

Stimulating Effect on Behavioral and Cognitive Functions of Mice at Silver Citrate Exposure

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Abstract

Influence of long-term daily oral exposure of C57BL/6 male mice during up to 180 days to 50 µg/day/ animal of silver citrate on their cognitive and behavioral functions was studied in the present work. The behavioral and cognitive functions' change can be expressed as the 3-staged process such as 1) anxiety increase, 2) susceptibility (sensitivity) increase, 3) tendency to improve long-term contextual memory at the background of decreased anxiety and locomotion increase. The observed effect of stimulation of behavioral and cognitive functions at silver citrate exposure at the final stage can be applied in Medicine for treatment of neurodegenerative diseases as well symptoms of mental illness. Silver citrate may potentially replace silver nanoparticles, which are well-known from scientific literature to be toxic.

Key words: silver citrate, silver chelate complex, behavioral functions, cognitive functions, stimulation, mammals

Introduction

Silver in different forms has been applied for medical purposes due to its antiseptic, anti-inflammatory and regenerative properties [1,2]. Nowadays silver is used in medicine mostly as silver nanoparticles. There are the silver-based preparations such as Protargol, which is applied for infectious diseases' treatment [3], Argosulfan ointment, which is used for wound and burn treatment [4], dressings with silver nanoparticles' coating are applied in purulent surgery [5], silver plating is used in pacemakers [6] and implants [7] to improve their biocompatibility. Silver nanoparticles are applied in dentistry [8] as well, in packing materials to extend the shelf life of food [9], in cosmetology for acne treatment [10], in textiles such as protective masks and respirators [11], actively used in agriculture for treatment of seeds and crops from pathogens [12].

At the same time, a many relevant scientific papers and presentations at scientific conferences during 2010-2020s discusses the problem of silver nanoparticles' toxicity. Silver nanoparticles demonstrate their toxicity in *in vitro* [13-15] and *in vivo* [16-18] experiments, change behavioral and cognitive functions of laboratory animals [19, 20], negatively impact fertility [21], show nephrotoxicity [22] and hepatotoxicity [23]. It was shown previously that silver nanoparticles are able to penetrate blood-brain barrier and interact directly with the nervous tissue of the brain [24, 25]. The problems of silver nanoparticles' safety and security as well approaches of their risk assessment are actively discussed [26].

In general, silver nanoparticles are known as hazardous for human health and environment. For that reason, the search of less toxic and more biocompatible silver compounds is relevant.

Colloidal silver (silver nanoparticles) and silver chelate complexes are permitted for use at production of food supplements for adults according to Uniform sanitary and epidemiological and

hygienic requirements for products subject to sanitary and epidemiological supervision (control) accepted by Eurasian Economic Union [27]. All chelate complex compounds are characterized by the fact that one of the terminal atoms of the polydentate ligand is bound to the central atom or complex-forming ion through unpaired electrons of a normal covalent bond, and the other is bound through a donor-acceptor bond. Metal chelate complexes are known to be highly bioavailable. Which form of silver is more biocompatible - whether it's silver chelate complexes or silver nanoparticles? Biocompatibility of silver chelate complexes is less studied than silver nanoparticles' toxicity. Therefore, the answer for the question is not clear today. Due to this, scientific studies on the problem of toxicity of silver chelate complexes are rather relevant and demanded.

The purpose of the present work was to study influence of long-term oral exposure of laboratory mice to silver citrate, as an example of such a silver chelate complex, on their behavioral and cognitive functions.

2.1. Materials

Silver citrate (SilverSalt, Saint Petersburg, Russia) was used as a silver chelate complex for exposure. Silver citrate was chosen due to its high commercial availability. It was a transparent water solution. The chemical formulae of silver citrate is $\text{Ag}_3\text{C}_6\text{H}_5\text{O}_7$.

The exposure solutions were obtained by dissolution of silver citrate by purified tap water (OOO Pharmsystemy, Besedy village, Moscow region, Russia) to the required concentration.

2.2. Animals

Male mice C57BL/6 ('Stolbovaya' branch of the Federal Medical Biological Agency of Russia, Moscow, Russia) were applied as a mammalian model. The animals were received from the supplier in the age of 2 months, with the average weight of 20-22 g. After the delivery into the National Research Center Kurchatov Institute the mice were allowed to adapt to the new environment during 2 weeks. The animals were kept in the individual cages in the room with automatically maintained temperature of $23 \pm 2^\circ\text{C}$ and a 12/12-h day/night cycle during the whole experiment. The animals had an unlimited access to food and water. All the manipulations with the animals were implemented according to Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes as well as the requirements of the Local Ethical Committee on Biomedical Research of the National Research Center Kurchatov Institute approved this research (Protocol No. 2, 07 October 2021).

2.3. Methods

Behavioral tests such as Open Field (OF), Elevated Plus Maze (EPM), Light-Dark Box (LDB) were used to assess behavioral functions of mice. Namely, locomotion, anxiety and exploration behavior were assessed in the tests. Susceptibility (sensitivity) was used as an additional behavior parameter. Contextual fear conditioning task (CFCT) was used for long-term contextual memory evaluation.

2.4. Experimental Scheme

Body weight of mice was measured weekly using electronic scales Adventurer Pro (Ohaus Corporation, Pine Brook, NJ, USA).

Experimental mice received silver citrate in composition of drinking water with unlimited access to it in the amount of 50 μg of silver/day/animal during 30, 60, 120 or 180 days. Control animals received purified water *ad libitum*. Thus, 4 groups of experimental mice ($n=12$ in each group: «Ag+ 30», «Ag+ 60», «Ag+ 120»; $n=14$ in «Ag+ 180») and 4 groups of control mice ($n=12$ in each group: «Control+ 30», «Control+ 60», «Control+ 120»; $n=14$ in «Control+ 180») took part in the study.

Assessment of behavioral and cognitive functions of mice was carried out after the cessation of the periods of silver citrate exposure since 31st, 61st, 121st and 181st days. Testing was implemented in the following order such as OF, EPM and LDB, by one test per day. Silver citrate administration was maintained during testing in order to exclude influence of silver elimination processes. Home cages were used for transportation of animals from the animal holding area to the testing area and back. The animals were returned to the home cages after each testing. The parameters and conditions of the tests are described below.

2.4.1. Open Field

A round arena with a diameter of 1200 mm, surrounded by 450 mm high walls, was used as OF. The arena floor and walls were made of gray polyvinylchloride. The illumination level of the arena was 115 lux. The walls and the floor were wiped with a 70% ethanol solution (ZAO Bryntsalov-A, Russian Federation) before placing of each subsequent animal in the arena.

Each animal was placed into the center of OF and was allowed to explore the OF freely during 300 s. Video recording of the animal's behavior during testing was performed using a WV-CP500G color analog video camera (Panasonic, Japan) installed above the center of the arena at a height of 2.5 m, and EthoVision XT 8.5 behavioral video recording system (Noldus Information Technology, the Netherlands). The video recordings of mice behavior were analyzed using the EthoVision XT 8.5 program.

2.4.2. Elevated Plus Maze

A maze with 4 arms (LxW: 620x50 mm) intersecting at an angle of 90°, and a central platform at the intersection of the arms (LxW: 50x50 mm) was used as EPM. The base of the EPM is made of gray plastic and is raised 700 mm above the floor. Two closed arms of the maze are surrounded on three sides by transparent plexiglass 160 mm high walls. A 5 mm high transparent plexiglass side is installed along the edges of the open arms. The illumination of the closed arms was 60 lux, illumination of the open arms it was 77 lux, and illumination of the central platform was 70 lux. The maze was wiped with a 70% ethanol solution (ZAO Bryntsalov-A, Russian Federation) before placing each subsequent animal in the EPM.

For testing, each animal was placed onto the central platform facing either open arm, and allowed to freely explore the maze for 300 s. Video recording of animal's behavior was carried out during testing with the use of analog video camera WV-CP500G (Panasonic, Japan) installed above the central platform of EPM at a height of 2.5 m, and the EthoVision XT 8.5 behavioral video recording system (Noldus Information Technology, the Netherlands).

2.4.3. Light-Dark Box

The test is used as a common model of anxiety behavior of rodents. The LDB consists of safe dark part and brightly illuminated open part, which forms a zone of disgust. The model creates a conflict situation for the mouse, which is inclined to explore the unfamiliar area, but initially wants to avoid the unknown circumstances, characterizing by neophobia. The interval of time spent in the dark compartment correlates with the level of anxiety, while the number of exits and the time spent exploring the illuminated compartment are indicators of risk-taking and exploratory activity.

The setup consists of two parts such as dark and light chamber (50x25x40 cm), which are connected by an opening (8x6 cm). The walls and floor of the chambers were wiped with a 70% ethanol solution (ZAO Bryntsalov-A, Russian Federation) before placing each subsequent animal in the LDB.

For testing, each animal was placed in the center of a light chamber and allowed to freely explore the setup for 600 s. Video recording of the animal's behavior in the light section of the setup during testing was performed using a WV-CP500G color analog video camera (Panasonic, Japan) installed above the center of the light chamber at a height of 2.5 m and an EthoVision XT 8.5 behavioral video recording system (Noldus Information Technology, the Netherlands). The resulting behavioral video recordings of animal's behavior were analyzed using the EthoVision XT 8.5 program.

2.4.4. Contextual fear conditioning task

The animals were trained and then tested in CFCT to assess silver citrate influence on forming and retention of long-term contextual memory using Video fear conditioning system (MED Associates Inc., USA) and Video Freeze software (MED Associates Inc., USA).

Video recording of the mice's behavior was performed and the number and duration of freezing acts were automatically determined during learning and during testing. Freezing was defined as a specific form of mouse behavior characterized by a complete absence of any movements other than breathing.

Mice of each group were divided into two subgroups such as active control (AC) (n=6 or 7) and fear conditioning (FC) (n=6 or 7). Mice of the AC subgroups were placed in the experimental chamber for 6 min to explore the environment, without electrical stimulation. Mice of the FC subgroups were placed in the experimental chamber for 6 min: 3 min to explore the environment, 3 electric stimulations with current strength of 1 mA, duration of 2 sec and an interval of 1 min to memorize the environment. The quality of learning of the mice was analyzed by assessing and comparing the percentage of freezing between the AC and FC subgroups of each group in 1 min after training. Intervals of interest were set in the Video Freeze program (0-178 sec and 300-360 sec of the experiment) and the time (percentage) of freezing was automatically calculated for these intervals as the ratio of the total duration of freezing acts in the interval to the duration of the interval for this purpose.

The animals were tested for determination of the level of long-term contextual memory, or, in other words, skill retention in 24 hours after training. For this, each animal was placed into the same experimental chamber for 3 min. An interval of interest (0-180 sec of the experiment) was set in the Video Freeze program, and the time (percentage) of freezing was automatically calculated for this interval as the ratio of the total duration of freezing acts in the interval to the total duration of the interval.

2.4.5. Parameters of assessment of mice behavioral and cognitive functions

The values of parameters characterizing behavioral, cognitive functions between experimental and control animals were compared within each period of exposure such as «Ag+ 30» and «Control+ 30», «Ag+ 60» and «Control+ 60», «Ag+ 120» and «Control+ 120», «Ag+ 180» and «Control+ 180».

These parameters are:

- 1.) OF: total distance moved; distance moved in Peripheral, Intermediate and Central areas; number of entrances into center; number of rearings;
- 2.) EPM: total distance moved; distance moved in open arms, closed arms, center; time spent in open arms, closed arms; number of head dips;
- 3.) LDB: number of lookings out from the dark chamber; number of transitions between chambers; distance moved in the light chamber; latency to exit from the light chamber.

4.) We suggest to introduce additional parameter to assess behavioral functions of mice such as freezing time in 1 min after learning in CFCT. Apparently, it characterizes sensitivity (susceptibility) of mice. Also it shows the quality of learning.

5.) Long-term contextual memory was assessed by the time of freezing (%) in 24 h after the learning in the CFCT.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.01 software (La Jolla, San Diego, USA). Nonparametric Kruskal-Wallis analysis of variance with Dunn's post hoc test for multiple comparisons or Mann-Whitney U test were used. Differences were considered to be significant at $p < 0.05$.

Results

The Figures 1-5 show statistically significant ($p < 0.05$) differences as well as a tendency ($0.05 < p < 0.07$) in the measured values. Reliable differences were observed only in EPM and CFCT.

EPM: A decrease of time spent in the open arms by «Ag+ 30» mice compared to «Control+ 30» was observed after 30 days of the experiment (Figure 1). It shows the enhanced level of anxiety in «Ag+ 30» mice.

Increase of time spent in open arms of EPM was observed in «Ag+ 120» compared to «Control+ 120» (Figure 1). It shows anxiety decrease in «Ag+ 120» mice. Decrease of time spent in closed arms of EPM was also observed in «Ag+ 120» compared to «Control+ 120» (Figure 2). It shows anxiety decrease in «Ag+ 120» as well.

Increases of total distance moved and distance moved in the center of EPM were observed in «Ag+ 180» group compared to «Control+ 180» (Figure 3). It indicates locomotion increase in the «Ag+ 180» group.

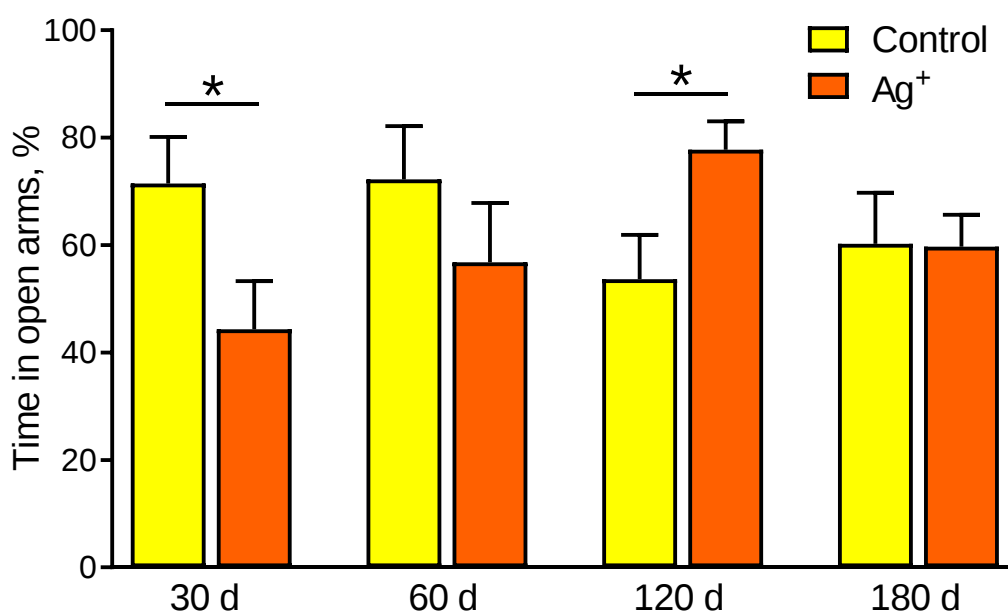


Figure 1. Time spent in open arms of EPM. Values are presented as mean $\pm$ SEM. * $p < 0.05$. Decrease of time spent in open arms was observed in «Ag+ 30» compared to «Control+ 30» and increase of the time was observed in «Ag+ 120» compared to «Control+ 120». It shows reverse in

the character of mice behavior at prolongation of silver citrate exposure periods. Increased anxiety is replaced by decreased anxiety.

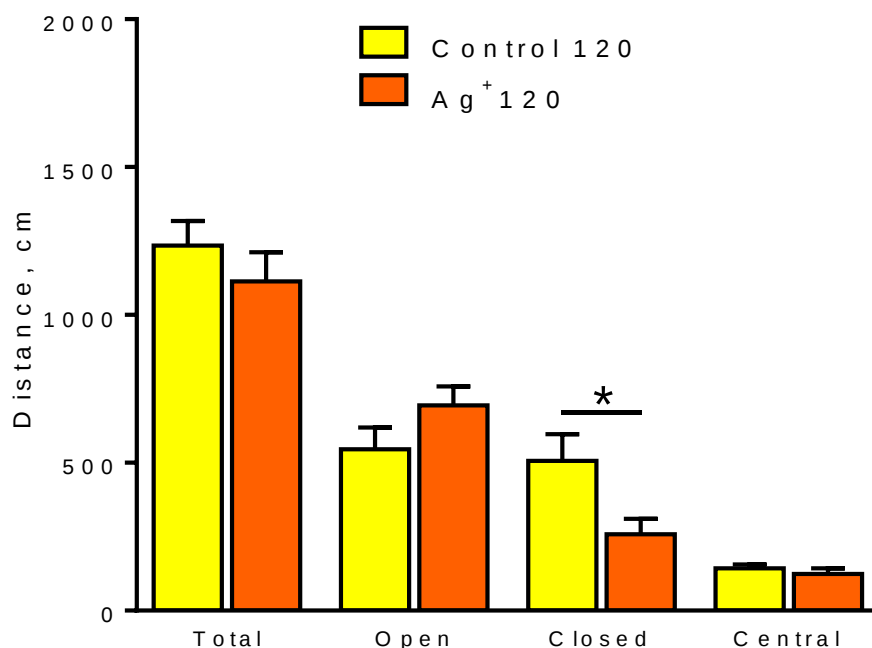


Figure 2. Distance moved in EPM by «Ag+ 120», «Control+ 120» such as total, in open arms, in closed arms, in center. Values are presented as mean $\pm$ SEM. * $p < 0.05$. Decrease of distance moved in closed arms was observed in «Ag+ 120» compared to «Control+ 120». It shows anxiety decrease in the mice exposed to silver citrate during 120 days.

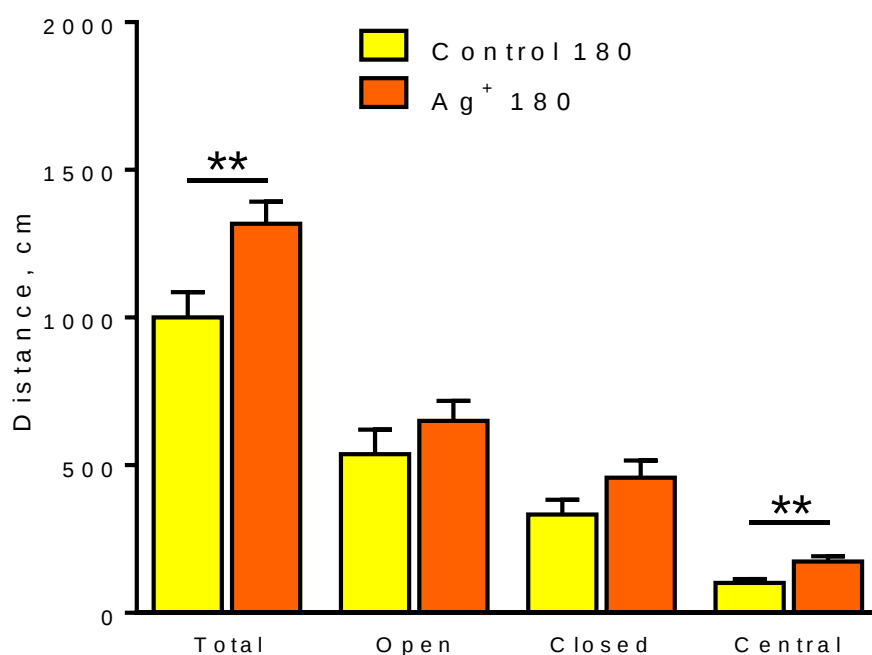


Figure 3. Distance moved in EPM by «Ag+ 180», «Control+ 180» such as total, in open arms, in closed arms, in center. Values are presented as mean $\pm$ SEM. * $p < 0.05$. Increase of total

distance moved and distance moved in center of EPM was observed in «Ag+ 180». It shows locomotion increase in the experimental animals.

CFCT: Increase of freezing time in «Ag+ 60» group compared to «Control+ 60» group was observed in 1 min after learning (Figure 4). The similar, however without significant differences, character of the ratios between experimental and control mice was observed in all the following by period of exposure groups as well. It may show increase of sensitivity (susceptibility) to the stressor in the form of negative experience of electric shocks in the mice exposed to silver citrate in comparison to the controls. Also, it shows that «Ag+ 60» mice learned better than «Control+ 60» mice. The trend remained for all the following by the period of exposure groups.

Tendency to increase of freezing time in «Ag+ 120» group compared to «Control+ 120» group ($p=0.0617$) was observed in 24 hours after learning in the CFCT (Figure 5). It demonstrates tendency to improve long-term contextual memory in «Ag+ 120» mice. Significant differences by the parameter were not observed for all the periods of exposure to silver citrate.

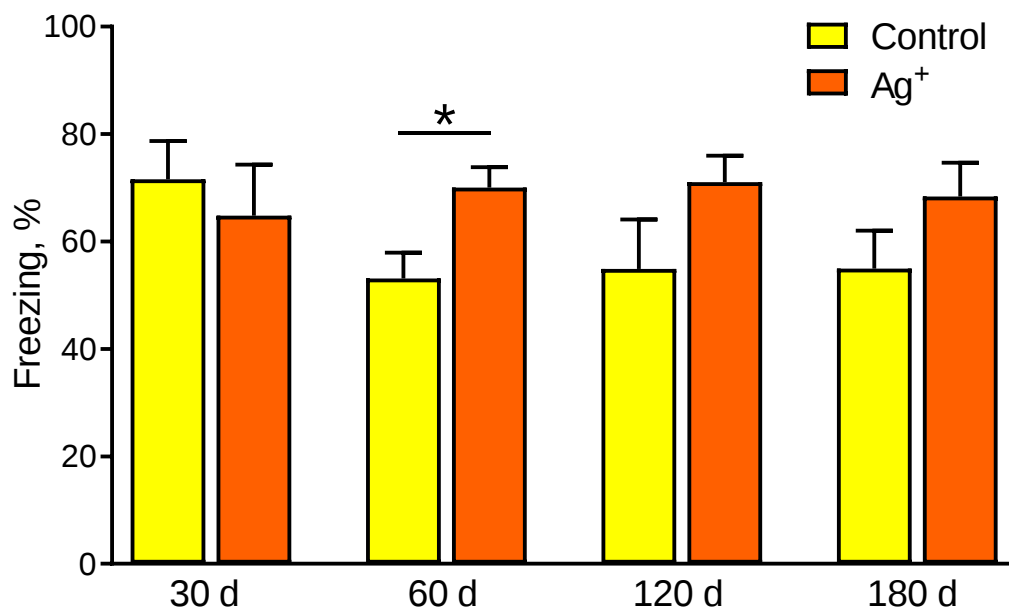


Figure 4. Freezing time in 1 min after learning in CFCT. Values are presented as mean $\pm$ SEM. * $p<0.05$. Increase of freezing time was observed in «Ag+ 60» compared to «Control+ 60». The similar character of the ratios between experimental and control mice, however without significant differences, was observed for all the following by time groups as well. It may show increase of sensitivity (susceptibility) in the experimental mice.

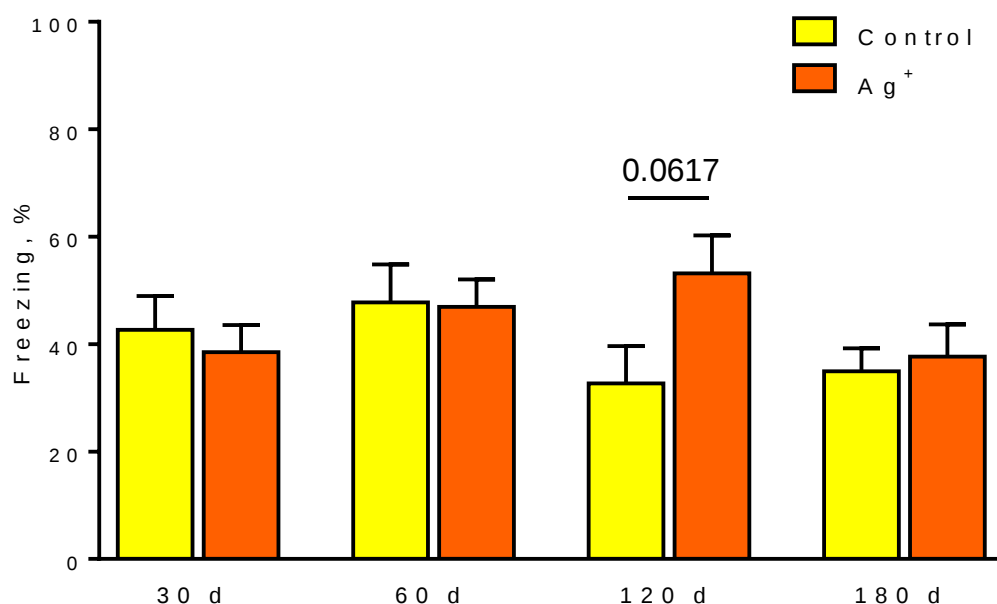


Figure 5. Freezing time in 24 h after learning in CFCT. Values are presented as mean $\pm$ SEM. * $p < 0.05$. Tendency to increase freezing time in 24 h after learning was observed in «Ag+ 120» compared to «Control+ 120». It shows tendency to improve long-term contextual memory in «Ag+ 120» mice.

Discussion

Changes of behavioral and cognitive functions at silver citrate exposure can be expressed as the 3-staged process such as 1) anxiety increase, 2) susceptibility (sensitivity) increase, 3) tendency to improve long-term contextual memory at the background of decreased anxiety and locomotion increase. Anxiety increase at the early stage is rather expectable and associated with response of the organism to stress induced by the exogenous substance such as silver citrate exposure. The tendency to improve long-term memory and locomotion increase are likely compensation to the anxiety increase observed at the early stage.

Susceptibility (sensitivity) increase may be regarded as the marker of successful compensation of anxiety, stimulating effect appearance.

We observed stimulating effect of behavioral and cognitive functions of mice at their long-term exposure to silver citrate in the present experiment. In our previous works on 60-days' exposure of mice to silver citrate we revealed absence of the influence in younger mice [28, 29] as well as increase of exploration behavior and decrease of anxiety in elder mice [29]. The result in elder mice shows stimulating effect of silver citrate as well. Herewith, equivalent dose and exposure period of silver nanoparticles increased anxiety in younger and in elder mice, while the level of anxiety was higher in the younger mice compared to the elder [30]. We have not found other works on silver citrate toxicity investigation in scientific literature. Let's consider the known studies on the toxicity of other silver salts such as silver acetate, which is a chelate complex as well, and silver nitrate, which is not a chelate complex.

There is a work on silver acetate and silver nanoparticles' influence on behavioral and cognitive functions of laboratory rats at daily intranasal exposure to them for 20 days [31]. Anxiety increase, locomotion and spatial memory disturbance were detected at exposure both to silver acetate and the nanoparticles. Herewith, silver acetate had greater toxic impact compared to the nanoparticles. A pronounced acidity of silver acetate and possible irritation of nose receptors that could strengthen the negative effect should be taken into account in this case. A relatively short

period of exposure such as 20 days should be taken into account as well. We observed impairment of behavioral functions after 30 days of exposure to silver citrate such as anxiety increase. Silver acetate influence on reproductive capacity, the offspring and the parents as well, which was introduced into male and female mice in different doses, was studied in [32]. A decrease of the consumed liquid and food by parents as well as their increase of locomotion were observed. Decrease of fertility, number of pups, and number of alive pups as well as pup's body weight was observed as well. The most pronounced changes compared to controls by the major number of parameters were found at the exposure to maximal dose of silver acetate such as 40 mg/kg bw. The authors believe that the effects are due to the binding of selenium by silver. Selenium is essential and vital element for mammals, which takes a key part in the processes, which are important for reproductive health, while its lack can cause different pathologies. Thus, granules of silver with selenium inclusions were observed in tissues after exposure of rats to silver acetate [33].

Toxicokinetics of silver powder, silver nanoparticles, silver acetate and silver nitrate after 28-day exposure of rats to them were studied in [34]. The highest bioavailability was found in the salts and the lowest related to the powder. The bioavailability of silver nanoparticles was in the middle.

Negative influence on long-term memory of 28-day exposure of rats to silver nitrate [35], as well as behavioral functions of fishes after 48-hour [36] and 72-hour [37] exposure were noted. Silver nitrate led to the fish lethality [36]. Silver acetate and silver nitrate were more cytotoxic and genotoxic compared to silver nanoparticles in *in vitro* experiment [38]. IC₅₀ for human hepatoblastoma inhibition by silver nitrate was 2-fold lower than IC₅₀ for silver nanoparticles [39]. It demonstrates higher toxicity of silver nitrate compared to silver nanoparticles.

Toxicity of the salt is determined by the properties of both ions – cation and anion [40]. Toxicity of silver chelate complexes strongly depends on each anion's toxicity itself. Silver salts demonstrated higher bioavailability as well as higher toxicity in comparison to silver nanoparticles as reviewed above. It should be taken into attention that solely short-term effects at silver compounds' exposure for no longer than 28 days were assessed in the previous works reviewed above. In the present study we esteemed effects of subchronic and chronic exposure to silver citrate from 30 to 180 days. Short-term effects are neutralized and long-term ones start to play significant role then. The long-term effects of silver nanoparticles onto living organisms are not sufficiently studied yet. However, we observed impairment of long-term contextual memory of mice after 180 days of daily exposure to silver nanoparticles [19].

The stimulating effect of silver citrate found in the present work can potentially be applied in Medicine, taking into account the existing permission for the use of silver chelate complexes in the production of food supplements for adults. Such a stimulating regenerative and wound healing effect of silver is known and successfully used surgery today [41]. There is a patent that demonstrates the effective wound healing and regenerative properties of copper-silver citrate composite [42].

Anti-neurodegenerative properties of silver nanoparticles such as possibility to treat Alzheimer's and Parkinson's diseases as well as multiple sclerosis are actively studied today [43-45]. Along this, the stimulating effect of silver citrate on behavioral and cognitive functions found in this work can be potentially used to treat such neurodegenerative diseases. Also, it is reasoned to consider the prospects of its use in the treatment of symptoms of mental illnesses such as depression, apathy as well as negative symptoms in schizophrenia. It is especially relevant according to the known difficulties and sometimes inefficiency in treatment of neurodegenerative and mental diseases by standard therapy. Herewith, the possible side effects should be minimized.

Influence of silver citrate and silver itself is definitely manifested via stress at the general organism level [46]. Thus, the concept of the mechanism underlying the proposed silver citrate

therapeutic action is in the words of well-known Canadian M.D. Hans Selye such as ‘Stress fallen on one system helps the other system to rest’ [47].

The possible terminological confusion should be pointed separately. Silver citrate and citrate coated silver nanoparticles are two completely different objects such as salt and nanoparticles. We encountered such a confusion during review of our previous manuscript in one European journal. The reviewers apparently using AI and having inattentively delved into our work stated the identity of these two objects. However, we should emphasize that these are principally different objects. Such a terminological confusion may bring to a wrong conclusion about the existing of sufficient knowledge on silver citrate toxic properties. In reality, however, there are enough data on citrate coated silver nanoparticles’ toxicity and just a few works on toxicity of silver citrate itself [28, 29].

Conclusions

We observed stimulating effect at long-term exposure to silver citrate such as tendency to improve long-term contextual memory and locomotion increase, which can be explained as compensation to anxiety increased at early stage and adaptation of the organisms of mice to the exogenous substance. Considering that silver chelate compounds are permitted for the production of food supplements for adults, such a stimulating effect may potentially be applied for a therapy of neurodegenerative diseases as well as symptoms of mental illness. Silver citrate may potentially replace the known to be toxic silver nanoparticles at the market.

Conflict of interest: Authors declare no conflict of interests

Ethics approval: The study was conducted according to GOST 33215-2014 “Guidelines for the Maintenance and Care of Laboratory Animals. Rules for Equipping Premises and Organizing Procedures” and approved by the Local Ethical Committee on Biomedical Research of the National Research Center “Kurchatov Institute” (Protocol No. 2, 07 October 2021).

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