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The effects of prenatal exposure to silver nanoparticles on the developing brain in neonatal rats

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An increasing number of studies indicate that oxidative stress is a mechanism in nanoparticle-mediated neurotoxicity which may lead to apoptosis and cause brain damage. However, there are relatively few studies investigating the developmental neurotoxic effects of nanoparticles. This study was designed to evaluate the potential of silver nanoparticles to induce toxicity on developing brains and determine the possible mechanism. Pregnant Wistar rats were divided into two groups. Animals in the experimental group were treated orally with a silver nanoparticle suspension (25 mg kg^{-1} BW) while animals in the control group were treated with deionized water. The offspring were sacrificed and their brains were collected. Silver accumulation, the amounts of malondialdehyde and glutathione, glutathione peroxidase activity, and the amounts of caspase 8 and 9 in the brains of offspring were determined using ICP-MS analysis, spectrophotometric assay, and ELISA, respectively. The results showed that maternal exposure to silver nanoparticles can induce oxidative stress and apoptosis in brains of their offspring. These findings indicate that silver nanoparticles act as a developmental toxicant and application of this particle during pregnancy may affect fetus embryogenesis.

Key words: apoptosis, embryogenesis, oxidative stress, silver nanoparticle.

INTRODUCTION

Nanoparticles, by definition, are small-scale particles ranging from roughly 1 to 100 nm in at least one axis. They often possess unique physicochemical characteristics. These properties are mostly attributed to inherent quantum size effects and high surface-to-volume ratio, which potentially results in high chemical reactivity (Toshima, 2003). For these reasons, nanoparticles are being increasingly employed in various fields including medicine, biology, pharmacology etc. (Nam & Lead, 2008; Warheit *et al*., 2008).

Silver nanoparticles (AgNPs) are among the most commercialized nanoparticles (Woodrow-Wilson database; www.nanotechproject.org). Because of their strong antimicrobial properties, silver nanoparticles are used in many cases, such as personal care products, medical instruments, clothing, wound dressings, and in food processing (Ahamed *et al*., 2010). They are also incorporated in filters for purification of drinking water and air (Tolaymat *et al*., 2010).

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Widespread application of silver nanoparticles leads to higher human and environmental exposures. Many reports unequivocally indicate that AgNPs can translocate into various organs, including liver, lung, and kidney. AgNPs can also cross through the blood-brain barrier (BBB) and reach the brain (Lockman *et al*., 2004; Lansdown, 2007; Kim *et al*., 2008). Tang and colleagues in two studies (Tang *et al*., 2008, 2009) indicated that subcutaneous injection of nanosilver can distribute to the rat's brain. Trickler *et al*. (2010) showed that interaction between nanosilver and primary rat brain microvessel endothelial cells (as an *in vitro* BBB model) can induce brain inflammation and increase BBB permeability. Recent studies have revealed that nanoparticles are able to transfer from pregnant females to their fetuses and accumulate in the embryonic tissues, especially brain, and are able to influence the development of CNS, due to incompletely formed blood-brain barrier in the embryogenesis stage (Takeda *et al*., 2009; Hougaard *et al*., 2010; Gao *et al*., 2011; Kulvietis *et al*., 2011). One study showed that prenatal exposure to diesel exhaust particles (DEs) alters the concentrations of monoamines in brains of male mice (Yokota *et al*., 2009). Furthermore, female offspring exhibited increased activity in the open field (an experiment used to assay general locomotor activity) after maternal exposure to DEs (Hougaard *et al*., 2008). Jackson *et al.* (2011) reported that offspring prenatally exposed to printex 90 exhibited small behavioral changes in the open field test. A recent study has revealed that maternal exposure to TiO_2 nanoparticles can cause changes in synaptic plasticity (Gao *et al*., 2011). Moreover, Ag^+ can switch neurodifferentiation to the dopamine phenotype at the expense of the cholinergic phenotype (Powers *et al*., 2010).

Silver in the brain has longer biological half-life than in other organs and may produce adverse effects (Yang *et al*., 2010). Rungby *et al*. (1987) reported that acute exposure to high levels of silver salts can induce oxidative stress in the brain of adult rodents. Wu *et al*. (2010) observed that maximum width of the optic tectum (as an indicator of midbrain development) was reduced in Japanese medaka (*Oryzias latipes*) embryos exposed to AgNP. When *in vitro* effects of monovalent silver ions on undifferentiated PC-12 cells were evaluated, Ag^+ inhibited cell replication, enhanced cell death, and retarded neurite formation (Powers *et al*., 2010). A significant decrease in mitochondrial function and increase in the production of reactive oxygen species have been shown in murine neuroblastoma cells after exposure to 25 μg ml^{-1} AgNP (25 nM) (Schrand *et al*., 2008). These results suggest that the impact of AgNP on the brain, and especially the developing brain, is not negligible. Unfortunately, information about developmental neurotoxicity of silver nanoparticles is lacking. Therefore, the aim of the current study was to evaluate the effects of prenatal exposure to silver nanoparticles on the developing brain in rats.

MATERIALS AND METHODS

Materials

Silver, dispersion nanoparticle, with particle size 20 ± 4 nm, 0.02 mg ml^{-1} in aqueous buffer, containing sodium citrate as stabilizer, based on the data sheet which was provided by manufacturer (Sigma-Aldrich Prod. No. 730793) were purchased from Sigma-Aldrich, Inc. The stock suspension of silver nanoparticle was diluted with deionized water and stirred on vortex before every use. The size of nanoparticles and agglomeration state after dilution with deionized water was determined using Transmission Electron Microscopy (TEM). No changes were observed in the state of the agglomeration and particle size compared with the manufacturer's information (figures not shown). The rat caspase 8 and 9 ELISA kits were purchased from Glory Science Co., Ltd. The other chemicals used in this study were also obtained from Sigma- Aldrich, Inc.

Animals and treatment

Adult male and female Wistar rats (180 ± 20 g) were purchased from Animal Center of Isfahan University and acclimated for three weeks before starting the experiment. The rats were housed under controlled environmental conditions with a 12 hr light/dark cycle, 23 ± 2°C temperature, and 50 ± 5% humidity. Distilled water and sterilized food for rats were provided ad libitum. Time-mated, pregnant rats (n = 90) were housed separately and randomly divided into two groups: control group and pregnancy AgNP administrative group. Based on numerous toxicity studies reporting that AgNPs at concentrations between 2.5-200 μg ml^{-1} (equal to 2.5-200 mg kg^{-1}) induce oxidative stress, mediated cell death, and apoptosis in cell lines (Hussain *et al*., 2005; Arora *et al*., 2008; Hsin *et al*., 2008; Yildirimer *et al*., 2011), we used 25 mg kg^{-1} AgNP in this study. Silver nanoparticles (25 mg kg^{-1} BW) were given to the pregnant administrative group by an intragastric administration from ninth day (roughly corresponding to the beginning of neurolation in

the rat) (Butcher, 1929) to the last day of pregnancy. The control group was treated with deionized water without AgNP. Per each litter with 6-8 pups, one male pup on the day after birth was randomly chosen from each group. After weighing, the pups were sacrificed and their brains were collected. The brains were rinsed in 0.1 M potassium phosphate buffer (pH = 7.4) and were quickly frozen at –80˚C. The permission for animal samples usage in the experiments was taken from the institutional review board of the University of Isfahan after considering the project and its aims.

Relative brain weight

The brains (n = 16, each group from pups) were excised and weighed accurately. The ratio of brain to body weight was calculated as the ratio of wet brain weight (mg) to wet body weight (g).

Histopathological analysis

For pathological studies, eight brains were removed from pups in each of the control and treated groups and then fixed with 10% neutral buffered formalin. Thereafter, the brains were embedded in paraffin. After paraffin embedding, 5 μm sections (four sections from each brain) were sliced and stained with hematoxylin and eosin (H&E). The slides were observed under a light microscope for evidence of brain damage. The numbers of microvacuolar structures were randomly counted by a microscope field view of 30 degrees and the averages were compared between groups.

Oxidative stress

Right cerebral hemisphere samples (n = 16, each group from pups) were weighed and homogenized (1:10) in ice-cold 0.1 M potassium phosphate buffer (pH = 7.4). The homogenate was centrifuged (3000 rpm) at 4˚C for 10 min and the supernatant was collected for assay of malondialdehyde (MDA) and glutathione (GSH) levels and glutathione peroxidase (GPX) activity.

Determination of GSH

For measurement of glutathione, 1 ml of the homogenate was centrifuged with 5% trichloroacetic acid to centrifuge out the proteins. A volume of 2 ml of phosphate buffer (pH = 8.4), 0.4 ml of double distilled water and 0.5 ml of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were added to 0.1 ml of this homogenate, vortexed and the absorbance read at 412 nm within 15 min.

GPX activity determination

The activity of glutathione peroxidase in the brain was spectrophotometrically measured. A volume of 0.2 ml of supernatant was mixed with 1 ml of 0.4 M phosphate buffer (pH = 7.0) containing 0.4 mM EDTA, 1 ml of 8 mM reduced glutathione (GSH) and 1 ml of 5 mM NaN_3. The reaction mixture was incubated at 37˚C for 10 min. Subsequently 1 ml of 8 mM H_2O_2 was added and incubated at 37˚C for another 10 min. Then, the mixture was centrifuged with 5% trichloroacetic acid to centrifuge out the proteins. A volume of 1 ml of phosphate buffer (pH = 8.4), 250 μl of DTNB and 200 μl of double distilled water were added to this homogenate and vortexed. The absorbance was read at 412 nm within 15 min. One unit of GPX is defined as the amount of enzyme required to oxidize 1 nmol GPX/min.

Determination of MDA

Malondialdehyde was measured by using the thiobarbituric acid method (Ohkawa *et al*., 1979). Briefly, 0.2 ml of supernatant was mixed with 1 ml of 50% trichloroacetic acid in 0.1 M HCl and 1 ml of 26 mM thiobarbituric acid. The samples were vortexed and then heated at 95˚C for 20 min. After centrifugation at 1000 ×g for 10 min, the supernatants were withdrawn and absorbance was read at 532 nm using a spectrophotometer.

Determination of cytokines

The concentrations of caspase 8 and 9 in the brain were determined using commercially available ELISA kits. Briefly, left cerebral hemisphere of the same samples (n = 16, each group from pups) were weighed and homogenized (1:10) in 1×PBS and stored overnight at –20˚C. After two freeze-thaw cycles to break the cell membranes, homogenates were centrifuged for 5 min at 5000 ×g. The supernatant was assayed and removed immediately. A volume of 50 μl of standard solution and 50 μl of streptavidin-HRP were added to standard wells. Forty μl of sample and then 10 μl of visfatin-antibody and 50 μl of streptavidin-HRP were added to test wells; then plates were incubated for 60 min at 37˚C. The plates were washed five times and 50 μl of chromogen solution A, then 50 μl of chromogen solution B were added to each well. After incubation for 10 min at 37˚C away from light, 50 μl of stop solution was added into each well to stop the reaction. The OD values of the samples were meas-

ured and calculated according to standard curves.

Silver content analysis

The brains (n = 8, group) were digested with perchloric acid and nitric acid (at a ratio of 1:4) for 48 hr. The solutions were incubated at 120°C to remove the remaining acids and until the solutions were colorless and clear. At the end, each sample was diluted with 1 ml distilled water and silver levels in the samples were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Statistical analysis

All data were expressed as means ± s.d. (standard deviation). The independent samples t- test was performed on the data for analysis of differences between the control and the treatment groups. The level of significance was set at $p < 0.05$, $p < 0.01$, and $p < 0.001$. The analyses were performed using the Statistical Package for the Social Sciences (SPSS) 16.0 software. Litter effects were avoided by using a maximum of one pup/litter in any one assay.

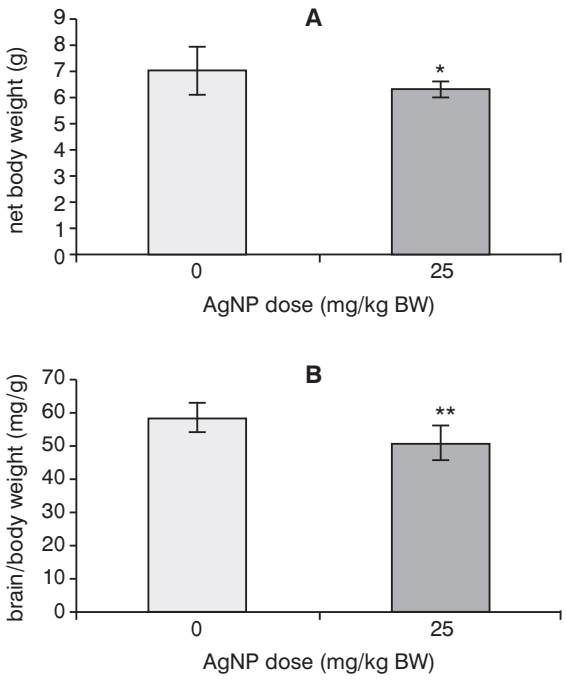

FIG. 1. Effect of AgNP on net weight (A) and brain/body weight ratio (B) of offspring rats. Bars marked with a star show significantly different from control group (* $p < 0.05$ and ** $p < 0.01$). Values are means ± s.d., n = 16.

RESULTS

Body weight and the ratio of brain/body weight

The average weight gain of mothers during pregnancy was approximately 40-50 g, but the body weights were not significantly different between groups (data not shown). Furthermore the gestation length was between 21 to 23 days similar to the control group. The pups were weighed on the day after birth. The average body weight (mean ± s.d.) of the control group (n = 16) was 7 ± 0.91 g, and for the treated group (n = 16) it was 6.28 ± 0.26 g. There were significant differences in the body weights among the groups ($p < 0.05$; Fig. 1A). In addition, the ratio of brain/body weight decreased significantly compared with that of the control ($p < 0.01$, Fig. 1B). These results indicate that AgNP (25 mg kg^{-1}) caused the brain damage during the prenatal period.

Histopathological findings

Brain histopathological morphology is illustrated in Figure 2. The microvacuolar structures were counted among the brain cells of the offspring groups. The average number of these structures in the brains from the treated group (159.37 ± 13.23) was higher than the control group (611.75 ± 20.52). This significant in-

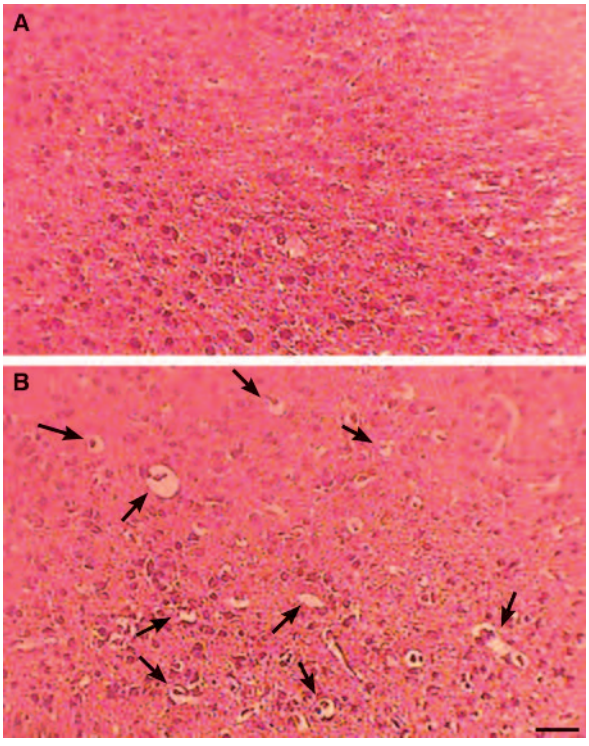

FIG. 2. Histopathology of brain tissues in offspring rats exposed prenatally to vehicle or AgNP. (A) control group; (B) treated group. Arrows indicate tissue damage. Scale bar is 20 μm.

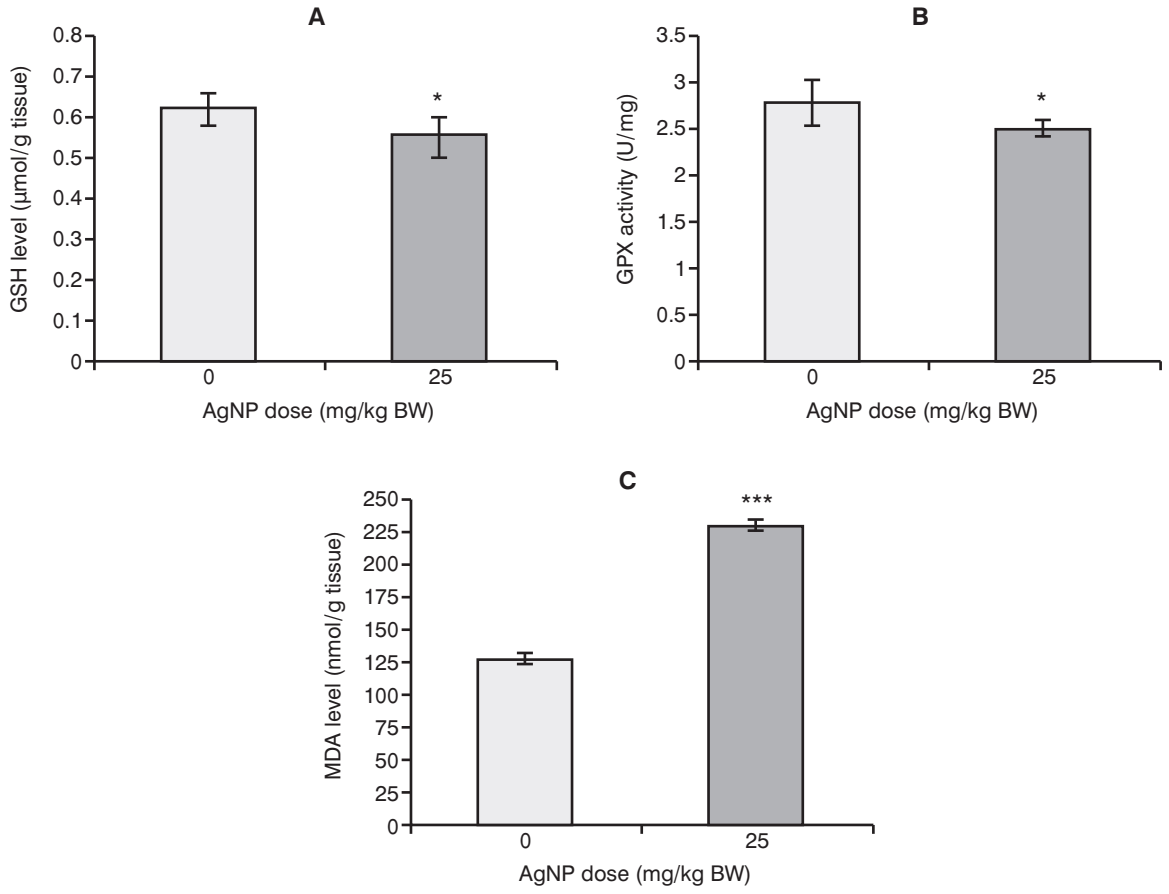

FIG. 3. Effect of AgNP on the GSH level (A), GPX activity (B), and MDA level (C) in the rat offspring brains that prenatally exposed to AgNP. Values represent means ± s.d., n = 16. * $p < 0.05$, *** $p < 0.001$ (significantly different from control group).

crease in the average number of microvacuolar structures ($p < 0.001$) indicated that AgNP caused brain damage.

Impact of silver nanoparticle on MDA and GSH level and GPX activity

To investigate oxidative stress after exposure to nanosilver, the concentration of MDA and GSH and the activity of GPX in the brains were measured. GPX and GSH act as an antioxidant enzyme and an non-enzymatic antioxidant, respectively against reactive oxygen species in the body. MDA is a biomarker of lipid peroxidation. A significant decrease in GPX activity and GSH level ($p < 0.05$, Fig. 3A & B) and an increase in MDA level ($p < 0.001$) were observed in the brains of treated pups compared with those in the control group (Fig. 3C).

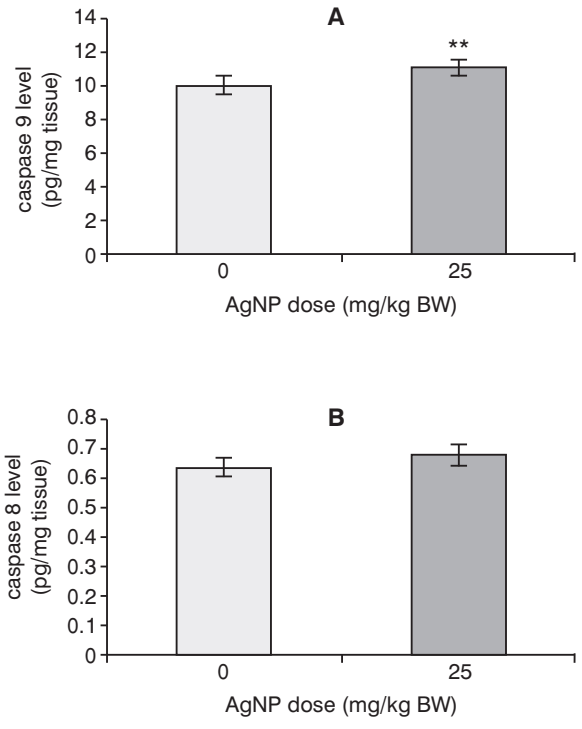

FIG. 4. Effect of AgNP on the caspase 9 (A) and caspase 8 (B) levels in the rat offspring brains that prenatally exposed to AgNP. Values represent means ± s.d., n = 16. ** $p < 0.01$.

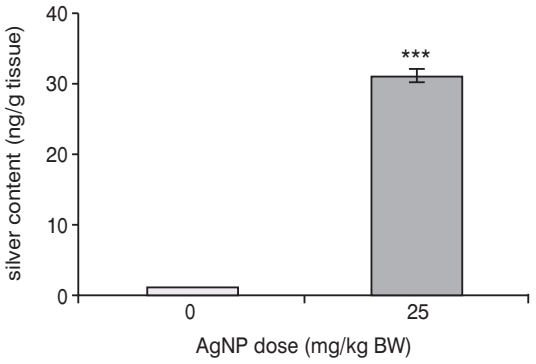

FIG. 5. Silver contents in rat brains after developmental AgNP exposure. Values represent means ± s.d., n = 8. *** $p < 0.001$ (significantly different from control group).

Effects of AgNP on caspase 8 & 9 levels

To investigate apoptosis induced by silver nanoparticle, the levels of caspase 8 and 9, which are cysteine proteases involved in cell death, were measured in the brains. As shown in Figure 4A, the level of caspase 9 in the brain of the treated offspring was higher than that of the control group ($p < 0.01$). There was no significant difference in the caspase 8 level among the groups (Fig. 4B).

Silver content in the brain

The contents of silver in the brain of offspring rats are shown in Figure 5. Silver concentration in the brain tissue from pups born by mothers exposed to AgNP were significantly higher ($p < 0.001$), while silver content in pups of unexposed group were not significantly different. These results indicated that the nanosilver traversed into the brain.

DISCUSSION

With regard to the use of silver nanoparticles in a variety of consumer products (e.g., nursing nipples, kitchen utensils, tooth paste, and reusable bottles), a potential exposure route is by oral ingestion (Wijnhoven *et al*., 2009). Recently, the size-dependent penetration of AgNPs to BBB after oral administration has been evaluated. Results showed that AgNP of ~22 nm in diameter accumulated at high levels in adult mice brain compared with larger particles with diameters of 32, 42, and 71 nm (Park *et al*., 2010a). So, we chose AgNP of 20 nm in diameter for the current project and evaluated neurotoxicity of this particle on the brain of offspring rats after maternal intragastric administration. Our study revealed that after AgNP maternal exposure, offspring body weight (BW) and relative brain weight decreased significantly compared with those in the control group. Significant spongiform vacuolation was observed in the brain tissues of treated offspring. Such morphological changes are a hallmark of neurodegeneration (Hansen *et al*., 1989; Rezaie & Lantos, 2001; Muthuraju *et al*., 2013). Therefore, we speculate that accumulation of silver nanoparticles in brain may be associated to an increased number of these structures. Previous studies have demonstrated that silver could be distributed and accumulated in the different regions of the adult rat and mouse brain (Rungby & Danscher, 1983; Tang *et al*., 2008, 2009; Park *et al*., 2010a). However, there has been little research on the accumulation of this particle in the brain of the offspring whose mothers were exposed to AgNP during pregnancy. In the present work, the results of the ICP-MS analysis showed the presence of silver in the brain of pups of the treated group.

It is well known that induction of oxidative stress by generation of reactive oxygen species (ROS) is a key mechanism for nanosilver toxicity (Nel *et al*., 2006; Liu *et al*., 2010). For example, an increase in the generation of ROS was observed in BRL3A rat liver cells treated with AgNP (Hussain *et al*., 2005). ROS includes all reactive forms of oxygen that are generated in low frequency under normal conditions. However, under conditions of extra ROS formation (such as nanoparticle exposure) (Nel *et al*., 2006), the defense mechanism of antioxidant that applies to neutralize the negative effects of ROS is inadequate, leading to oxidative stress (Halliwell & Gutteridge, 1999).

In the embryonic stage the brain is highly vulnerable to oxidative stress due to its higher oxygen consumption, high metabolic rate associated with growth, low levels of antioxidants and protective enzymes, and high content of polyunsaturated fatty acids (Gupta, 2004). GSH is the most concentrated non-enzymatic antioxidant in the brain (Funchal *et al*., 2010). Under conditions of oxidative stress, GSH is depleted while oxidized glutathione (GSSG) accumulates. GSH also is a co-enzyme for activity of GPX antioxidant enzyme (Halliwell & Gutteridge, 1999). In our study, a significant decrease in GPX activity and GSH level was observed in the offspring brain of the group exposed to AgNP. Furthermore, since brain tissue is rich in polyunsaturated fatty acids, which are extensively susceptible to ROS damage, lipid peroxidation (another oxidative stress marker) was evaluated by measurement of MDA level in the brain. A significant increase in MDA level was observed in the brain of pups of

the treated groups. Our findings suggest that silver induced oxidative stress. In line with our investigation, it has been reported that the Ag-25 nanoparticle has the potential to induce oxidative stress in the caudate, frontal cortex, and hippocampus of mice via changes in expression of the oxidative stress and antioxidant defense genes, such as Fmo and Gpx2 (Rahman *et al*., 2009). Moreover, an increase in malondialdehyde level, a decrease in superoxide dismutase activity, GPX activity, and GSH content was observed in the brain of the adult male rats after exposure to 23 and 29 nm silver nanoparticles cupped with polyamide hydroxyl urethane (Hritcu *et al*., 2011). Arora and colleagues (Arora *et al*., 2008) in an *in vitro* study indicated that the interaction of 7-20 nm silver nanoparticles with HT-1080 and A431 cells could lead to oxidative stress, resulting in an increase in lipid peroxidation and decrease in superoxide dismutase activity and GSH content.

Several researchers (Foldbjerg *et al*., 2009; Gopinath *et al*., 2010; Park *et al*., 2010b) have reported that nanosilver has the potential to induce DNA damage and apoptosis via oxidative stress and lipid peroxidation. There is evidence that AgNP significantly increases cell death through oxidative stress-related mechanisms in different mammalian cell lines such as human keratinocytes and fibroblast cultures (Burd *et al*., 2007; Kim *et al*., 2010).

During neurogenesis, apoptosis is essential for the regulation of neuronal cell number and correct synaptogenesis (Gordon, 1995; Porter & Janicke, 1999). Abnormal apoptosis can disrupt the developmental processes. Several studies have reported that exencephaly, craniofacial malformations, dramatic alterations of the lens, retina and eye vascular system, and defects in inner ear development were caused by defective apoptosis (Yoshida *et al*., 1998; Cecconi *et al*., 2008). Excessive apoptosis can also delete important cell lineages, potentially leading to embryonic malformation (Byrne *et al*., 1999). In *in vitro* models, excessive apoptosis has been previously shown to occur in response to treatment with AgNP in mouse embryonic stem cells, embryonic fibroblasts (Ahamed *et al*., 2008), RAW264.7 (the mouse peritoneal macrophage cell line) (Park *et al*., 2010b), and PC-12 cells (Powers *et al*., 2010).

In this experiment, concentrations of caspase 8 and 9 were measured in offspring brains to analyze apoptosis induction. Our findings indicated that while the caspase 8 level in pups whose mothers were treated with AgNP did not change significantly, caspase 9 level in the same pups increased significantly compared with pups in the control group. Caspases are cysteine proteases involved in cell death. There are two major pathways leading to apoptosis that have been named the extrinsic and intrinsic routes. The activation of an initiator caspase is the first step in initiating activation of the caspase cascades that in turn, induces apoptosis. The initiator caspase in the extrinsic pathway is caspase 8. Caspase 9 is the initiator caspase in the intrinsic pathway that is activated by events occurring at mitochondrial membranes (Rastogi-Richa & Sinha, 2009). Shi *et al*. (2010) indicated that the induction of apoptosis in BEAS-2B cells treated with TiO_2 nanoparticle occurred via the activation of the intrinsic apoptosis pathway independent of caspase 8/t-Bid pathway. In another study, it was shown that caspase 9 level, but not caspase 8, increased in the mouse hippocampus after titanium dioxide nanoparticle exposure. These data indicated that hippocampal apoptosis may not be mediated via the extrinsic pathway (Hu *et al*., 2011). It has been recently demonstrated that the possible mechanism of silver nanoparticle-mediated apoptosis is the release of proapoptotic factors through perturbation of mitochondrial permeability (Braydich-Stolle *et al*., 2005; Hussain *et al*., 2006; Yacobi *et al*., 2007). For example, Hsin *et al*. (2008) reported that apoptosis in fibroblasts was caused by the release of cytochrome C into the cytosol and the translocation of Bax protein to mitochondria. Hence, we speculated that AgNP may stimulate apoptosis, probably via the intrinsic pathway in the developing brains.

Collectively, the results of this study indicate that AgNP contributes oxidative stress which may consequently lead to apoptosis induction. However, it is unclear whether the observed toxicity is due to the intrinsically toxic nature of nanosilver, release of the Ag^+ ions or a combination of both. Some investigators suggest that silver nanoparticle acts as an effective delivery vehicle for Ag^+ ions and many aspects of AgNP toxicity can be influenced by these ions (Foldbjerg *et al*., 2009; Kim *et al*., 2009; Miura & Shinohara, 2009; Johnston *et al*., 2010; Liu & Hurt, 2010). Ag+ ions could be released from silver nanoparticles in the aqueous media, for example after preparation of dosing suspensions (Park *et al*., 2010b; Loeschner *et al*., 2011). Furthermore, Ag+ ion releasing rates could be increased by a reduction in pH which involves AgNP reaction with O_2 (Liu & Hurt, 2010). This change has also been observed after exposure of AgNP to human synthetic stomach fluid (Rogers *et al*.,

2010; Mwilu *et al*., 2013). Thus, presumably in this study, following dilution of AgNP with deionized water and oral administration, Ag+ ions may be released and cause brain toxicity. However, further experiments are required to unravel such a phenomenon.

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