administration showing excess deposition of collagen fibres in the stroma and around the follicles(arrow) (Masson's Trichrome x 100)

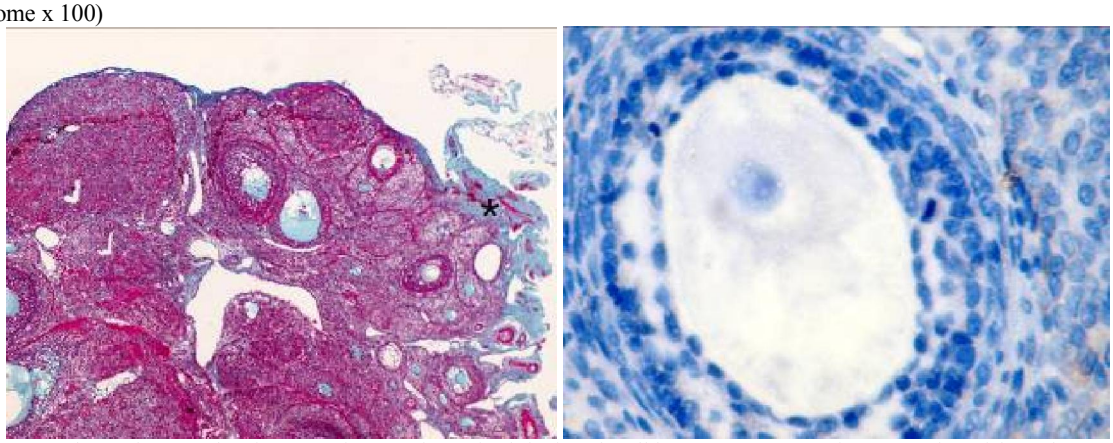

Figure 6 A photomicrograph of an ovarian section of adult female albino rat ,group5, after 4 weeks of low dose of silver nanoparticles administration showing excess collagenous fibres deposition in the stroma (*)(Masson's Trichrome x 100)

Figure 7 A photomicrograph of an ovarian section of adult control female albino rat group 1 showing negative immunoreactions for Caspase 3 in the surface epithelium and ovarian tissue (immunoreactions for Caspase 3 x 1000)

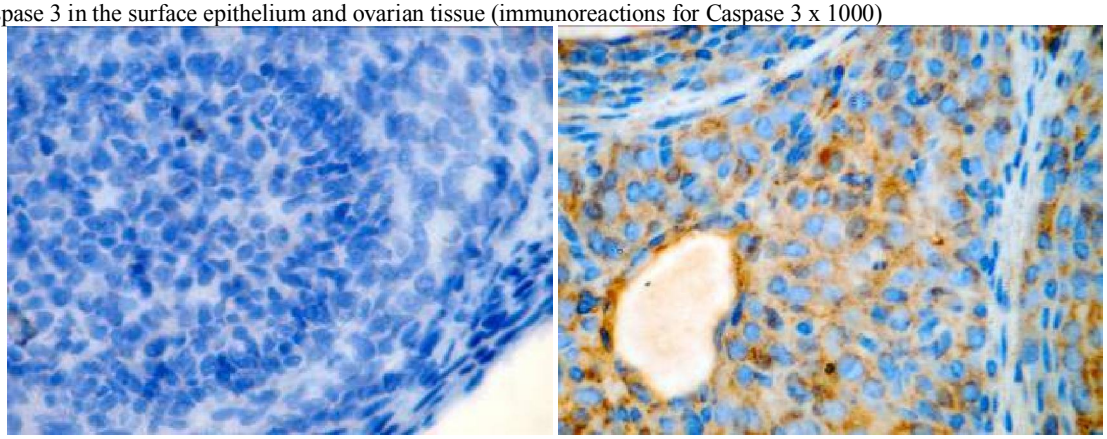

Figure 8 A photomicrograph of an ovarian section of adult control female albino rat group 2 showing negative immunoreactions for Caspase 3 ovarian tissue (immunoreactions for Caspase 3 x 1000)

Figure 9 A photomicrograph of an ovarian section of adult female albino rat, group 4, after 2 weeks of high dose of silver nanoparticles administration showing moderate to strong immunoreactions for Caspase 3 in the follicles (Immunoreactions for Caspase 3 x 1000)

4. Discussion

The present study showed that there were highly significant decreases in the mean body weight gain in all experiment groups when compared to the control groups. These findings agree with **Kim *et al.*, 2010** who stated that there was a significant decrease ($P < 0.05$) in the body weight of rats after 4 weeks of oral administration of silver nanoparticles with low-dose (30 mg/kg), middle-dose (125 mg/kg), and high-dose (500 mg/kg). On the other hand, **Park *et al.*, 2010** mentioned that there were no significant differences in the body weight between control group and silver treated groups when a dose of 1mg/kg given orally for 14 days, but the dose used in their study was much lower than those used in the present study**.**

In the present study, the light microscope examinations of all experimental groups of silver nanoparticles showed congested blood vessels in the stroma with inflammatory mononuclear cell infiltration. Also, there were areas of congestion in the stroma with extravasations of blood which were dose and duration dependant. Excess depositions of collagen fibres were seen in all groups which indicate the presence of fibrosis. Another study reported that after oral administration of silver nanoparticles for 28 days, inflammatory responses were significantly induced with increased cytokine production, increased B cell distribution, and inflammatory cell infiltrates **(Park *et al.*, 2010).** Also, higher incidence of bile-duct fibrosis, pigmentation, hyperplasia and necrosis were observed by histopathological examination in treated animals after oral exposure to silver nanoparticles **(Kim *et al.*, 2008).** It was concluded by many studies that exposure to silver nanoparticles induces inflammation which is a sign of toxicity mediated through oxidative stress **(Carlson *et al.*, 2008; Folkmann *et al.*, 2009; Su *et al.*, 2009).**

In our study, there were positive reactions for Caspase3 which indicate the presence of apoptosis. These positive reactions in ovarian tissue increased in a manner which is dose and duration dependent. The presence of apoptosis in silver nanoparticles fed mice was explained in other studies by the alteration in apoptosis related genes **(Cha *et al.*, 2008; Rahman *et al.*, 2009).**

Also, Silver nanoparticles up-regulate the expression levels of stress genes, *Ho1* and *Mt2A* **(Miura & Shinohara, 2009)** and these genes were induced by oxidative stress **(Sato & Bremner, 1993; Kondoh *et al.*, 2001;Takahashi *et al.*, 2004).** Oxidative stress was reported to be one of the causes inducing apoptosis to cells **(Ott *et al.*, 2007; Rana, 2008).** In addition, ROS was generated in vitro cells that were exposed to silver nanoparticles **(Hsin *et al.*, 2008).** A number of in vitro studies that examined the effect of silver nanoparticles on different tissues such as testicular cells **(Asarea *et al.*, 2012)**, monocytes **(Foldbjerg *et al.*, 2009)** and germ cells **(Braydich-Stolle *et al.*, 2005)** reported that the toxicity of nanoparticles includes apoptosis, necrosis and decrease of proliferation in a manner that is concentration and time dependent. In 2005, Braydich-Stolle and colleagues assessed nanotoxicity of sliver nanoparticles of size 15nm on male mice germline using spermatogonial stem cell. And the results showed that apoptosis of cells was dose dependent and there was reduced mitochondrial function and increased membrane leakage **(Braydich-Stolle *et al.*, 2005).**

In the present study, two doses of silver nanoparticles were given orally (30 and 300mg/kg) and both of them showed toxic effects on the ovary in the form of inflammation, congestion, fibrosis and apoptosis. Another study observed that when rats were injected with a dose of 62.8 mg/kg of silver nanoparticles, blood-brain barrier (BBB) destruction, astrocyte swelling and neuronal degeneration were induced **(Tang *et al.*, 2009).** In 2011, Tiwari and colleagues used 4 doses of silver nanoparticles 4, 10, 20 and 40 mg/kg which were injected intravenously and they concluded that silver nanoparticles in doses less than 10mg/kg are safe for biomedical application and has no side effects, but doses more than 20mg/kg are toxic **(Tiwari *et al.*, 2011).** However**,** another study mentioned that after 2 weeks of intravenous administration of silver nanoparticles in mice no obvious acute toxicity were observed with the dose of 30mg/kg, while inflammatory reactions in lung and liver cells were induced in mice treated with the dose of 120mg/kg **(Xue *et al.*, 2012).** Also, after 28 days of oral administration of more than 300mg/kg of silver nanoparticles to Sprague-Dawley rats slight liver damage may result, but low-dose (30 mg/kg) and middle-dose (300 mg/kg) showed no side effects on the rats **(Kim *et al.*, 2008).**

The cytotoxic behaviour of the silver nanoparticles may be attributed to their ability to attach to cell membrane, changing its permeability and leading to intracellular ROS accumulation and oxidative stress **(Sondi &Salopek-Sondi, 2004; Lok *et al.*, 2006)**. In normal conditions there is high oxidised/reduced glutathione ratio (GSH/GSSG ratio), when tissues are exposed to silver nanoparticles it depletes the glutathione (GSH) level leading to an increase of ROS generation and reduction of the antioxidant defence system (**Rahman *et al.*, 2009)** leading to oxidative stress **(Nel *et al.*, 2006).** GSH is the key antioxidant defence mechanism that is responsible for maintaining cellular oxidation-reduction homeostasis **(Sies, 1999)**. At mild conditions of oxidative stress, antioxidant enzymes are induced to restore cellular redox

homeostasis. As the oxidative stress continues, inflammatory responses are induced followed by perturbation of the mitochondria and disruption of electron transfer resulting in cellular apoptosis **(Nel *et al.*, 2006)**

In 2005, Hussain and colleagues reported GSH depletion in liver cells exposed to silver nanoparticles of sizes 15nm and 100nm. In their opinion the low levels of GSH is due to either binding of silver nanoparticles directly to GSH or by inhibiting enzymes involved in GSH synthesis and this was directly correlated with high level of ROS and oxidative stress **(Hussain *et al.*, 2005).** This was also supported by **Kim *et al.*, 2009** who stated that the toxic effects are due to silver nanoparticles not only due to the release of silver ions**.**

The ROS is uniquely associated with 15nm silver nanoparticles **(Carlson *et al.*, 2008)** compared to similar sizes of other nanoparticles such as aluminum **(Hussain *et al.*, 2005).**A study reported that after 24 hours of exposure to 15nm silver nanoparticles, a significant inflammatory response was observed by the release of TNF-α, MIP-2, and IL-1β**(Carlson *et al.*, 2008).** This is because the inflammatory responses escalate with increasing oxidative stress **(Rouse *et al.*, 2008).**

Conclusion

Silver nanoparticles showed evidences of inflammation, congestion, extravasations of red blood cells, fibrosis and apoptosis in cells of the ovary in all examined doses. Further studies are required with doses lower than 30mg/kg.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgments

This work was supported by the National Institute of Laser Enhanced Sciences (NILES) and National Research Centre (NRC)

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5/12/2013