**Reviewer’s Comments**

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**Nasal Administration of Silver Nanoparticles and Silver Ions Reduced Cognitive Performance in Adult Rats**

**Abstract**

**Background:** Silver nanoparticles (AgNPs) have shown adverse effects and a toxic impact on human cell bodies. Passing the blood-brain barrier and increasing oxidative stress can change different parts of the brain such as the hippocampus. Therefore, the purpose of this investigation was to explore the possible neurotoxicity of AgNPs in adult rats. **Method:** Neurotoxicity was created in rats by treating them with 15 mg/kg NP3 and silver ions (AgAc) intra-nasally every two days for 20 days. The animals were divided into five groups: control, vehicle, NP3, NP15, and AgAc. Behavioral assessments such as Morris water maze and elevated plus maze, and assessment of biomarkers such as malondialdehyde assay were used to evaluate cognitive impairment and molecular changes induced by silver. **Result:** The results revealed that NP15 and AgAc significantly impaired spatial memory. Moreover, NP3, NP15, and AgAc increased anxiety in animals. Additionally, MDA was significantly increased by NP3 and NP15. **Conclusion:** The findings showed that AgNPs and AgAc, particularly NP15, result in neurotoxicity and behavioral impairments.

**Keywords**: Silver nanoparticles, Silver ions, Neurotoxicity, Learning and Memory, Anxiety-like Behaviors.

**1. Introduction**

In recent years, the use of metal nanoparticles (NPs) such as silver has increased wildly for different purposes including sterilization and creating anti-bacterial agents, food additives, and medicine. The unique electronic, optical, mechanical, magnetic, and chemical properties of these NPs are so different from the bulk metals that a variety of applications have been found for them 1,2. Thus, there are many who are worried about the spread of AgNPs in the human living environment 2. AgNPs can enter the body through different ways (ingestion, inhalation, etc.), and lead to harmful effects 3.

Various biological models have been used to evaluate the effects of NPs on living organisms. They adversely affect the reproductive systems and embryonic growth of animals such as mice and zebrafish 4,5. Once the NPs enter the body, they can reach different organs through the systemic circulation. Depending on their size, shape, and chemical reactivity, they can also cross the blood-brain barrier and penetrate the brain via axonal transport along the olfactory nerve. 6.

 Many current in vivo and in vitro research studies have demonstrated that AgNPs have adverse effects and a toxic impact on human cell bodies 7,8. Furthermore, NPs show a tendency to aggregate in different body organs and cause oxidative damage by producing free radicals and inflammatory responses, create changes in gene-expression, cause over-proliferation of glial and hippocampal cells, and increase apoptosis, genotoxicity, and DNA damage 9-11. Studies have shown that AgNPs can pass the blood-brain barrier and accumulate in the brain 12. These mechanisms may provide pathological conditions that may cause brain damage 12. Moreover, there is evidence that AgNPs disrupt the transporting neurotransmitters such as dopamine, norepinephrine, and serotonin in neural pathways  13,14, and these changes in neurotransmitters can affect cognitive and behavioral mechanisms, especially learning and memory 15.

Studies have demonstrated that gradual declines in spatial learning and memory functions are inversely correlated with ROS values in the brain. Moreover, recent evidence suggests a direct correlation between oxidative stress and anxiety behavior in animals 16. Besides, after passing through the blood-brain barrier, AgNPs produce oxidative stress (similar to ROS) in different parts of the brain such as the hippocampus, which can cause the dysfunction in learning and memory mechanisms 17,18. Studies have shown that maternal exposure to AgNPs impaired cognitive behavior in the Morris water maze (MWM), but no difference was observed in anxiety-like behavior in the elevated plus maze (EPM) test 19. Furthermore, one study demonstrated that these NPs have an adverse effect on short-term memory in animals 17, while other studies did not indicate a significant difference in spatial memory in animals treated with AgNPs 17.

This study was conducted to investigate the adverse effects of AgNPs and AgAc particles through inhalation administration on learning, memory, and anxiety-like behaviors using MWM and EPM tests. In this study, the bulk size of AgNPs was used for observing the difference in toxicity between bulk- and nano-sized particles.

**2. Material and Method**

**2.1. Animals**

For this investigation, 50 adult male Wistar rats (weighing 200-230 g) were used, which were acquired from the animal house facility of Shahid Chamran University of Ahwaz. The animals were kept at room temperature (22 ± 2 °C) and a 12 h light/dark cycle with proper ventilation and ad libitum access to food and water.

**2.2. Experimental protocol**

The Wistar rats were divided into five groups (n = 10) at random. Group 1 was considered as the control group. Group 2 was used as the vehicle group that received sodium citrate (0.12 M) every two days for 20 days. Group 3 received silver acetate (4.6 mg/kg) every two days for 20 days. Groups 4 and 5 received AgNPs (3 and 15 mg/kg, respectively) every two days for 20 days. Moreover, all administrations were done intra-nasally.

**2.3. Synthesis of nanosized silver particles**

2.3.1. Preparation of AgNPs: Hydrazine hydrate and sodium citrate were used as reductants to synthesize nanosized silver particles. Firstly, two mixtures of silver nitrate (0.02 M) (1 ml) and sodium citrate (0.12 M) (400 and 600 μL, respectively) had to be prepared. Then, distilled water (2 ml) and hydrazine hydrate (1 μL) were added. After that, the mixtures were placed in a dark environment at room temperature for two hours. Finally, they were centrifuged for 15 minutes at 10,000 rpm that produced a somewhat yellow solution separated from the sediment, which was AgNPs.

2.3.2. Determination of the concentration: UV light absorption spectrum was used to measure the concentration of the samples by Beer-Lambert and molar extinction coefficient.

**2.4. Behavioral tests**

 On the 14th day after the last administration, behavioral tests were performed including MWM and EPM.

**2.4.1. Morris water maze**

The MWM test was done to evaluate the spatial learning and memory of the animals. A circular pool (160 cm in diameter and 80 cm in height) with four quadrants is filled with water at a temperature of 25 °C, which makes the maze. A square-shaped platform (10 cm in diameter) is submerged 1.5 cm at the northeast quadrant of the pool. During the learning phase, the rats were trained for four days to find the platform. Each block comprised four sequential trials. In each trial, 60 seconds was set for a rat to find the concealed platform and it was allowed to stay on it for 30 s. The trial was terminated when the platform was discovered and the rat climbed and positioned itself on top of it. If the rat could not find the platform during 60 s, the experimenter would put the rat on the platform. To evaluate spatial memory, a “probe trial” was performed twenty-four hours after the last session. To perform the probe trial, the experimenter removed the platform, and the rat was supposed to find the target quadrant in 60 s 20. After that, a visible platform test was performed using the rats. In the next step, an aluminum foil was used to cover the platform, which was elevated above the water for just 2 cm, in order to decide if there was an interfering factor with motor and sensory functions 21. A video tracking system was used to keep records of the time spent and distance moved by the rats in the target quadrant in addition to the velocity of movement.

**2.4.2. Elevated plus maze**

The EPM test is commonly performed to check anxiety-like behavior in rodents. It is made of two open arms (30 × 5× 15 cm) and two closed arms (30 × 5 × 15 cm). For performing this test, the rats were placed at the center point of the maze and their behavior was recorded for 5 min. The numbers of entering the open and closed arms and the time spent in them were recorded for data analysis 22.

**2.5. Malondialdehyde assay**

 The appropriate method to investigate the malondialdehyde (MDA) in blood samples is thiobarbituric acid (TBA). Firstly, 1 % potassium iodide and 0.1 % butylated hydroxytoluene were combined with the samples, which was followed by incubation at 50 °C for 20 min. Next, 0.4 % TBA was added followed by incubation at 60 °C for 60 min. Then, isobutyl alcohol was used to run high performance liquid chromatography with fluorescence detection to assess the samples.

**2.6. Statistical analysis**

 The data is presented as mean ± SEM. For EPM and the probe test in MWM, calculations were done by the one-way analysis of variance (ANOVA). Moreover, for making comparisons between the groups in the learning phase of MWM, calculations were done by the two-way ANOVA. For significant differences among the groups, *p* values < 0.05 were considered statistically significant. GraphPad Prism 8.0 software program was used for statistical analysis.

This study was approved by the Ethics Committee of Shahid Chamran University of Ahvaz, Iran.

**3. Results**

**3.1. Effects of AgNPs on learning and memory in MWM**

Spatial learning and memory in all the groups were evaluated through the MWM test. Both path length and escape latency were reduced to discover the hidden platform during four days. The two-way ANOVA demonstrated that there were no significant differences in terms of the total traveled distance and escape latency among the control, vehicle, NP3, NP15, and AgAc rats (figure 1(a) and figure 1(b)). Consequently, 24 h after the last session, the probe test was done to evaluate spatial memory by the mean percentage of distance traveled and time spent in the target quadrant. Interestingly, it revealed significant differences in the traveled distance percentages in the target quadrant for the NP15 (*p* < 0.01) and AgAc rats (*p* < 0.05) compared to the control group. Additionally, the time percentage spent in the target quadrant was significantly lower in the NP15 group (*p* < 0.01) compared to the control group (figure 2(a) and figure 2(b)).

**3.2. Effects of AgNPs on anxiety in EPM**

One-way ANOVA showed that there was a significant difference between the control group and NP3 (*p* < 0.05), NP15 (*p* < 0.01), and AgAc (*p* < 0.05) groups regarding the average percentage of time spent in the open arms, while no significant difference was observed among the groups in the average percentage of entries into the open arms (figure 3(a) and figure 3(b)). However, the average percentage of time spent in both open and closed arms showed significant statistical differences in NP15 (*p* < 0.001) and AgAc (*p* < 0.01) groups compared to the control group (figures 3(c)).

**3.3. Effects of AgNPs on malondialdehyde level in MDA assay**

The one-way ANOVA showed that there was a significant difference in MDA level between the control group and NP3 (*p* < 0.05) and NP15 (*p* < 0.01) groups (figure 4).

**Discussion**

Results of the current study show the adverse effects of AgNPs and AgAc on some parameters of memory and anxiety. The EPM results showed that OAT percentages in NP15, NP3, and AgAc groups were decreased significantly compared with the control group, and total arms entries were decreased in NP15 and AgAc groups. These results demonstrated an increase in anxiety-like behavior and problems in locomotion. Moreover, MWM was used to evaluate spatial learning and memory in Rats and its results demonstrated that there were no significant differences in the travelled distance among the experimental groups. This result may indicate that learning is not affected by AgNPs and AgAc. Increases in swimming speed were observed in NP15 and AgAc groups that may show the dose-dependent toxicological effect of NP. Moreover, decrease in target quadrant travel percentages in NP15 and AgAc groups in the probe trials may be indicative of the adverse effect of these materials on the memory processes in animals. Whereas equal dosage was used in NP3 and AgAc, these results may indicate that AgAc have more toxicological potential compared to nano sizes. In line with these results, Kvitek et al. (~~2011~~) Showed that AgNPs at a high concentration (60 mg/l) are toxic to mammalian cells, while AgAc cause this toxicity at a low concentration (1 mg/l) 23. Loeschner et al. (~~2011~~) demonstrated that after oral administration of AgAc and AgNPs, the concentrations of AgAc in the brain and plasma were significantly higher compared with NPs 24. Together, these results showed that AgAc have more toxicological effects compared to AgNPs.

These results are in line with other studies that had demonstrated that exposure of zebrafish to AgNPs during the developmental period may lead to anxiogenic effects as well as negative effect on cognitive function and behavioral performance 25. Hritcu et al. (~~2011~~) demonstrated that intranasal administration of AgNPs leads to spatial memory problems that may be related to the increase of ROS in the hippocampus 26. In contrast to this study, Liu et al. (~~2013~~) did not find a significant difference in memory after exposure to these particles compared with the control group (Liu et al., ~~2013~~). These controversial results may be due to differences in shapes, surface coatings, and size, or difference in administration methods 17.

Studies have shown that intranasal administration of AgNPs caused them to accumulate in the olfactory bulb and ventricles and led to inflammation and increased tissue GSH levels, and these results may be proof of NPs transport from nose to brain 27. Several ways of transport have been considered to allow NPs to enter the brain through the nasal passages such as olfactory neural pathway and trigeminal pathway as well as the paracellular transport 28. In addition, oral administration of these NPs increased IL-1, TNF-ą, IL-6, and ROS in brain tissues, which induced apoptosis and changes in brain genes 29. Moreover, another study showed that different shapes of AgNPs induced histopathological changes in different brain parts like amygdala and hippocampus, which are important brain areas in regulation of anxiety, stress behaviors, and memory 30. Increased intracellular accumulation of AgNPs has been shown to cause inflammation and oxidative stress, and trigger the activation of antioxidant defense mechanisms in cells 31. Due to the low oxidative capacity of the brain, it is more susceptible to oxidative stress than other organs, and an increase of oxidative stress in the brain causes striatal and amygdala damages 32. Moreover, AgAc can spread to different organs like liver, kidney, and the CNS by the lymphatic system, and it can also be transferred by neural axons in the CNS 33. Similar to the AgNPs, AgAc have toxicological effects in brain cells, but through different mechanisms. This may be due to differences in the regulatory mechanisms of gene expression and mRNA synthesis related to oxidative stress factors 10. The toxic effects of silver may be due to the binding of AgAc to important functional proteins, while AgNPs are attached to vesicles and organelles such as lysosomes and collagen, which have little or no effect. In general, the results of these studies indicated that AgAc and AgNPs have toxicological effects on the CNS through different mechanisms that have crucial roles in cognitive functions, and these results confirmed that AgAc have more effect compared with AgNPs in similar doses.

Changes in neurotransmitters is another possible mechanism that may be related to behavioral problems after exposure to these materials. Silver nanoparticles and AgAc impair the differentiation of nerve cells and their ability to produce dopamine 13,14. Hadrup et al. (2012) reported that exposure to AgAc and AgNPs increased dopamine but had different effects in other neurotransmitters: AgAc had a significant effect on noradrenaline but NPs affected 5-HT to a greater degree. These neurotransmitters have critical roles in cognitive mechanisms 34. Studies have demonstrated that an imbalance between serotonin and norepinephrine can lead to anxiety and hypersensitivity. Moreover, an imbalance between norepinephrine and dopamine causes impulsive behavior and reward processing problems 35. Behavioral response to anxiety and stress is mediated by many neurotransmitters including dopamine, norepinephrine, serotonin, and acetylcholine 36. In addition, investigations about memory have shown that dopamine and norepinephrine have critical roles in different aspects of memory, in a way that norepinephrine depletion causes a slight impairment of working memory during delayed tasks, and reduction of dopamine in substantial area is associated with a deficit in memory 37.

In general, these studies aimed to evaluate the effects of AgNPs on anxiety-like behaviors as well as learning and memory. Results showed deficiency in memory and anxiety-like behavior after treatment with AgAc and AgNPs, and based on other studies, this may be related to change in serotonin, dopamine, and norepinephrine neurotransmitters and the toxicological effect of these materials.

**Conclusion**

Silver can impair spatial memory and induce anxiety-like behavior in rats. Further studies are needed for determining the molecular mechanism of these NPs.

**Limitations and future studies**

The findings of this study are limited by the use of only male animals and these effects can also be studied on female rats in future studies. Moreover, chronic toxicological effects of these materials could be examined as well.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**AUTHOR’S CONTRIBUTION**

ST conceptualized this study, all authors drafted the review, and JF and ST drafted the final article.

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**Figure 1**: Morris water maze. The total distance traveled and escape latency by rats showed no significant difference among groups (a, b).





**Figure 2**: Probe test. The distance percentages in target quadrant was decreased significantly in NP15 rats (*p* < 0.01) and AgAc rats (*p*< 0.05)compared to the control group. The data is presented as mean ± SEM.\*\**p*< 0.01 vs. NP15. \**p*< 0.001 vs. AgAc (a).The time percentage spent in the target quadrant was significantly less in the NP15 group (*p*< 0.01) compared to the control group. The data is presented as mean ± SEM. \*\**p*< 0.01 vs. NP15(b).







**Figure 3:** Elevated plus maze. There was a significant difference between the control group and NP3, NP15, and AgAc groups regarding the average percentages of time spent in the open arm. The data is presented as mean ± SEM. \**p*< 0.05 vs. NP3. \*\**p*< 0.01 vs. NP15.\**p*< 0.05 vs. AgAc (a). The average percentages in entries into the open arm demonstrated no significant differences between groups (b). The average percentages of time spent in both open and closed arms showed no significant differences among groups(c).

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**Figure 4:** Malondialdehyde assay**.** There was a significant difference between the control group and NP3 and NP15 groups regarding the malondialdehyde level. The data is presented as mean ± SEM. \**p*< 0.05 vs. NP3. \*\**p*< 0.01 vs. NP15.