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RESEARCH PAPER

The effects of indirect exposure of nanosilver on caspase-8 and caspase-9 levels in liver and brain of suckling rats

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ABSTRACT

Objective(s): The adverse health effects of nanosilver (AgNp) on adult animal models have been well documented. However, data is scarce regarding the toxic effects of AgNp on sensitive developmental stages. The present study aimed to investigate the effects of maternal milk exposure to AgNp on apoptosis induction in the liver and brain of the offspring of rats.

Materials and Methods: Lactating Wistar rats were intragastrically exposed to the vehicle (deionized water) or two doses of AgNp (25 and 100 mg/kg) for 21 days. Liver and brain samples were collected from the male pups of the mothers on postnatal day 21. The silver content and levels of caspase-8 and caspase-9 in the tissues were measured using the ICP-MS analysis and ELISA assay, respectively. For histopathological examinations, the tissue sections were stained using the hematoxylin-eosin (H&E) stain and examined by light microscopy.

Results: A significant, dose-dependent increase was observed in the silver content of the liver and brain of the pups and maternal milk exposed to AgNp. In addition, the level of caspase-9 significantly increased in the liver and brain in the pups exposed to the high dose of AgNp (100 mg/kg-1), while no significant changes were observed in the level of caspase-8 in the experimental groups compared to the controls. Histopathological studies also demonstrated tissue damage in the liver and brain of the pups exposed to the high dose of AgNp.

Conclusion: According to the results, lactational exposure to AgNp may induce apoptosis via the intrinsic pathway in the offspring tissues of rats. However, further investigation is required in order to document these findings.

Keywords: *Apoptosis, Caspase, Nanosilver*

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INTRODUCTION

Nanosilver is used globally in cosmetic, pharmaceutical, and medical industries owing to its excellent antimicrobial properties [1]. Considering various applications of nanosilver, its potential hazardous risks to the environment and human health must be investigated.

Several *in-vitro* and *in-vivo* studies have demonstrated the toxic potential of silver nanoparticles (AgNps), reporting that these particles could reduce cell viability in normal and cancer cell lines [2-7]. Moreover, it is well established that nanoparticles could induce reactive oxygen species (ROS) generation, thereby leading to DNA damage and cell apoptosis due to oxidative stress induction [8, 9]. For instance, Kim *et al* (2012) exposed PC12 cells to AgNp, observing an up-regulated matrix *metalloproteinases-3* and *heme oxygenase-1* (oxidative stress-related genes) and cell apoptosis. Therefore, it was suggested that AgNp-induced oxidative stress led to apoptosis [10]. In another study, nanosilver was reported to increase ROS production, as well as the proportion of apoptotic cells in primary cerebral cortical neurons [11]. Furthermore, the overexpression of the genes related to the antioxidant defense system (*sad1, cal, gpx1a,* and *ppara*) and up-regulation of proapoptotic genes (*Bax, puma,* and *apaf1*) have been reported in the zebrafish embryos exposed to zinc oxide nanoparticles (10-120 mg/ml) [12].

Caspases are a group of intracellular cysteine-dependent, aspartate-specific proteases, which are classified as upstream and downstream caspases. Upstream caspases (caspase-8 and

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caspase-9) promote the activity of the cascade of downstream caspases (caspase-3, caspase-6, and caspase-7), ultimately leading to apoptosis [13, 14].

Pathological apoptosis could be triggered by various exogenous stimuli (e.g., hypoxia, radiation, nanoparticles, and chemotherapeutic drugs), predisposing humans to various diseases, especially during the fetal stage and early postnatal period due to the high vulnerability of embryos and infants to the stress stimuli caused by the immature immune system, high metabolic rate, lack of protective enzymes, and low infiltration rate [15, 16].

Some *in-vivo* studies conducted on animal models have indicated that nanoparticles are able to transfer from lactating and pregnant mothers to the offspring [17-20]. However, data is scarce regarding the toxicity of these particles on developing organs.

Considering that maternal milk is the predominant route of exposure to nanoparticles in newborn mammals, the present study aimed to assess the effects of maternal exposure to nanosilver on apoptosis induction by measuring two apoptosis biomarkers (caspase-8 and caspase-9) in the liver and brain of the rat offspring with exposure during the lactation period.

MATERIALS AND METHODS

Materials

Rat caspase-8 and caspase-9 ELISA kits were purchased from the Glory Science Co., Ltd (Taiwan). The silver dispersion nanoparticle (20±4 nm; 0.02 mg/ml in an aqueous buffer) containing sodium citrate as the stabilizer (No. 730793) and the other chemicals used in this study were obtained from Sigma-Aldrich Inc. (USA).

Animals exposure

In this study, 45 pregnant Wistar rats (weight: 180-200 g) were purchased from the animal center of Isfahan University, Iran and observed until delivery. The rats were housed at the controlled temperature of 23±2ºC and relative humidity of 55±5% in constant photoperiodic conditions (12-hour light/dark cycle). Food and water were provided *ad libitum*. After explaining the research objectives to the authorities, the required permit for the use of animal samples in the experiments was obtained from the institutional review board of the University of Isfahan (No. 301/28185).

Lactating females and the litters were randomly divided into three groups of control, low-dose treatment, and high-dose treatment. The dams were gavaged (intragastric administration) with the silver nanoparticle stabilizer at the concentrations of 0.02 mg/ml in the control group, 25 mg/kg in the low-dose treatment group, and 100 mg/kg in the high-dose treatment group. The AgNp solution was administered on postnatal days 1-20 [21]. One day after the last exposure (day 21), the male pups were isolated from each group (6-8 pups per each litter), weighed, and decapitated. Livers and brains were dissected, weighed, and rinsed in 0.1 M potassium phosphate buffer (pH: 7.4). The ratio of the liver and brain to the body weight was calculated as the ratio of the wet liver and brain weight (mg) to the wet body weight (g).

Silver content determination

The silver content in the samples was measured using inductively-coupled plasma mass spectrometry (ICP-MS) [22]. In brief, the liver, brain, and milk inside the stomach of the pups were removed in the control and treatment groups (n=10). Afterwards, 150 milligrams of each sample was digested in 2.5 milliliters of nitric acid for three days, followed by the addition of 0.25 milliliter of hydrogen chloride for two days. The solutions were incubated at the temperature of 120ºC in order to remove the remaining acids until the solutions were colorless and clear. Afterwards, the solutions were diluted with deionized water to the total volume of 12.5 milliliters, and the silver content was analyzed using the ICP-MS.

Histopathological analysis

The liver and brain of the pups (six per each group) were removed and fixed in 10% neutral buffered formalin solution for 24 hours and dehydrated with ethanol (70%, 80%, 90%, 95%, and 100%). The samples were clear with two changes of xylene, impregnated with two changes of molten paraffin wax, and embedded. Paraffin-embedded sections of the tissues (5 µm) were stained with hematoxylin-eosin (H&E), and the slides were observed using a light microscope for the histopathological evaluation of tissue injuries.

Determination of caspase concentrations

The concentrations of caspase-8 and caspase-9 in the liver and brain tissues were determined using the ELISA assay in accordance with the

instructions of the manufacturer. In brief, the livers and brains (10 per each group of pups) were weighed, and after two freeze-thaw cycles to break the cell membranes, they were homogenized in 1× phosphate buffered saline (1:10). Following that, the homogenates were centrifuged at 5,000×g for five minutes, and the supernatants were immediately removed.

At the next stage, 40 microliters of the supernatant, 50 microliters of streptavidin (HRP), and 10 microliters of the anti-visfatin antibody were added to the test wells. Moreover, 50 microliters of the standard solution and 50 microliters of HRP were added to the standard wells.

After incubation at the temperature of 37ºC for 60 minutes, the plates were washed five times. Following that, 50 microliters of chromogen solution A and 50 microliters of chromogen solution B were added to each well. After incubation at the temperature of 37ºC for 10 minutes, 50 microliters of the stop solution was added to interrupt the reaction. The optical density (OD) values of the samples were calculated based on the standard curves.

Statistical analysis

Data analysis was performed in SPSS version 16, and the data were expressed as mean and standard deviation. The control and treatment groups were compared using one-way analysis of variance (ANOVA), and Tukey's post-hoc test was applied for multiple comparisons at the significance levels of P≤0.05, P≤0.01, and P≤0.001.

RESULTS

Body distribution of silver content

In order to analyze the transfer of AgNp from the nursing dams to the suckling pups, the silver concentration was measured in the milk inside the stomach of the newborns. A significant increase (P≤0.001) was observed in the silver content of the milk inside the stomach of the animals in the treatment groups compared to the control group (Fig 1-A).

Figs 1-A and 1-B show the silver concentration in the liver and brain of the pups. In addition, a significant increase (P≤0.001) was observed in the silver content of the liver and brain of the pups in the treatment groups compared to the controls. It is notable that the organ distribution pattern of silver was dose-dependent. Furthermore, the highest and lowest silver content was observed in the milk inside the stomach and brain of the infants of the exposed rats, respectively.

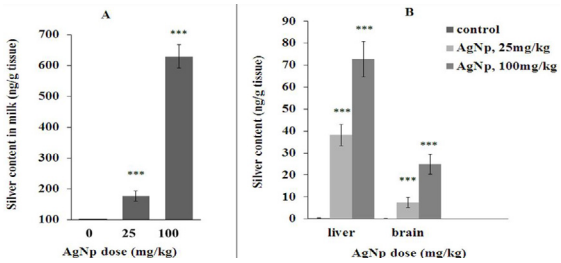

Fig 1. Ag accumulation in milk in stomach: A) Liver and brain of rat pups; B) After exposure of dams to AgNp during lactation (values expressed as mean±SD; ***P<0.001: significantly different with controls; n=10)

Body weight and body weight ratio

The body weight and liver/body weight in the offspring with maternal exposure to AgNp during lactation were lower compared to the controls (Fig 2-A). However, no significant difference was observed between the groups in terms of the body weight. A significant reduction (P≤0.01) was denoted in the brain/body weight ratio in the high-dose treatment group (100 mg/kg^{-1}) compared to the control group (Fig 2-B).

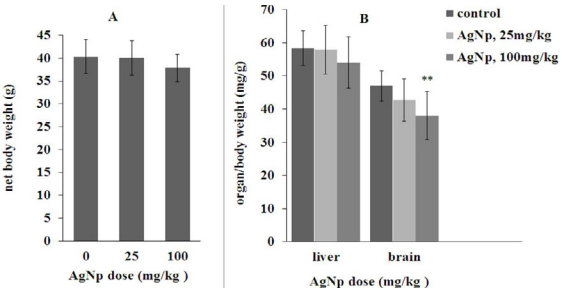

Fig 2. Net weight A) Liver/body and brain/body weight ratio; B) Results in rat pups after exposure of dams to AgNp during lactation (values expressed as mean±SD; **P<0.01: significantly different with controls; n=10)

Histological changes in the liver and brain tissues

Liver and brain morphology and pathology were evaluated in the sampled pups. The microscopic findings after H&E staining are depicted in Fig 3. Specimen A in Fig 3 exhibits a central vein with normal hepatocyte cords, and the specimen of the low-dose treatment group (Fig 3-B) was comparable to the control group. On the other hand, the liver specimen in the high-dose treatment group exhibited multiple foci of inflammatory cell infiltration, as well as dilated and congested central vein with hepatocyte disarray,

and congested hepatic sinusoids containing red blood cells (Figs 3-C and 3-D).

The histopathological observations of the brain tissue sections revealed cell apoptosis and vascular degeneration in the high-dose treatment group (Fig 3-G) compared to the control group (Fig 3-E). Although apoptosis was also observed in the low-dose treatment group, the effect was sporadic compared to the high-dose treatment group (Fig 3-F).

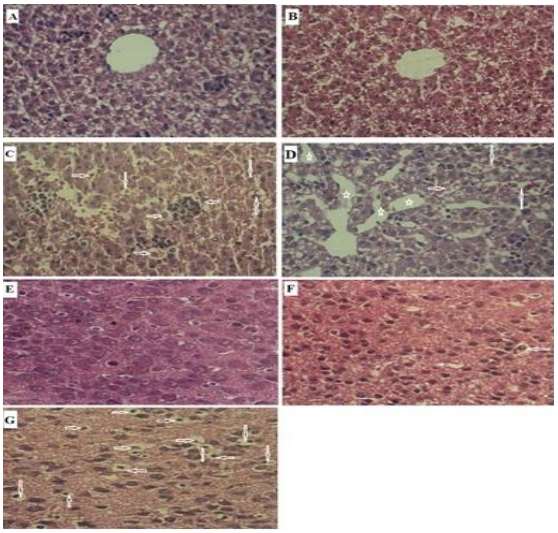

Fig 3. H&E stained liver and brain sections of rat pups after exposure of dams to AgNp during lactation A) Liver of controls with normal hepatocytes surrounding central vein; B) Liver of low-dose treatment group compared to controls; C-D) Liver of high-dose treatment group with cell swelling and hepatocyte disarray; C) Dilatation of hepatic sinusoids (asterisks) containing red blood cells (arrows); D-E) Brain of controls with normal tissue; F) Brain of low-dose treatment group with a small number of cells with shrunk nucleus; G) Brain of high-dose treatment group with a large number of cells with shrunk nucleus (arrows) (Images were taken by light microscopy with 400× magnification)

Effects of nanosilver on the caspase-8 and caspase-9 Levels

In order to investigate the induction of apoptosis, caspase-8 and caspase-9 concentrations were measured in the liver and brain of the pups. As is shown in Fig 4-A, AgNp at the concentration of 100 mg/kg rather than the concentration of 25 mg/kg could increase the level of caspase-9 in the liver (P≤0.001) and brain (P≤0.01) of the treatment groups compared to the controls. However, no statistically significant differences were observed in the caspase-8 levels of the liver and brain between the treatment groups and controls (Fig 4-B).

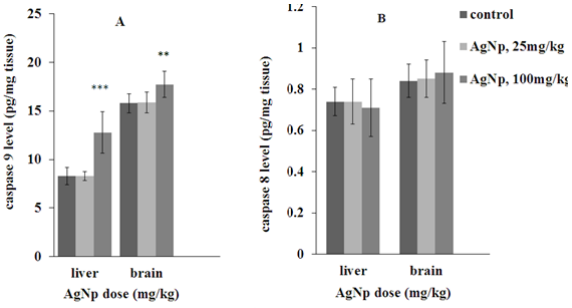

Fig 4. A) Caspase-9 and B) Caspase-8 levels in liver and brain of rat pups after exposure of dams to AgNp during lactation (values expressed as mean±SD; **P<0.01, ***P<0.001: significantly different with controls; n=10)

DISCUSSION

In the present study, the possibility of the transition of nanosilver from breastfeeding to the pups was investigated through the measurement of the silver content in the milk inside the stomach of the pups using the ICP-MS technique. According to the findings, the silver content significantly increased in the milk inside the stomach of the pups (P≤0.001), which confirmed the transmission of silver from the blood-milk barrier.

In a study in this regard, Melnik *et al.* (2013) exposed lactating female rats to nanosilver labeled with the ^{110m}Ag radioactive isotope at a dosage of 1.69-2.21 mg/kg during days 14-16 of lactation, observing the penetration of the ^{110m}Ag-labeled NPs into the maternal milk [17]. In another research, the distribution of nanosilver was observed in breast milk after the intravenous and oral exposure of lactating mice to this nanoparticle [23].

In this regard, Zhang *et al.* (2015) observed titanium nanoparticle accumulation in the mammary glands of lactating mice after intravenous administration through the tail vein at the dosages of 2, 6, and 8 mg/kg. Therefore, it was suggested that the TiO_2 nanoparticle could lead to the oxidative stress-induced disruption of the tight junction by the loss of the tight junction proteins (e.g., ZO-1) and occluding [18]. The tight junctions between the basal (blood) and apical (milk) sides of the alveolar epithelial cells of the mammary glands prevent the direct paracellular exchange of substances between the blood vessels and milk contents [24]. As such, it seems that in the current research, the main cause of silver aggregation in milk was the destruction of the tight junctions in the blood-milk barrier by this

nanoparticle. Furthermore, it has recently been proved that increased blood pressure toward the mammary glands could facilitate the transition of nanoparticles through the blood into these glands [18, 25].

In the current research, the silver content in the liver and brain of the pups was also evaluated, and the findings indicated a significant increase in the silver content of both tissues. Moreover, comparison of the mean data on the silver content indicated that silver was denser in the liver compared to the brain. In line with the results of the present study, Morishita *et al*. (2016) reported the presence of silver in the brain, liver, and lungs of breastfed mice pups after the exposure of maternal milk to AgNp [23]. Moreover, some studies have indicated that liver is the main organ affected by silver nanoparticles, followed by the spleen and lungs although larger silver nanoparticles may aggregate in the spleen more frequently than the other organs [9]. Silver aggregation in the liver is expected since the liver is the first organ to receive the blood from the digestive system, and liver sinusoids are highly permeable to various factors. On the other hand, the blood-brain barrier is a strong barrier against the penetration of various factors into the brain. Therefore, the penetration of silver into the brain in the present study could be due to the direct transmission of nanosilver to the brain [26], destruction of the blood-brain barrier due to the induction of oxidative stress or inflammation by nanosilver [27, 28] or incomplete formation of this barrier in the early stages of growth [19].

In the current research, body weight was observed to decrease, while the reduction was not considered significant, in the pups of the treatment groups compared to the controls. In addition, evaluation of the ratio of the liver and brain weight to the body weight indicated a significant decrease in the ratio of brain weight to the body weight only at the nanosilver concentration of 100 mg/kg ($P \leq 0.05$). Our findings regarding the body weight and ratio of the liver and brain weight to the body weight are consistent with some studies, while inconsistent with some research on toxicity. This could be due to the fact that various elements (e.g., type, shape, size, and charge of nanoparticles and cell type and animal species) affect the type and intensity of toxicity [9]. It is speculated that the weight loss of the brain to body in these pups could be due to widespread cell apoptosis; however, no changes were observed in the ratio of the liver weight to the body weight in this group despite the cell death, which could be attributed to the aggregation of inflammatory cells in cell death sites and hyperemia in the liver sinusoids. To confirm this hypothesis, the histopathological changes in the liver and brain of the pups were evaluated. According to the results, cell apoptosis occurred in both tissues of the treatment group receiving 100 mg/kg of nanosilver. Furthermore, the liver slides showed dilated, congested liver sinusoids with hepatocyte disarray and inflammatory cell infiltration into the liver lobules, while the brain slides showed microvacuolar structures among the brain cells in this treatment group, which was the hallmark of neurodegeneration. Therefore, the accumulation of AgNp in the liver and brain may be associated with changes in these tissues.

In two studies, after exposure to nanosilver, bile duct hyperplasia and inflammatory cell infiltration were induced in the liver of the rats due to the accumulation of silver in the liver tissues [29, 30]. In another research, Heydarnejad *et al.* (2014) treated the wounds on the back of rats with bandage containing 50 microliters of nanosilver solution (10 ppm) for 14 days. After the treatment, dilation was observed in the central vein, which was associated with the increased number of Kupffer and inflammatory cells and cell swelling in the liver [31].

In an *in-vitro* study, the effects of AgNp (20 nm) at various concentrations (1, 5, 10, and 50 µg/ml) were examined on the cultured cortical neurons of rats. The results indicated that nanosilver not only reduced the cell viability of neurons and glial cell, but it also induced the degeneration of neuronal processes and inhibited neurite outgrowth through inducing the loss of β-tubulin and filamentous actin [32].

In this regard, Skalska *et al*. (2015) observed ultrastructural changes, such as the enhanced density of synaptic vesicles clustering in the presynaptic segment, blurred synapse structure, and liberation of synaptic vesicles into the neuropils, in the hippocampal region of the brain of rats after oral exposure to silver nanoparticle [33].

Considering the observed changes in the tissues in the current research, the possibility of apoptosis induction was assessed by measuring the levels of caspase-8 and caspase-9. The findings indicated no changes in the concentration of caspase-8 in

the liver and brain of the pups of the treatment groups compared to the controls. However, the level of caspase-9 in the liver and brain of the pups whose maternal milk was exposed to 100 mg/kg of nanosilver increased significantly. The increase in caspase-9 concentration in the present study could represent death cell induction due to apoptosis. However, the invariability of the caspase-8 levels suggested the possible effects of nanosilver on the intrinsic apoptotic pathway rather than the extrinsic pathway.

Apoptosis is regulated by two different pathways. In the extrinsic pathway, caspase-8 is the initiator protease, while in the intrinsic pathway, caspase-9 is the initiator protease, which activates the cascade of the effector caspases, thereby leading to apoptosis induction [13].

In the present study, the interaction of nanosilver with the thiol group of antioxidant defense system was probable [34], especially with glutathione (GSH), which could lead to the depletion of this factor, thereby inducing ROS generation. ROS accumulation in cells leads to the destruction of the mitochondrial membrane, cytochrome C release, and activation of caspase-9, which is the initiator protease in the intrinsic pathway.

In a study in this regard, we exposed pregnant rats to nanosilver (20 nm) at the concentration of 25 mg/kg and observed oxidative stress induction, along with the reduced GSH level, glutathione peroxidase activity, and increased malondialdehyde concentration. In addition, despite the increase in the caspase-9 level, no changes were observed in the level of caspase-8 in the brain of the offspring [35].

Using a confocal microscope, Hsin et al. (2008) examined the release of cytochrome C and connection of *Bax* to the mitochondria in NIH3T3 cell fibroblasts after incubation with nanosilver [36]. In another research, Cha et al. (2008) observed a significant increase in the expression of the genes related to apoptosis via RNA microarray analysis in the liver cells three days after nanosilver exposure (13 nm) in rats [27]. Moreover, Zhu *et al*. (2016) investigated the effects of silver nanoparticles on human hepatocellular carcinoma HePG-2 cells, reporting the inhibition of cell proliferation through apoptosis induction with caspase-3 activation and DNA damage-mediated *p53* phosphorylation [37]. In another study, cultured cortical neurons were exposed to nanosilver (0.4, 2, and 10 µg/ml) for six, 12, and 24 hours. In the mentioned research, apoptosis induction was performed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and a DNA ladder assay, and the expression of caspase-3 was observed to increase in a time-dependent manner [11].

Govender *et al.* (2013) treated the lung carcinoma cell line using the silver nanoparticles synthesized from *Albizia adianthifolia* overnight, reporting a reduction in cell viability. The results of the mentioned study also demonstrated 2.5 times reduction in the activity of caspase-8, 1.8 times increase in the activity of caspase-3 and caspase-7, and 1.4 times increase in the activity of caspase-9. Furthermore, the expression of *p53*, *Bax*, and *PARP-1* was reported to increase, which caused death cell induction [8]. These findings confirm that silver nanoparticles could induce apoptosis *via* the intrinsic pathway.

CONCLUSION

According to the results, nanosilver could cross the milk-blood barrier and penetrate into the maternal milk and body of suckling pups. This nanoparticle could also aggregate in the liver or even the brain, and the aggregation of nanosilver in these tissues could cause apoptosis induction *via* the intrinsic pathway. It is recommended that further investigation be conducted in this regard in order to determine the other influential factors in apoptosis and confirm these results.

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