Original Article

Induced adverse effects of prenatal exposure to silver nanoparticles on neurobehavioral development of offspring of mice

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ABSTRACT — Steadily increased use of silver nanoparticles (Ag-NPs), has increased the amount of its exposure to humans and animals. Current scarce knowledge about the influences of prenatal exposure to Ag-NPs on postnatal outcomes, motivated us to investigate whether being exposed to it during pregnancy has any effects on neurobehavioral development of the adult offspring. Thirty virgin female NMRI mice were mated and treated subcutaneously once every three days from gestation day 3 until delivery, by 0, 0.2 and 2 mg/kg of bodyweight (BW) of Ag-NPs. Behavioral functions of adult offspring including spatial memory, passive avoidance learning, stress, anxiety-like behaviors and locomotor activities were assessed by commonly used neurobehavioral paradigms and the results were compared according to treatment and sex. Prenatal exposure to Ag-NPs significantly impaired their cognitive behavior in the Morris water maze. Although no evidence was observed indicating more anxiety-like behaviors in the treated offspring in the elevated plus maze, the number of defecations and leanings in the open field assay and number of passages in the light-dark box were greater in groups prenatally treated by Ag-NPs. Most of the impairments were more apparent in the offspring which had been prenatally exposed to high doses of Ag-NPs, particularly female ones. The present study indicated that the exposure of pregnant animals to Ag-NPs may lead to various neurobehavioral disorders in their offspring. Thus, more attention should be paid to avoid exposure to Ag-NPs, especially from pregnant females.

Key words: Silver nanoparticle, Prenatal exposure, Neurobehavior, Mice

INTRODUCTION

Developments in nanotechnology have led to a huge growth in application of nanomaterials, which has increased the risk of human exposure to them (Kisin *et al.*, 2007). Silver nanoparticles (Ag-NPs) are one of the most used nanomaterials (Chen and Schluesener, 2008). Silver and Ag-NPs are widely used in consumable and health care products (Chen and Schluesener, 2008; Edwards-Jones, 2009). Increase in application of Ag-NPs has intensified their environmental exposure.

Subcutaneously administered Ag-NPs will translocate into the circulation and get distributed throughout the main organs; including kidneys, liver, spleen, brain and lungs (Tang *et al.*, 2009). Ag-NPs residue could last for more than 4 months in the body of mice and they have the abil-

ity to cross the placental barrier and accumulate in fetuses (Wang *et al.*, 2013). Lee *et al.* (2012) detected Ag-NPs in various organs, including the brain of 4 day-old pups following oral administration to pregnant dams. Subcutaneously injected Ag-NPs can cross the blood-brain barrier (BBB) and traverse the brain in the form of particles and accumulate in the brain over a long period of time (Tang *et al.*, 2008). On the other hand, Ag-NPs lead to BBB inflammation and increase of permeability and, they induce brain toxicity (Tang *et al.*, 2009, 2010; Trickler *et al.*, 2010). Accumulation of Ag-NPs in the brain of pup rats, following the prenatal exposure to them, has been reported before (Fatemi *et al.*, 2013). It seems that nano-sized materials penetrate into fetal brains with more ease (Shimizu *et al.*, 2009; Umezawa *et al.*, 2012). Furthermore, some neurological disorders that become appar-

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ent after birth have their origin in fetal life (Kumar *et al.*, 2011; Kenna *et al.*, 2011).

Prenatal exposure to nanoparticles may impact the neurobehavioral development of offspring (Cui *et al.*, 2014; Mohammadipour *et al.*, 2014). Oxidative stress and inflammation which occur following the passage of nanoparticles from circulation to placenta, endometrium, yolk sac, or fetus, lead to placental dysfunction, retarded neonatal growth, fetal malformations, and neurotoxicity in offspring; furthermore inflammatory cytokines that are induced by nanoparticles in the dam, following their entrance, will affect the brain development of fetuses (Li *et al.*, 2014). It is believed that Ag-NPs mediate their detrimental effects primarily through producing oxidative stress (Rahman *et al.*, 2009; Park *et al.*, 2010; Kim *et al.*, 2014). Collectively, Ag-NPs may produce neurotoxicity by inducing oxidative stress, altering gene expression and producing apoptosis (Rahman *et al.*, 2009; Yin *et al.*, 2013; Xu *et al.*, 2015). Since the biological half-life of silver in the central nervous system (CNS) is longer than in other organs, exposure to Ag-NPs may induce severe pathological consequences in the mammalian brain (Liu *et al.*, 2009). Overall, the above mentioned effects may eventually lead to maternal and embryonic toxicity.

Despite the fact that many studies have been conducted worldwide to investigate the effects of Ag-NPs, their developmental effects have not yet been fully examined, especially the neurobehavioral development of offspring and its different effects on different sexes. Therefore, this study was designed to evaluate the influences of prenatal exposure to Ag-NPs on neurobehavioral developments of mouse offspring and to compare the severity of its possible effects between sexes by employing certain neurobehavioral paradigms such as Morris Water Maze (MWM), Passive Avoidance Learning (PAL), Elevated Plus Maze (EPM), Light-Dark Box (LDB), and Open Field (OF). This complex set of various organized tests enabled us to evaluate the influences of prenatal exposure to Ag-NPs on neurobehavioral functions of offspring in widespread aspects including spatial memory, passive avoidance learning, stress, anxiety-like behaviors, and locomotor activities.

MATERIALS AND METHODS

Nanoparticle

Two prepared solutions, one without nanoparticles and the other containing stabilized suspended Ag-NPs with a concentration of 1,000 mg/mL and purity of 99.9% were purchased from Neutrino Corporation (Tehran, Iran). The nanoparticles had been prepared by the sol-gel method in

which silver ions were reduced by sodium borohydride in the presence of citrate as stabilizer. Different concentrations of the Ag-NPs were prepared by adding the vehicle to the stock suspension. The Ag-NPs morphology was studied by a scanning electron microscope (SEM) (LEO 1455 VP, Firma Zeiss, Oberkochen, Germany), and their sizes were measured by counting one hundred Ag-NPs in SEM photographs (Fig. 1).

Animals

Six- to seven-week-old NMRI mice (30 virgin females and 10 males), were purchased from the Laboratory of Animal Facility of Jundishapur University of Ahvaz, Iran. Male and female mice were housed separately under controlled room temperatures ($23 \pm 2^\circ\text{C}$), with 12:12 hr light/dark cycle. Animals had *ad libitum* access to normal pellet diet (Pars Animal Feed Co, Tehran, Iran) and tap water.

After one week of acclimatization, the female mice were divided in three groups ($n = 10$ in each group): Control, NP0.2 and NP2 that subcutaneously received equal volumes of 0.2 mL of sodium citrate suspension (0.1 M) which contained 0, 0.2 and 2 mg/kg of bodyweight (BW) Ag-NPs, respectively. These doses of Ag-NPs which were selected according to a study conducted by Austin *et al.* (2012) were to transfer to most of the maternal organs, extra-embryonic tissues and embryos. Since the absence of any external anomalies is the hallmark of a true neurobehavioral teratogen (Powers *et al.*, 2011a), these doses were checked in a preliminary study in pregnant mice to avoid any malformation which may take place because of the administrated Ag-NPs. Since nanomaterials have this tendency to aggregate at higher concentrations in biological situations, which may reduce their bioavailability (Braydich-Stolle *et al.*, 2005; Oberdörster *et al.*, 2010; Puvanakrishnan *et al.*, 2012) and decrease the occurrence of malformations, we used low doses of Ag-NPs. On the other hand, subcutaneous injection delays the spread and clearance of nanoparticles, which would minimize the amount of animal handling during pregnancy. Administration of Ag-NPs was only done in female mice from gestation day 3 and repeated once every three days until parturition. Vaginal plug detection was considered the day 0 of pregnancy. Finally, 20 offspring including 10 males and 10 females were randomly selected from different colonies of each group for behavioral tests. All experiments were done on adult offspring. The used protocol and animal ethics were approved by the Research Council of Veterinary Medicine Faculty of Shahid Chamran University of Ahvaz, Iran.

Morris water maze (MWM)

The spatial learning and memory ability of offspring were tested by MWM when they were 45 to 49 days old. Briefly, the system included a circular pool (height 50 cm, diameter 150 cm) which was filled with water ($24 \pm 1^\circ\text{C}$) to a depth of 25 cm and a video tracking device captured the navigational pathway by a computerized Software program (Borj Sanat Co, Tehran, Iran). A hidden square platform (10 cm in diameter) was placed in the center of the southwest quadrant, submerged 1 cm below the surface of the water. The maze was geographically divided into four quadrants; northeast (NE), northwest (NW), southeast (SE) and southwest (SW), and four starting positions including north (N), south (S), east (E) and west (W), which were equally spaced around the perimeter of the pool. In the training trials, all the mice experienced a daily session of four trials for four consecutive days and the swim paths of every mice was recorded for 90 sec. During the training trials, swim speed, escape latency and distance traveled to reach the hidden platform were recorded by the software. In the probe trial which was 24 hr after the end of the training trials, spatial memory of the mice was evaluated in the maze with no platform. At this point, mice's activity was recorded for 60 sec to compute the percentages of the distance traveled and the time spent in the target quadrant as measures of retention and memory performance of the mice (Wang *et al.*, 2014).

Passive avoidance learning (PAL)

The PAL of offspring was evaluated when they were 53 to 54 days old. Passive avoidance apparatus (Shuttle box) consisted of a stimulator (Borj Sanat Co) and two plexiglass chambers ($20 \times 20 \times 20$ cm) which were connected to each other by a guillotine door (5×5 cm). The floor of the dark chamber ($20 \times 20 \times 20$ cm) was composed of 20 parallel stainless steel rods, 2 mm in diameter, which were spaced 0.5 cm apart and were used to deliver a foot shock. The apparatus was cleaned with ethanol (70%) after each test. Thirty minutes prior to a training trial, each mouse was given a two-stage adaptation, with 2-minute intervals. Thirty minutes after the second adaptation stage, the mouse was gently placed in the light chamber for the training trial, and the door between the two chambers was opened 5 sec later. Every time the mouse entered the dark chamber, the door would be closed and an electric foot shock (0.1 mA) was to be delivered to it through the stainless steel rods for 2 sec. This process was to be repeated again every 2 min, until the delay before entering the dark chamber reached to 120 sec. Twenty-four hours after the training trial, the mouse was placed again in the light chamber

for a retention trial to record step-through latency and the time spent in the dark chamber as a measure of retention performance. The step-through latency and the time spent in the dark chamber were recorded to a maximum of 300 sec (Shahidi *et al.*, 2004).

Elevated plus maze (EPM)

Anxiety-like behavior of offspring was evaluated when they were 56 days old. The EPM is comprised of two open arms ($30 \times 5 \times 0.25$ cm) and two closed arms ($30 \times 5 \times 15$ cm), which are connected by a common 5×5 cm central square. Mice were placed in the central square, facing an open arm and were allowed to explore the apparatus for 5 min. The maze was cleaned with ethanol (70%) after each test. A video tracking device captured mice's movement pathways by a computerized Software program (Borj Sanat Co) and measured: a) total time spent in open arms, b) total time spent in closed arms, c) number of entries into open arms and d) number of entries into closed arms. Finally, the following parameters were calculated: 1) percentage of time spent in the open arms relative to the total time spent in the maze 2) percentage of entries into open arms relative to the total number of entries into any arms (Fujimoto *et al.*, 2013).

Light-dark box (LDB)

Anxiety-like behavior and stress-like behavior of offspring were evaluated when they were 58 days old. The LDB test is based on the innate aversion of rodents from illuminated areas and, on the spontaneous exploratory behavior of them in response to mild stressors, that is, novel environment and light (Crawley and Goodwin, 1980). The apparatus consists of two light and dark plexiglass chambers ($20 \times 20 \times 20$ cm) which are connected to each other by a 5×5 opening, and was cleaned with ethanol (70%) after each test. Each mouse was placed individually in the light chamber and recordings were made over a 5-min period. A video tracking device captured the movements of the mice in the light chamber by a computerized Software program (Borj Sanat Co), counting the time spent in the light chamber and the number of passages between the two light and dark chambers (Clément *et al.*, 2007). In the LDB test, the percentage of time spent in the light chamber is influenced by two conflicting desires: trying to avoid the brightly lit chamber, and seeking for a new environment (Wahlsten, 2011). Mice which are low in anxiety tend to discover the environment more completely and make many passages (Wahlsten, 2011).

Open field (OF)

Offspring were tested in the OF when they were 60 days old. The OF was a wooden box ($80 \times 80 \times 50$ cm). Mice were continuously filmed for 5 min with a video tracking device (Borj Sanat Co) which could capture and analyze locomotion via a computerized system. The OF arena was subdivided into two squares; a large square (80×80 cm) for assaying peripheral locomotion, and a small square (50×50 cm) in the center of the large square to assay central locomotion. Total, peripheral, and central locomotions were recorded and analyzed by the system's software program. The number of leanings (one or two paws in contact with the wall), rearings (the mouse standing on its two hind paws without touching the walls), grooming (face cleaning, paw licking, fur licking, head scraping, and rubbing), and defecations were also recorded as we were watching the video recordings from the next room. At the end of each test, the whole area was cleaned with 70% ethanol (Pardon *et al.*, 2000). Total locomotion and difference in the distances that subjects kept from the center of the OF can be used as preliminary indicators of anxiety, as highly anxious mice tend to avoid the center of an OF (Crawley and Paylor, 1997). Leaning is an indicator of anxiety-related behavior (Lipkind *et al.*, 2004). Grooming is considered as an adaptational behavior to stressful conditions (Kametani, 1988). It consists of a number of voluntary movements which may be decreased during intense excitation (Barros *et al.*, 1994).

Bodyweight and brain weight

Bodyweight and brain weight of 5 male and 5 female offspring that were selected randomly from each group were measured at the end of the study.

Data analyses

Data were statistically analyzed by the software SPSS, version 16 (SPSS; Chicago, IL, USA). In the MWM test, traveled distance, the escape latency to reach the hidden platform and swim speed were each analyzed using repeated-measures analysis while considering treatment (control, NP0.2 and NP2) and sex (male, female) as between-subject factors and the training session (1-4) as the within-subject factor. Other variables were compared using the two-way ANOVA with considering treatment and sex as fixed factors. If the response to treatment was statistically sex dependent, the one way analysis of variance was used to compare the effects of treatment inside the two subgroups of males and females, while each contained three treatment groups. When an effect was statistically significant ($p < 0.05$), means of the groups were

compared by the Least Significant Difference (LSD) multiple comparison test.

RESULTS

The used Ag-NPs were spherical and their diameters were 32 ± 6.6 nm (Fig. 1). The size distribution of Ag-NPs was 5-70 nm.

MWM

Statistical analyses of the data obtained during the four consecutive training trials by repeated measures, showed that the performance of mice were improved during training sessions regardless of treatment or sex [$(F_{3,156} = 24.12$; $P = 0.000$, for traveled distance) and $(F_{3,156} = 28.69$; $P = 0.000$, for escape latency)]; but the trend of the changes were statistically different [session $\times$ treatment: $(F_{6,156} = 2.788$; $P = 0.013$, for traveled distance) and $(F_{6,156} = 2.25$; $P = 0.045$, for escape latency), (Figs. 2A and 2B)]. Furthermore, the means of the distances traveled to reach the hidden platform and the escape latency to get to it in the training sessions 1, 2, and 4 were larger in the NP2 group compared to control and the NP0.2 group ($P < 0.05$). The average swim speed was increased during consecutive training sessions ($F_{3,156} = 11.21$; $P = 0.000$), but its trend was not statistically different between the groups [session $\times$ treatment: $(F_{6,156} = 0.670$; $P = 0.674$, Fig. 2C)]. Furthermore, the effects of Ag-NPs were not sex dependent at the training trials [$(F_{3,156} = 0.30$; $P = 0.822$, for traveled distance), $(F_{3,156} = 0.495$; $P = 0.688$, for escape latency) and $(F_{3,156} = 0.499$; $P = 0.718$, for swim speed)].

Data obtained from the probe trial were statistically analyzed by two-way ANOVA (treatment $\times$ sex). Treat-

Fig. 1. A photograph of silver nanoparticles taken by a scanning electron microscope (SEM).

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Fig. 2. Effects of prenatal exposure to 0 (control), 0.2 (NP0.2) and 2 mg/kg (NP2) of Ag-NPs in four consecutive sessions of training trials in the MWM, (A) traveled distance to reach the hidden platform, (B) escape latency to reach the hidden platform, (C) swim speed. Data are represented as mean $\pm$ S.E.M. According to repeated measures test, trend of changes in the distance traveled and the escape latency to reach the hidden platform were significantly different between groups ($P < 0.01$ and $P < 0.05$, respectively). *: indicates the significant differences of the NP2 group compared to control and the NP0.2 groups in each training session (*: $P < 0.05$ and **: $P < 0.01$). There were no significant sex-related effects on the recorded variables of any group. ($n = 20$ in each group, which means 10 males and 10 females).

ment regardless of sex, had a significant effect on the performance of mice [$(F_{2,52} = 3.40; P = 0.041$, for the percentage of traveled distance in the target quadrant) and ($F_{2,52} = 3.19; P = 0.049$, for the percentage of time spent in the target quadrant)]. The percentages of the distance traveled and the time spent in the target quadrant were statistically lower in the NP2 group compared to control ($P < 0.05$, Figs. 3A and 3B). An important difference between the effect of Ag-NPs on male and female offspring was observed [treatment $\times$ sex: ($F_{2,52} = 6.41; P = 0.003$, for the percentage of traveled distance in the target quadrant) and ($F_{2,52} = 6.28; P = 0.004$ for the percentage of time spent in the target quadrant)]. Comparison of the three male groups using ANOVA revealed that there were no significant differences in their performance

at the probe trial [$(F_{2,25} = 2.26; P = 0.129$, for the percentage of traveled distance in the target quadrant) and ($F_{2,25} = 1.13; P = 0.339$, for the percentage of time spent in the target quadrant)], although these values were significantly different between female groups [$(F_{2,27} = 7.71; P = 0.002$, for the percentage of traveled distance in the target quadrant) and ($F_{2,52} = 7.57; P = 0.002$, for the percentage of time spent in the target quadrant)]. LSD post hoc showed that percentages of traveled distance in the target quadrant and time spent in the target quadrant were significantly lower in female NP2 and NP0.2 groups compared to female control ($P < 0.01$ and $P < 0.05$, respectively; Fig. 3). These results show that the performance of Ag-NPs treated groups was decreased dose dependently at the probe trial, which suggests that the offspring's learn-

Fig. 3. Effects of prenatal exposure to 0 (control), 0.2 (NP0.2) and 2 mg/kg (NP2) of Ag-NPs on mice's behavior in the probe trial of the MWM, (A) percentage of distance traveled in the target quadrant, (B) percentage of time spent in the target quadrant. Data are represented as mean $\pm$ S.E.M. *: indicates the significant differences compared to the control group (*: $p < 0.05$; **: $P < 0.01$). Since the effects of Ag-NPs were sex dependent ($P < 0.01$), the data related to males and females were analyzed separately ($n = 20$ in each group, including 10 males and 10 females).

Fig. 4. Effects of prenatal exposure to 0 (control), 0.2 (NP0.2) and 2 mg/kg (NP2) of Ag-NPs on mice's behavior in the PAL, (A) the number of shocks at the training trials, (B) step-through latency and time spent in the dark chamber at the retention trial. Data are represented as mean $\pm$ S.E.M. There were no significant sex-related effects on recorded variables of any group. ($n = 20$ in each group, consisted of 10 males and 10 females).

ing and memory were impaired, due to prenatal exposure to Ag-NPs.

PAL

Analyses of data obtained from PAL by two-way ANOVA (treatment $\times$ sex) showed that Ag-NPs, regardless of sex, had no effect on the variables measured in passive avoidance learning ($(F_{2,54} = 1.15$; $P = 0.325$, for the number of shocks at the training trial), ($F_{2,54} = 0.01$; $P = 0.994$ for the step-through latency time) and ($F_{2,54} = 2.735$; $P = 0.074$, for the time spent in the dark chamber);

(Figs. 4A and 4B)]. Furthermore, the responses to the treatment were not sex dependent [treatment $\times$ sex: ($F_{2,54} = 1.15$; $P = 0.325$, for the number of shocks at the training trial), ($F_{2,54} = 0.50$; $P = 0.609$, for the step-through latency time) and ($F_{2,54} = 0.003$; $P = 0.997$, for the time spent in the dark chamber)].

EPM

Analyses of data obtained from the EPM by two-way ANOVA (treatment $\times$ sex) showed that Ag-NPs, regardless of sex, had no significant effects on the behavior of

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Fig. 5. Effects of prenatal exposure to 0 (control), 0.2 (NP0.2) and 2 mg/kg (NP2) of Ag-NPs on mice's behavior in the EPM, (A) percentage of time spent in open arms and percentage of open arm entries, (B) number of total arm entries. Data are represented as mean $\pm$ S.E.M. There were no significant sex-related effects on recorded variables of any group. (n = 20 in each group, including 10 males and 10 females).

Fig. 6. Effects of prenatal exposure to 0 (control), 0.2 (NP0.2) and 2 mg/kg (NP2) of Ag-NPs on mice's behavior in the LDB, (A) time spent in the light or dark chambers, (B) number of passages between the two chambers. Data are represented as mean $\pm$ S.E.M. *: indicates the significant differences from the control group, (p < 0.05). There were no significant sex-related effects on recorded variables of any group. (n = 20 in each group, including 10 males and 10 females).

mice in EPM [$(F_{2,54} = 0.02; P = 0.983$, for the percentage of time spent in the open arms), ($F_{2,54} = 0.804; P = 0.453$, for the percentage of open arm entries) and ($F_{2,54} = 0.017; P = 0.983$, for the number of total arm entries; Figs. 5A and 5B)]. Furthermore, responses to the treatment were not sex dependent [treatment $\times$ sex: ($F_{2,54} = 0.351; P = 0.706$, for the percentage of time spent in the open arms), ($F_{2,54} = 0.461; P = 0.633$, for percentage of open arm entries) and ($F_{2,54} = 0.176; P = 0.839$, for number of total arm entries)].

LDB

Analyzing the data obtained from LDB by two-way ANOVA (treatment $\times$ sex) showed that Ag-NPs, regardless of sex, had no significant effects on the time spent in the light chamber ($F_{2,54} = 0.818; P = 0.447$), but had a significant effect on the number of passages between the two chambers ($F_{2,54} = 3.84; P = 0.028$), so that it was significantly lower in the NP2 group compared to the control (p < 0.05, Figs. 6A and 6B). Responses to the treatment were also not sex dependent [treatment $\times$ sex: ($F_{2,54} = 0.351; P = 0.214; P = 0.808$, for the time spent in

Fig. 7. Effects of prenatal exposure to 0 (control), 0.2 (NP0.2) and 2 mg/kg (NP2) of Ag-NPs on mice's behavior in the OF, (A) peripheral, central and total locomotion, (B) percentage of central locomotion, (C) locomotion speed. Data are represented as mean $\pm$ S.E.M. There were no significant sex-related effects on recorded variables of any group. ($n = 20$ in each group, i.e. 10 males and 10 females).

the light chamber) and ($F_{2,54} = 0.607$, for the number of passages between the two chambers)].

OF

Analyses of the data obtained from OF by two-way ANOVA (treatment $\times$ sex) revealed that Ag-NPs, regardless of sex, had no significant effects on locomotion [($F_{2,54} = 2.509$; $P = 0.091$, for total locomotion), ($F_{2,54} = 0.79$; $P = 0.459$, for the percentage of central locomotion), and ($F_{2,54} = 2.505$; $P = 0.091$, for locomotion speed); Fig. 7] and it was not influenced by sex [treatment $\times$ sex: ($F_{2,54} = 0.479$; $P = 0.622$, for total locomotion), ($F_{2,54} = 1.89$; $P = 0.16$, for the percentage of central locomotion), and ($F_{2,54} = 0.478$; $P = 0.623$, for locomotion speed). Furthermore, the number of rearings and groomings were not significantly influenced by Ag-NPs treatment [($F_{2,54} = 2.701$; $P = 0.077$, for the number of rearings) and ($F_{2,54} = 0.005$; $P = 0.994$, for the number of groomings)], and there were

no particular influences from different sexes [(treatment $\times$ sex: $F_{2,54} = 1.864$; $P = 0.166$, for the number of rearings) and ($F_{2,54} = 2.862$; $P = 0.067$, for the number of groomings); Table 1]. But the numbers for leaning and defecation were significantly different between groups [($F_{2,54} = 3.216$; $P = 0.049$, for the number of leanings) and ($F_{2,54} = 4.380$; $P = 0.018$, for the number of defecations)], and they were greater in the NP2 group compared to the control group ($P < 0.05$, Table 1). These results were not sex dependent for defecation (treatment $\times$ sex: $F_{2,54} = 1.230$; $P = 0.301$) but the effect of prenatal Ag-NPs treatment on leaning was different between the two sexes (treatment $\times$ sex: $F_{2,54} = 5.491$; $P = 0.007$), in a way that the number of leanings was increased significantly, only in the female NP2 group ($P < 0.01$, Table 1).

Bodyweight and brain weight

Analyses of the data obtained from measuring body-

Table 1. Effects of prenatal exposure to Ag-NPs on the numbers of defecation, leaning, rearing and grooming of offspring in the OF test.

Open Field	♂+♀			♂			♀		
Parameters	Control	NP0.2	NP2	Control	NP0.2	NP2	Control	NP0.2	NP2
Defecation number	1.1 ± 0.4	1.7 ± 0.3	2.7 ± 0.3 [#]	0.4 ± 0.6	1.5 ± 0.5	2.8 ± 0.5	1.9 ± 0.6	1.8 ± 0.5	2.6 ± 0.5
Leaning number**	18 ± 2.7	22.8 ± 2.4	27.1 ± 2.4 [#]	14.6 ± 3.8	19.5 ± 3.4	14 ± 3.4	21.4 ± 3.8	26 ± 3.4	40.3 ± 3.4 ^{ss}
Rearing number	4.1 ± 0.6	2.2 ± 0.5	3 ± 0.5	5 ± 0.9	1.5 ± 0.8	3 ± 0.8	3.1 ± 0.9	2.8 ± 0.8	3 ± 0.8
Grooming number	2.8 ± 0.45	2.7 ± 0.4	2.8 ± 0.4	3.6 ± 0.6	3.7 ± 0.6	2.5 ± 0.6	1.9 ± 0.6	1.7 ± 0.6	3 ± 0.6

Data are represented as mean ± S.E.M. #: indicates the significant differences between groups ($p < 0.05$); *: indicates any significant effect of sex on treatment responses (**: $p < 0.01$) ^s: indicates the significant differences within female groups (ss : $p < 0.01$). Assessment of significant differences within the male and female groups was done separately, only if sex had any significant effects on treatment response. (n = 20 in each group, i.e. 10 males and 10 females).

Table 2. Effects of prenatal exposure to Ag-NPs on bodyweight and brain weight of the offspring.

Groups (♂+♀)	Control	NP0.2	NP2
Bodyweight (g)	34.5 ± 0.8	35.1 ± 0.8	35.3 ± 0.8
Brain weight (mg)	514 ± 5	529 ± 8	539 ± 6

Data are represented as mean ± S.E.M. No significant differences were detected. (n = 10 in each group, i.e. 5 males and 5 females).

weight and brain weight, by two-way ANOVA (treatment × sex) revealed that Ag-NPs without respect to the sex, had no significant effects neither on the weight of the body ($F_{2,24} = 0.246$; $P = 0.784$) nor the brain ($F_{2,24} = 3.296$; $P = 0.055$). Sex also had no effects on the response to the treatment [treatment × sex: ($F_{2,24} = 0.246$; $P = 0.784$, for bodyweight) and ($F_{2,24} = 0.697$; $P = 0.508$, for brain weight) Table 2)].

DISCUSSION

In our study, no external anomalies or growth retardation were observed in the offspring that were subcutaneously exposed to Ag-NPs before birth. Subcutaneously exposure of dams for evaluation of nanoparticles' effects on the brain of the offspring, has been used by other researchers including Shimizu *et al.* (2009), Takahashi *et al.* (2010), Umezawa *et al.* (2012), Okada *et al.* (2013), and Cui *et al.* (2014). Moreover, Ag-NPs can cross the blood-brain and placental barriers and accumulate in the brain and fetal organs (Tang *et al.*, 2008; Lee *et al.*, 2012; Wang *et al.*, 2013; Fatemi *et al.*, 2013). We used Ag-NPs with the diameter of 32 nm, because smaller sizes have more toxic effects with respect to permeating and damaging cerebral microvascular structures, in comparison with

larger particles (Trickler *et al.*, 2010). However the placental damage caused by nanoparticles may potentially lead to developmental defects or retardation of the fetus (Sun *et al.*, 2013).

Ag-NPs significantly impaired the performance of treated offspring in a dose dependent manner in the MWM. Although no evidence was observed indicating more anxiety-like behaviors in the treated offspring in the EPM, the number of defecations and leanings in the OF assay and number of passages in the LDB were increased. It seems like fetuses can be affected in different ways; directly by particle transport through the placenta, disorder in the functions of placenta or indirectly, by circulating cytokines or other substances produced from inflammatory processes in the mother (Hougaard *et al.*, 2011; Li *et al.*, 2014).

To the best of our knowledge, there is no information related to the effects of prenatal exposure to Ag-NPs on behavioral performance of the offspring. Hritcu *et al.* (2011) have reported that exposure to Ag-NPs significantly affects short-term memory. It has been reported that exposure to Ag-NPs in rats causes spatial memory impairments in the MWM test by increasing the reactive oxygen species (ROS) in hippocampal homogenate (Liu *et al.*, 2012), while some others reported that exposure to Ag-NPs does not affect cognitive functions (Sharma and Sharma, 2007; Sharma *et al.*, 2009; Liu *et al.*, 2013). "ROS-mediated oxidative stress is an important factor for Ag-NPs-induced apoptosis in cell culture (Kim *et al.*, 2014)". This is while Skalska *et al.* (2014), and Xu *et al.* (2013) reported that Ag-NPs lead to more severe synaptic degeneration, mainly in the hippocampal region of the brain which will cause the impairment of cognitive processes in adult rats. However, calcium imbalance, destabilization of mitochondrial functions and ROS production and consequently, activation of N-methyl-d-

aspartate (NMDA) receptors are predicted mechanisms for the neurotoxicity induced by Ag-NPs (Ziemińska *et al.*, 2014). We did not witness any significant variations in swim speed at the training trials which is indicative of normal physical activity of the Ag-NPs treated groups in the MWM.

There were no significant differences in the performance of any group in the PAL. However, interpretation of PAL results should be with caution because changes in the pain threshold of the subjects may affect the way that the tasks involving foot shock are performed (Crawley and Paylor, 1997).

Absence of any obvious differences in locomotion in the OF arena between the Ag-NPs treated groups and the control group in the present study suggests that Ag-NPs could not induce adverse effects on locomotor activities. More leanings and defecations in the NP2 group and sex differences in the effects that Ag-NPs had on leaning, suggest more stress and anxiety in the Ag-NPs treated groups, especially in females compared to the control group. Although number of passages of the NP2 group was lower in the LDB test, there were no significant differences in the total time spent in the light chamber. In our study, the LDB findings are in accordance with the findings from the OF, however, we did not find any sign of anxiety in the EPM.

Ag-NPs can impair cell functions and cause brain damage (Tang *et al.*, 2008; Fatemi *et al.*, 2013; Huang *et al.*, 2015), which may lead to cell death of certain cells, such as hippocampal neurons (Liu *et al.*, 2012; Ataei and Ebrahimzadeh-bideskan, 2014; Skalska *et al.*, 2014). It is shown that Ag-NPs and ionic silver impair neuronal cell differentiation and their capability to produce dopamine (Powers *et al.*, 2010, 2011b). It has been reported that dopamine has had been increased in the brain of rats, following oral administration of Ag-NPs and silver ion in the form of silver acetate; furthermore Ag-NPs have increased serotonin (5-hydroxytryptamine; 5-HT) level (Hadrup *et al.*, 2012). Visuospatial tasks are dependent on activation of dopamine circuitry in the hippocampus and midbrain of rodents and non-human primates (Tran *et al.*, 2008), especially when learning is triggered by reward or punishment (Schultz, 2010). The 5-HT also plays a critical role in reward-based learning (Kranz *et al.*, 2010). Thus, it can be deduced that Ag-NPs might have detrimental effects on the CNS development which ultimately influences neurobehavioral functions including spatial memory, passive avoidance learning, stress, anxiety-like behaviors, and locomotor activities.

Prenatal exposure to Ag-NPs carries the potential to induce oxidative stress in the brain of newborn rats

(Fatemi *et al.*, 2013), as well as the ability to increase ROS formation in the caudate, frontal cortex, and hippocampus of mice via the changes it induces in the expression of the genes involved in antioxidant defense (Rahman *et al.*, 2009). Oxidative stress leads to DNA damage and apoptosis (Park *et al.*, 2010; Liu *et al.*, 2012; Kim *et al.*, 2014). The observed toxicity of Ag-NPs may be due to the intrinsically toxic nature of Ag-NPs, release of the Ag⁺ ions or a combination of both (Johnston *et al.*, 2010; Liu and Hurt, 2010).

Some of the effects of prenatal exposure to Ag-NPs in our study were more serious in the female offspring. It has been shown that sexual dimorphisms occur in different neurotransmitter systems, including 5-HT, dopamine, noradrenaline, GABA (γ -amino butyric acid), acetylcholine, vasopressin, and opioids (Madeira and Lieberman, 1995; Craft, 2003; Rhodes *et al.*, 2009), as well as sex differences which arise during development (Galanopoulou, 2005). These results indicate that sex differences in brain neurochemistry are much greater than what is thought to be (Cahill, 2006). These differences may be responsible for sex-dependent effects. However several studies have shown prenatal exposure to various types of nanoparticles (nanosized titanium dioxide, diesel exhaust particles and carbon black particles) is related to behavioral defects in locomotor activity and patterns of habituation of females, a lot more than males (Hougaard *et al.*, 2008, 2010; Jackson *et al.*, 2011).

Lack of any differences in bodyweight or brain weight of the offspring was indicative of normal growth in the Ag-NPs treated groups. However, Fatemi *et al.* (2013) have reported bodyweight reduction and relative brain weight reduction as result of prenatal exposure to 25 mg/kg of Ag-NPs in rats' offspring. Also Zhang *et al.* (2013) reported that Ag-NPs decreased bodyweight and locomotor activity in adult male rats.

Overall, the results of the present study demonstrate that subcutaneous injection of Ag-NPs during pregnancy can induce adverse influences on neurobehavioral development of adult offspring because of the damages it causes to spatial cognition and, increase in stress from being in a novel environment. It seems that some impairments are sex dependent, with greater susceptibility of female offspring. These findings are a warning sign to females who are pregnant or are planning to get pregnant to avoid exposure to Ag-NPs.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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