Maternal Exposure to Silver Nanoparticles in Mice: Effects on dams' Reproductive Performance and Pups' Neurobehavioral Ontogeny

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ABSTRACT

Introduction: - Significant increase in usage of silver nanoparticles (AgNPs) in consumer products, has increased its exposures to human and animals. Although there are some reports regarding the effects of AgNPs on complex organisms, no report was found as to its effects on neurobehavioral ontogeny.

Methods: To investigate the effects of maternal exposure to AgNPs on development of neurobehavioral reflexes as well as physical indexes of growthand development during pre-weaning period, virgin female NMRI mice were treated with zero, 5 and 50 µg/mouse AgNPs. AgNPs were injected subcutaneously (S.C) to female mice, immediately following male exposure and at once every three days until parturition. Reproductive performance of dams were assessed and home cage activity and developmental landmarks of all pups were observed daily. Gross necropsy was performed on the 28th day.

Results: No significant differences were observed in dams' and pups' weight. Survival rate was decreased ($p<0.05$) in NP5 group and a clear hyperactivity was observed in NP50 group. Prenatal exposure to AgNPs delayed the development of the some pups' neurobehavioral reflexes which were supposed to take place during the first four days following birth. The weight of spleen was decreased in AgNPs treated mice and the weight of liver was increased in male offspring who was exposed to AgNPs before birth.

Conclusion: This study revealed that prenatal exposure to AgNPs delayed neurobehavioral development during the early stages of the pre-weaning period. Furthermore, more attention should be paid to alteration of lymphoid organ and liver weights of offspring.

Key Words:
Silver Nanoparticles,
Neonatal Period,
Neurobehavioral,
Ontogeny.

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1. Introduction

Nanoparticles (NPs) have widespread commercial applications and might therefore be released into environment and effect on living things (1, 2). The use of AgNPs in medical applications is quickly expanding due to the beneficial physiochemical features they offer (3, 4). Recently, AgNPs have gained considerable interest in medicine as antimicrobial agent, such as wound dressing. If they will be released from dressings, they also have the potential to cross biological compartments (5). Currently AgNPs are the most widely used engineered nanoparticles (6, 7).

Subcutaneously injected AgNPs will translocate into the circulation and get distributed throughout the main organs; including kidney, liver, spleen, brain and lungs (8). AgNPs may produce toxicity by inducing oxidative stress, altering gene expression, inflammation, and by producing apoptosis (9, 10). AgNPs retention time in the body of mice is more than 4 months and they have the ability to cross the placental barrier and accumulate in fetuses (11). AgNPs have been detected in kidney, liver, lung and brain of 4-day-old pups following the oral administration to pregnant dams (12). 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 (13). It has been reported that prenatal exposure to AgNPs can result in their accumulation in the brain of pup rats (14).

Reproduction and developmental toxicity of AgNPs were assessed by single (15) or repeated (7) oral administration of AgNPs to CD-1 mice (10-1000 mg/kg) and Sprague-Dawley rats (62.5- 250 mg/kg), respectively. None of these studies reached the conclusion that oral exposure to AgNPs during pregnancy has any important toxic effects on dams and fetuses. In general, oral administrations of AgNPs have not shown significant toxicity, even at high doses (16, 17). Although repeated oral doses of AgNPs during days 6-19 of gestation in rats caused oxidative stress in dam hepatic tissues, it did not cause developmental toxicity in fetuses at doses of up to 1000 mg/kg/day (18). Since the relative low toxicity was observed, using orally administered AgNPs may be related to the bioavailability of oral AgNPs treatment. (7) Hong et al. have suggested a direct delivery of AgNPs by injection.

Although single intra peritoneal injection of 0.4 and 0.8 mg/kg of AgNPs to pregnant Wistar rats at 8th or 9th gestational day (GD) had some minor effects on pup's body weight and placental weight, it had no effect on skeletal system development (19). To our knowledge, this is the only study carried out so far to address the reproductive/developmental toxicity following parenteral administration.

Since there are few published reports on the effects of fetal exposure to AgNPs, the primary aim of the present study was to investigate whether subcutaneous exposure to low levels of AgNPs during mating and pregnancy has any effect on the reproductive performance of dams and neurobehavioral milestones of pups. To the best of our knowledge, no systematic research exists to explain if AgNPs alter the neurobehavioral development of exposed pups. In order to evaluate minor neurobehavioral defect, reflex ontogeny were assessed according to the battery of Fox (20), Tsujii and Hoshishima (21) and Tamashiro et al. (22) The assessment indicate developmental disorders even in the absence of teratogenic effects.

2. Materials and Methods

Nanoparticle: prepared solution without nanoparticles and solution which contains stabilized suspended AgNPs with a diameter of 10 nm, concentration of 1000 mg/ml and purity of 99.9% were purchased from Neutrino Corporation (Iran). The nanoparticles have been prepared by sol gel method in which silver ions were reduced by sodium borohydride in the presence of citrate as stabilizer. Different concentrations of the AgNPs were prepared by adding the vehicle to the stock suspension. The AgNPs morphology was studied by transverse electron microscopy (TEM) (LEO-906E, resolution 0.34), and their size were measured by counting one hundred AgNPs in TEM photographs (Figure 1).

Animals: 5-6-week-old NMRI mice (30 virgin female and 10 male), were purchased from Center for Laboratory Animals of Ahvaz Jundishapur University of Medical Sciences. Male and female mice were housed separately in standard plexiglass cages (6 mice/cage), and maintained in controlled room under the temperature of (22 ± 2 ◦C), with 12:12 h light/dark cycle. During the experiment, all animals had ad libitum access to normal pellet diet (Pars Animal Feed Co, Iran) and tap water. After one week of acclimatization, the female mice were divided in three groups (n=10 for each group): Control, NP5 and NP50 groups were injected subcutaneously with equal volumes of 0.2 ml of sodium citrate suspension (0.1 M) which contained

zero, 5 and 50 µg AgNPs, respectively. The minimum doses which transfer to most maternal organs, extra-embryonic tissues and embryos (23) were selected to avoid any malformation which may take place due to AgNPs feto-toxicity. Since the absence of the any external anomalies is the sign of a true neurobehavioral teratogen (24), this matter was checked in a preliminary study. Subcutaneous injection delayed spread and clearance of nanoparticles, which consequently minimized animals handling during pregnancy by reducing injections number. On the day 17, the female ones were also separated from each other and transferred to individual cages to provide enough space before delivery. Each individual cage was inspected at 10 and 16 o'clock in order to record time of parturition and all the pups' tests were recorded between these two intervals. The - next day parturition was considered as the post natal day 1 (PND1).

Reproductive performance of dams: The body weights of dams were measured during pregnancy and lactation period. The litter size, litter weight and pup anomalies were inspected at PND1. The litter size was adjusted in 4-day-old to maximum number of 8, to ensure equal conditions for all colonies. Only one colony in NP5 group with 7 pups was accepted. No pup exchange was done between colonies. The body weights of the pups were taken on PNDs 1, 4, 7, 14 and 21 (weaning).

Sex distributions (number of male pups/number of total pups), gestation index (number of pregnant females with alive pups/total number of females) and post-natal survival rate (number of pups alive on PND4/the number of living pups on PND1) were evaluated using a modified version of the methods of Ratnasooriya et al. (25) as described by Ivani et al. (26).

Neurobehavioral and developmental milestone: All pups of each group were observed daily for their home cage activity and developmental landmarks such as fur development, pinna unfolding, ear opening, eye opening, lower incisor eruption, and descent of testes. The absence and presence of different developmental landmarks were investigated by two blind raters from PND1 to PND25.

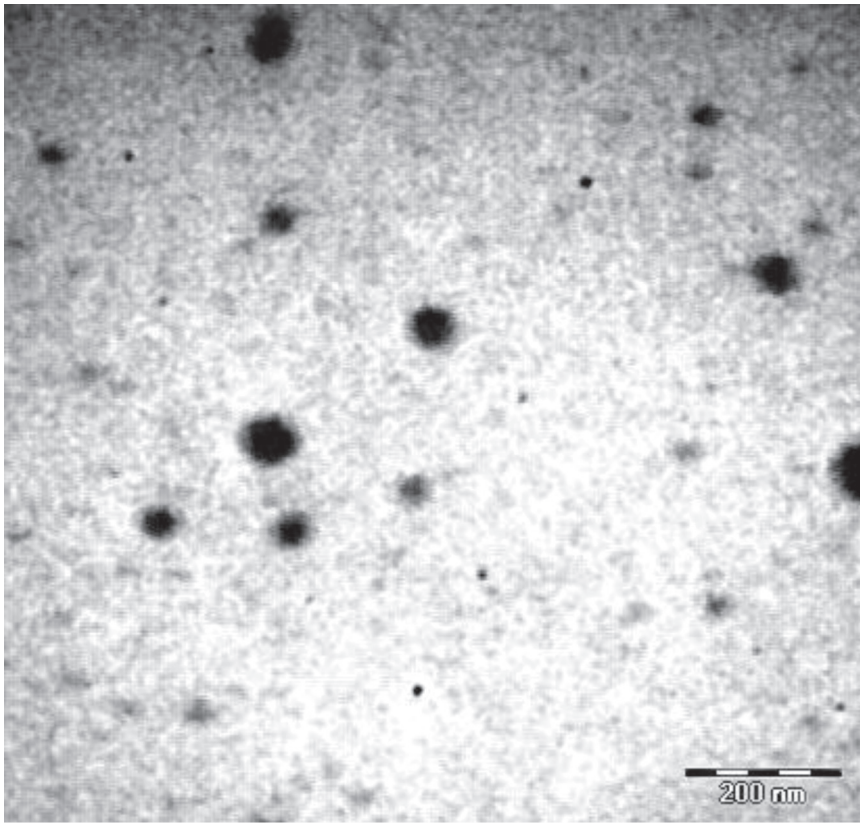

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Figure 1. Transverse electron microscope (TEM; LEO-906E, resolution 0.34) photograph of , specially nano-particle image is so largev. Particles are spherical, with a diameter of 32±6.6 nm.

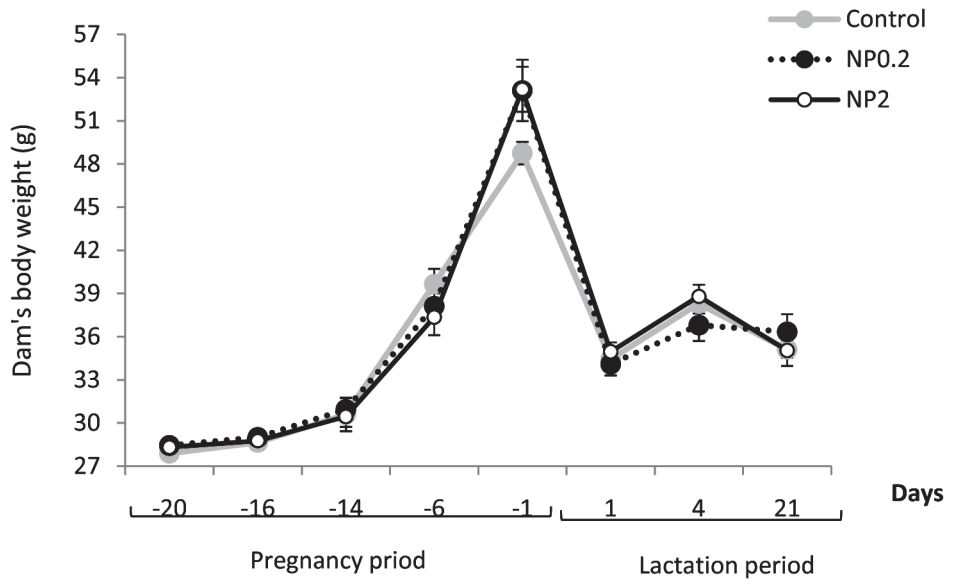

Figure 2. Dam's body weights (g) during pregnancy and lactation period. Data shown as mean ± standard error of mean; Control, NP5 and NP50 groups were treated by suspension contained zero, 5 and 50 µg of AgNPs, respectively. Negative and positive numbers below the horizontal axis indicate days before and after delivery, respectively (n =10 for each group).

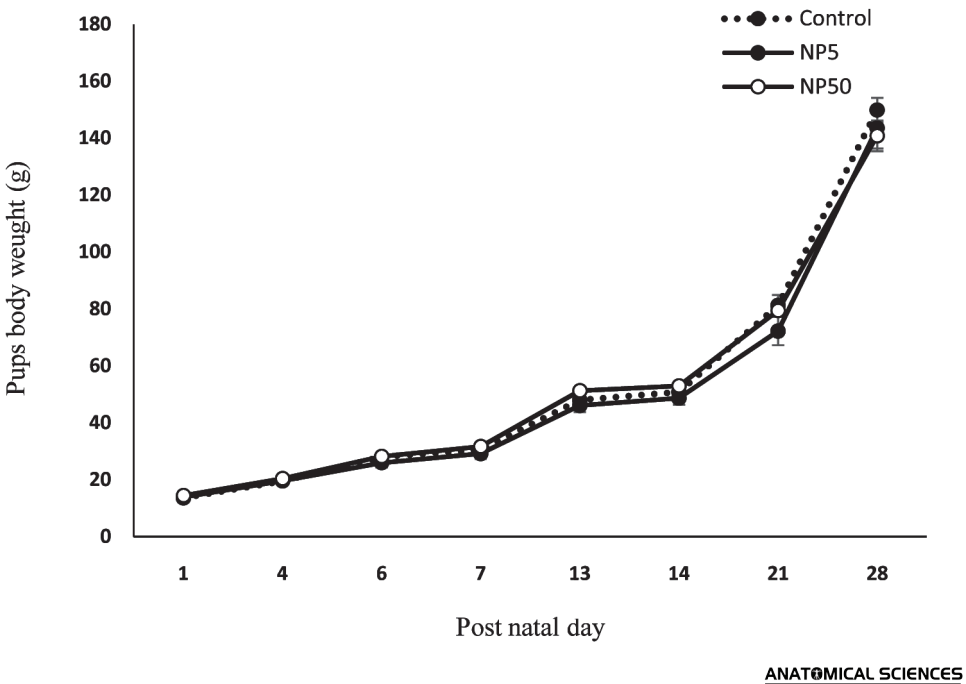

Figure 3. Litter weight (g), from delivery to PND 28. Data shown as mean ± standard error of mean; Control, NP5 and NP50 groups were treated by suspension contained zero, 5 and 50 µg of AgNPs, respectively (n =10 litter in each group).

both (28, 29). Ag-NPs can impair cell functions and cause brain damage (13, 14), which as a result may lead to death of certain cells, such as hippocampal neurons (30, 31).

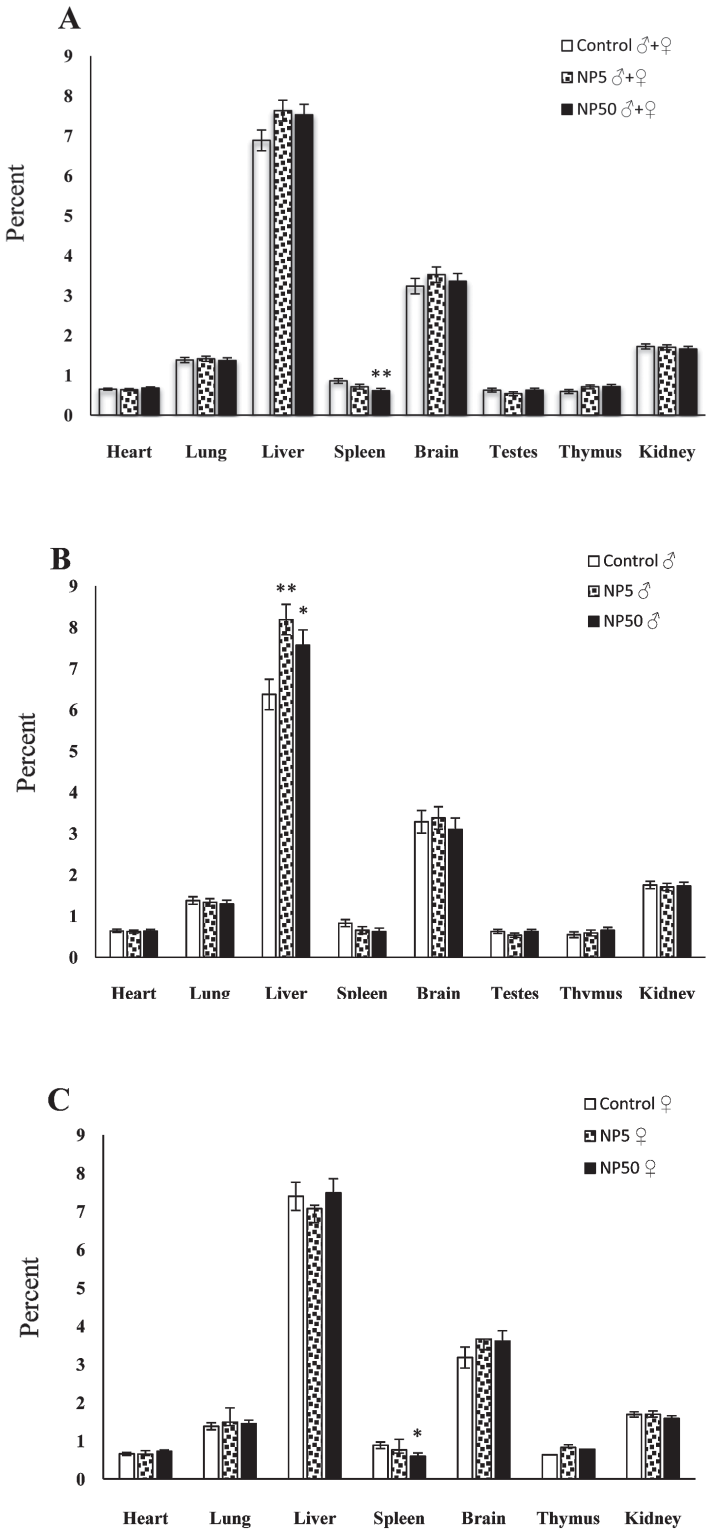

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Figure 4. Pups relative organs weight (organ/body weight × 100) at PND 28. Relative organ weight of male+female (A), males (B), and females (C). Data shown as mean ± standard error of mean. Control, NP5 and NP50 groups were treated by suspension contained zero, 5 and 50 µg of AgNPs, respectively; *: Indicate significant differences with control group (*: $p<0.05$, **: $p<0.01$). (n = 10 in each group, i.e. 5 males and 5 females).

The first day of expression of each reflex ontogeny was assessed according to the battery of Fox (20), Tsujii and Hoshishima (21) and Tamashiro et al. (22); surface righting reflex: pups were gently held on supine position and then released to right up throughout 30 s, with all four paws on the ground; cliff aversion test: pups turn and begin to crawl away from the edge of a flat surface during 30 s when its snout and forepaws are placed over the cliff; level or vertical stick reflex: pups grasp onto a wire mesh while gently pulled across the mesh by the tail. If he/she displayed resistance the test was considered positive; negative geotaxis: pups place head down on a 45 degree incline to turn 180 degrees and begin to crawl up the slope in 30 s; Rooting: head turns toward the side of the face being stroked with the tip of a swab; bar holding reflex: pups grip a thin rod with its forepaws while being suspended for at least 5 s; vibrissae placing reflex: pups were suspended by the tail and lowered so that the vibrissae makes contact with a solid object, the head is raised and the fore limbs are extended to grasp the object; visual placing response: pups were suspended by the tail and lowered toward a solid object (e.g. a bar or table top) raises its head and extend, the fore limbs in a placing response; air righting reflex: pups ability to turn around mid-air to land ventrally after being dropped from a prostrate position, 35-cm above a cotton wool pad; auditory startle reflex: pups show a whole-body startle response in response to a loud clap of the hands which occurs less than 15 cm away; pivoting and walking: pivoting and walking in

Table 1. Reproductive performance of dams.

Reproductive Parameters	Control	NP5	NP50
No. time-mated females	10	10	10
Male exposure to delivery interval (day)	23.30±1.40	21.22±2	23.91±1.03
Gestation index	1	1	1
Litter size	9.6±0.5	10.44±0.67	9.82±0.4
Post-natal survival rate	0.97±0.03	0.84±0.05 *	1.00±0.00
Sex distribution	46.1±10.7	48.1±12.5	52.4±21.8

Data shown as mean ± standard error of mean; Control, NP5 and NP50 groups were treated by suspension contained zero, 5 and 50 µg of AgNPs, respectively. *: Indicate significant differences with two other groups ($p<0.05$).

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Table 2. The PND which a physical landmark developed in 100% of the pups of a litter.

physical landmark	Control	NP5	NP50
Pinna unfolding	4.25±0.25	4.44±0.34	4.1±0.25
Fur development	7.00±0.19	7.56±0.29	8.00±0.27*
Incisor eruption	10.75±0.25	10.89±0.2	11.18±0.23
Ear opening	12.38±0.38	12.67±0.29	12.18±0.12
Eye opening	14.63±0.26	15.00±0.44	15.18±0.12
Testes descent	22.50±0.91	22.78±0.52	22.36±0.49

Data shown as mean ± standard error of mean; Control, NP5 and NP50 groups were treated by suspension contained zero, 5 and 50 µg of AgNPs, respectively; *: Indicate significant differences with control group (*: $p<0.05$). (n =10 litter in each group).

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a straight line are two locomotor activities observed at varying ages. The first PND at which a physical landmark or neurobehavioral reflex developed in 50% and 100% of the pups of a litter were considered as PND50% and PND100%, respectively.

Necropsy: Gross necropsy was performed in each group on 5 male and 5 female pups that were selected randomly at schedule termination on 28th day (PND 28). Maximum one male and one female were selected from any litter. Euthanasia was induced by chloroform; the mice were weighed and decapitated. Brain, liver, spleen, heart, lung, thymus, kidneys and testes, were removed and weighed to - determine absolute and relative organ weights (organ/body weight × 100).

Table 3. The PND which a neurobehavioral developed in 50% or 100% of the pups of a litter.

Behavior		Control	NP5	NP50
Surface righting reflex	50%	1.38±0.18	2.11±0.20	1.27±0.14
	100%	2.88±0.35	3.11±0.26**	2.64±0.51
Cliff aversion test	50%	1.00±0.00	1.44±0.18	1.27±0.19
	100%	1.75±0.31	2.11±0.26	4.18±0.24
Level Stick Reflex	50%	2.63±0.26	2.56±0.18	2.27±0.14
	100%	3.00±0.19	2.67±0.17	2.27±0.14
Negative Geotaxis	50%	2.62±0.46	3.33±0.37*	3.00±0.40*
	100%	2.88±0.30	4.22±0.40	4.27±0.36
Rooting	50%	1.63±0.38	2.33±0.44	2.36±0.39*
	100%	1.75±0.41	3.56±0.38*	2.55±0.41
Bar holding	50%	3.5±0.33	3.89±0.31**	3.73±0.33**
	100%	4.5±0.33	4.89±0.33	4.82±0.35
Vibrissae Placing Reflex$	50%	-	-	-
	100%	5.25±0.16	5.67±0.29	5.27±0.33
Air Righting Reflex$	50%	-	-	-
	100%	10.5±0.33	10.22±0.43	9.18±0.33*
Auditory Startle Reflex$	50%	-	-	-
	100%	13.38±0.32	13.56±0.41	13.81±0.35
Pivoting	50%	1.00±0.0	1.67±0.17*	1.64±0.20*
	100%	1.13±0.13	1.78±0.15*	1.64±0.20*
Walking	50%	9.63±0.46	10.89±0.45	10.18±0.18
	100%	10.0±0.42	11.11±0.39	10.18±0.18
Visual placing reflex	50%	14.5±0.19	15±0.44	14.9±0.16
	100%	14.8±0.25	15±0.44	14.9±0.25

Data shown as mean ± standard error of mean. Control, NP5 and NP50 groups were treated by suspension contained zero, 5 and 50 µg of AgNPs, respectively; *: Indicate significant differences with control group (*:p<0.05, **p<0.01); $: since the results of 50% and 100% were very close to each other, only the results of 100% were reported. (n =10 litter in each group).

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Data analysis: Statistical analysis were performed by comparing the treatment groups with the control groups using SPSS, version 16 (SPSS; Chicago, IL, USA). The data were presented as mean ± SEM. Variance in the numerical data was checked using Levene's test. If the variance was homogeneous, the one-way analysisofvariance test (ANOVA) was conducted to determine which pairs of comparison group were significantly different. When an effect was statistically significant ($p<0.05$), mean comparisons were done by post hoc comparisons with LSD multiple comparison test. To compare the effects of ANPs treatment on organs weight of two genders, Univariate test in GLM procedure was used, and treatment and gender were considered as fix factors. Organs weight of three male/female subgroups were compared using One Way ANOVA. Litters as a statistical unit were used for statistical analysis except for evaluation of organs weight.

3. Results

The used Ag-NPs were spherical and their diameters were 32±6.6 nm (Figure 1). Between the counted particles there were few particles greater than 100 nm which were not considered in the calculation of mean particles size.

Reproductive performance of dams: Weight changes were not significantly different between the AgNPs-treated and control dams during gestational and lactation periods (Figure 2). The post-natal survival rate of NP5 group had markedly decreased in comparison with the NP50 and control groups (Table 1; $p<0.05$). There were no statistically significant differences in gestation index and sex distribution between the groups ($p>0.05$).

Neurobehavioral and developmental milestones: Pup's body weight were not statistically different among the groups (Figure 2; $p>0.05$).

The results of physical landmark development in 100% of pups are shown in Table 2. The age of pinna unfolding, incisor eruption, ear opening, eye opening and testes descent were not different across the Ag-NPs-treated and control animals, but fur development were delayed significantly in NP50 compared to the control group ($p<0.05$).

Although no specific tests were used to assess the level of pups' activity, some findings based on the observation of pups' behavior in the cage (more jumping and running) and during reflex assessments (escape behavior) indicate hyper activity in NP50 group during the study.

Surface righting reflex 50% were delayed in both Ag-NPs treated group, but its delay was not significant. Surface righting reflex 100% were delayed significantly in NP5 group compared to NP50 and control groups ($p<0.01$). Negative geotaxis and bar holding were delayed in both AgNPs treated groups, although the delay was only significant at 50% ($p<0.05$). Compared to the control group, Rooting 50% and 100% was significantly delayed in NP50 and NP5 groups, respectively ($P<0.05$). Significant delay was observed in pivoting 50% and 100% in both AgNPs treated groups compared to control ($P<0.05$). Air righting reflex 100% was significantly earlier in NP50 group compared to the control and NP5 groups ($P<0.05$). In other words, no significant differences were found across groups (Table 3).

Necropsy finding: As the results of pups' necropsy at PND28 was shown in Figure 4, there were no significant differences in body weight (Figure 3), as well as relative weight of heart, lung, brain, testes and kidneys (Figure 4A). The effect of treatment on the relative weight of the liver was significantly different on male and female pups ($P<0.05$), such that the relative weight of the liver increased in male subjects of NP5 and NP50 groups ($P<0.01$ and $P<0.05$, respectively), while there were no significant differences between the relative weights of female pups in AgNPs treated and control groups (Figure 4B and 4C). The relative weight of spleen decreased significantly in NP50 group compared to the control group ($P<0.0$; figure 4A).

4. Discussion

Oxidative stress and inflammation which occur following nanoparticles passage from circulation to placenta, endometrium, yolk sac, or fetus, lead to placental dysfunction, retarded neonatal growth, fetal malformations, and neurotoxicity or reproductive toxicity; furthermore, inflammatory cytokines that are induced by nanoparticles in the dam, following their entrance, will affect the brain development of fetuses (27). Oxidative stress has been reported in the brain of newborn rats prenatally exposed to Ag-NPs (14). AgNPs (25 nm) toxicity alters gene expression and reactive oxygen species (ROS) in the caudate, frontal cortex, and hippocampus of mice (9). The AgNPs toxicity may be due to the - toxic nature of AgNPs, release of the Ag+ ions or a combination of

Changes of dams' body weight during pregnancy and following delivery until weaning of pups were almost similar in the studied groups. Dams' body weight during these periods could be a criterion of dams' health (32). Postnatal survival rate had decreased in NP5 group, which according to our observation, it was mostly related to infantiphagia behavior. In accordance to our finding repeated oral administration of AgNPs had no effects on weight gain of pregnant rats and reproductive indexes, including the gestation period, number of corpora lutea, implantation, delivery rate, number of living and dead pups, sex ratio, survival rate etc. Similarly, oral (15) and intra peritoneal (19) administration of AgNPs to pregnant mice and rats did not induce apparent sign of maternal toxicity.

Index of pups' general health was obtained by measuring body weight and recording observations on any abnormal physical features (32). The pups' body weight was almost similar in the studied groups. These data indicate the normal health of pups in AgNPs treated groups. Although a delay in fur development was observed in NP50 group, the lack of significant differences in pups' body weight and other physical landmarks could represent the approximate normal body growth and development in AgNPs treated groups during pre-weaning period. We did not find any report concerning the effects of AgNPs on these indexes during pre-weaning period. Only Hong et al. (7) reported similar weight of 4-day-old pups which had been exposed to AgNPs during prenatal period. However, no significant effects on growth or mortality were observed when earthworms were exposed to AgNPs coated with oleic acid or polyvinylpyrrolidone, but significant decrease in reproduction was seen (33). Developmental success decreased dose dependently in Drosophila who were treated by AgNPs (0.005–0.5%) (15). In the case of AgNPs in zebrafish (Danio rerio), decreased survival, increased malformations in the embryo, and delayed hatching were observed (34-36). These controversies may be due to the differences in the AgNPs quality and quantity, variable experimental protocols as well as species sensitivity.

Review of the behavioral changes related to the activity level and pathological changes in the brain following to the prenatal exposure to nanoparticles suggested that nanoparticles may affect neurobehavioral development (37, 38), but some other studies have shown that exposure to nanosize particles do not have negative effects during development (39, 40). Pups' cage activity and jumping behavior increased following prenatal exposure to high dose of AgNPs. However it should be noticed that pups' activity were not recorded systematically. It is well established that attention deficit hyperactivity disorder (ADHD) is associated with low level of dopamine in brain (41). The remarkable thing about this matter is that AgNPs (15 nm) significantly have reduced dopamine, dopamine metabolite concentrations, and the expression of genes related with the dopaminergic system on PC-12 cells (42, 43). AgNPs and ionic silver impair neuronal cell differentiation and their capability to produce dopamine (44, 45), whereas in the brain of zebrafish, ionic silver has elevated both dopamine and serotonin (5-hydroxytryptamine; 5-HT) turnover (46). Hadrup et al. (47) reported that dopamine has increased in the brain of rats, following oral administration of Ag-NPs and silver ion.

The Fox battery of tests created an assessment of development through the neonatal period because these behaviors are each expressed at different periods during the first 21 days of life (20). In our study, treatments with AgNPs delayed the development of the surface righting reflex, negative geotaxis, rooting, bar holding and pivoting, but accelerated air righting reflex. Thus 6 reflexes out of 12 were affected by prenatal treatment of low or high dose of AgNPs. The righting reflex is used more to evaluate the postural reflex (32). This reflex requires complex coordination between head, trunk, and paws, which is also observable in the human newborn around 6 months of life (48). Righting and grasping reflexes require complex sensorimotor coordination between different bodies segments (48). Fox have divided the behavioral development of the mice in 5 periods; perinatal (birth-3 days), neonatal (3-9 days), postnatal/transition (9-15 days), post infantile (15-26 days) and juvenile (>29 days until sexual maturity) (20). We think it is an interesting and important finding which all of the delayed responses belonged to the first or the early second round of Fox classification, but the accelerated response had to do with the early third stage. This may indicate a delay in neuromuscular coordination at early stages, which was finally compensated. We did not find any report indicating the effects of neonatal exposure to AgNPs on neurobehavioral reflex ontogeny in mammals. Exposure of developing zebrafish to Ag+ and AgNPs on days 0–5 post-fertilization indicates that AgNPs are less potent than Ag+ with respect to dysmorphology and loss of viability; nevertheless, they produce neurobehavioral effects that highly depend on particle coating and size, rather than just reflecting the release of Ag+ (24).

According to the necropsy results, we evaluated the adverse effects of AgNPs on the organ weights on 28-day-old offspring. The significant increase in male relative liver weight of AgNPs treated groups indicates that this effect is sex dependent, which may be related to metabolic differences. AgNPs can be accumulated in some organs, e.g. liver, kidney, lung, spleen, which can lead to toxicity by inflammatory responses and oxidative stress (16, 49, 50). Since AgNPs have antimicrobial effects, the reduction in relative weight of spleen may be related to reduction in microbial population which decreases immune system stimulation. To our knowledge, there is no information related to the effects of maternal exposure to AgNPs on offspring's organ weight. However, there are some reports which have indicated minor effects of oral exposure of AgNPs on organs weight of mature rats (7, 51). Similar to our results, an increase in the relative weight of liver was observed by Hong et al. (7), in male, not female, rats treated orally with 250 mg/kg AgNPs.

In conclusion, this is the first report which claims that SC administration of AgNPs during mating and pregnancy did not lead to adverse effects on pregnant pups growth. We showed that prenatal exposure to AgNPs delays the development of pups' neurobehavioral reflexes in the first days after birth, while no problems were found later in the infancy. Although this study showed that prenatal exposure to AgNPs does not overtly affect dams and pups, the delay in the appearance of some neurobehavioral reflexes and changes in relative weight of liver should be given more attention. These findings are warning to females who are pregnant or are planning to get pregnant to avoid exposure to AgNPs.

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