ANIMAL EXPERIMENT

Effects and mechanisms of scalp acupuncture on learning and memory in mice with lead poisoning

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Abstract

Objective: To study the effects and mechanism of scalp acupuncture on learning and memory ability in mice with lead poisoning.

Methods: From March 2018 to December 2018, 30 Kunming mice were randomly divided equally into the control group and the intervention group after intraperitoneal injection of lead acetate The intervention group received scalp acupuncture on the first day of the model establishment; the model group only received conventional feeding without treatment. At the same time, a control group of 15 rats was given the intraperitoneal injections of normal saline for 8 consecutive days, and only after routine feeding, no treatment was given. Determination of lead in blood was detected by Graphite Furnace Atomic Absorption Spectrometry, the Morris water maze test was used to detect the learning and memory function of mice, hydroxylamine colorimetric method was used to measure acetylcholinesterase (AChE) activity, and TUNEL staining was used to detect the apoptotic cells in the hippocampus.

Results: The results showed that the blood lead level of the model group (231.42±12.53μg/L) was significantly higher than that of the control group (20.43±4.62μg/L) (P<0.05); and there was no significant difference in blood lead content between the intervention group (228.12±5.21μg/L) and the model group. The Morris water maze test showed that from the fourth day of the orientation navigation experiment, the escape latency of the model group (22.2±4.10s) was longer than that of the control group (13.64±2.93s) (P<0.05); besides, from the third day, the escape latency of mice in the intervention group (13.52±9.18s) was significantly shortened compared with the model group (19.95±3.52s). In the space exploration experiment, in terms of passing through the platform, the distance (1.57±0.49m) and time (15.54±3.72s) of mice in the model group were longer than that of mice in the control group (0.73±0.44m, 3.24±2.24s) (P<0.05), the distance (0.41±0.28m) and time (3.0±1.93s) of mice in the intervention group were shorter than that of mice in the model group, and the difference was statistically significant (P<0.05). The apoptosis rate of hippocampus in the model group (8.79±0.37%) was significantly higher than that in the control group (3.56±0.44%) (P<0.05), and the apoptosis rate of hippocampus in the intervention group (4.36±0.12%0 was significantly lower than that in the model group (P<0.05). The expression of AchE in the model group (0.5±0.13U/ug) was significantly higher than that in the control group (0.23±0.04U/ug), but there was no significant difference in the AChE activity between the intervention group and the model group.

Conclusion: In conclusion, scalp acupuncture can improve the learning and memory ability of mice with lead poisoning, and the decrease of hippocampal apoptotic cells may be a possible mechanism for the improvement of learning and memory function.

Keywords: Scalp acupuncture, Lead poisoning, Learning and memory, Apoptotic cells, Acetylcholinesterase.

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Introduction

Lead, a heavy metal known to cause neurotoxicity, is currently the most common industrial poison in the environment, which has a serious impact on human beings and their living. Lead pollution comes from industries, school supplies and toys, drinking water, food and other industries. It can be said that lead pollution is everywhere. Lead can enter the body through food chains, air, water and soil, causing damage to multiple systems. Therefore, this study is of great significance for observing the effects of lead poisoning.

Lead poisoning damages the body, in which the most harmful damage is to the central nervous system. Especially, among the children, if the lead load in their systems is slightly increased, it will cause damage to their nervous systems, resulting in poor growth, mental decline, unresponsiveness, and concentration disorders.[1] The scalp acupuncture, also known as the scalp-acupuncture therapy, is a treatment that stimulates specific sites in the hairline area of the head to cure diseases; it is widely applied to cognitive disorders including vascular dementia and Alzheimer's disease and

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achieved satisfied therapeutic effects. The Morris water maze test, including the orientation navigation experiment and the space exploration experiment, is often applied to evaluate the learning and memory functions of rodents.[2,3]

The experiment explores the possible mechanism of scalp acupuncture affecting the learning and memory behaviours through establishing the mice models poisoned with lead acetate, treating the models with scalp acupuncture, evaluating the effects of scalp acupuncture on the learning and memory abilities of mice with lead poisoning through the Morris water maze test, as well as detecting the blood lead level, the hippocampus apoptotic cells and the acetylcholinesterase (AchE) activity. This study will also analyze the effects and mechanism of scalp acupuncture on learning and memory ability in mice with lead poisoning.

Materials and Methods

The study was conducted and completed at the Hangzhou Red Cross Hospital from March 2018 to December 2018. The experimental operations of this study were approved by the Ethics Committee of the Hangzhou Red Cross Hospital.

A total of 65 healthy male CL Kunming mice weighing 20-30g were provided by Jiangsu Changzhou Cavens Lab Animal Corporation, animal certificate No. SCXK (Su) 2011-0003. Feeding environment: temperature was 18-22°C and humidity 50-60%.

TUNEL apoptosis detection kit (Jiangsu Keygen Biotech), DAPI cell colorant (Shanghai Beyotime Biotechnology), acetylcholinesterase (AchE) test kit (Nanjing Jiancheng Bioengineering Institute) was utilized.

In accordance with the methods of Zhang Ji et al., the 50 healthy CL Kunming mice were poisoned by intraperitoneal injection of lead acetate (7mg/kg, 8 consecutive days).[4] The criteria of success model were that the blood lead level was >200ug/L and significantly higher than that of the normal control group and the orientation navigation and space exploration abilities of mice decreased in the Morris water maze test, i.e. the learning and memory behaviours were damaged. The sampling capacity of the actual experiment was 50, of which 40 models were successfully established (80%).

A total of 30 successful mice models were randomly selected as the experimental objects and divided into the model group and the intervention group, with 15 mice in each group; meanwhile, a total of 15 healthy CL Kunming mice were selected as the control group. The scalp acupuncture was given to the intervention group; in accordance with the Experimental Acupuncture Science, Baihui and Dazhui acupoints, which were selected as the position for the acupuncture treatment. The scalp acupuncture was given 20 minutes each day for 7 consecutive days. The model group was given routine feeding with no other treatment. The control group was given the intraperitoneal injections of the equivalent dosage of saline for 8 consecutive days; then the mice were kept on routine feeding with no other treatment.

All the mice were submitted to the water maze test 8 days after the treatment was finished, and the escape latency was tested. The platform was placed into the center of a random quadrant for the orientation navigation experiment to evaluate the spatial memory acquisition ability of mice: all the mice were successively placed into the water tank from entry point A, B, C, and D with their faces facing to the tank wall, the time spans that the mice took to find and climb up to the platform within 60 seconds were observed and recorded (the escape latency). If the mouse failed to find the platform within 60 seconds, it was led to the platform and stayed on the platform for 15 seconds, and its escape latency was recorded as 60 seconds. The space exploration experiment would evaluate the accurate memorizing ability of the mice in terms of memorizing the platform area. On the 5th day of the water maze test, after the orientation navigation experiment, the platform was removed; facing to the tank wall, the mice were placed into the water from the entry point in quadrant II, the times that mice passed through the area of platform and the distances that mice swam were observed and recorded.

After the Morris water maze test was finished, the mice were executed by chloral hydrate; 1.0mL of mice whole blood was taken and anti-coagulated with heparin; the Graphite Furnace Atomic Absorption Spectrometry was applied to assay the blood lead levels of mice.

Both the left and right cerebral hemispheres of the executed experimental mice were taken and soaked into the pre-cooled physiological saline immediately to remove the blood. The filter papers were used for draining the water and the blood vessels of mice cerebral pia mater were removed. The left brain was weighed and placed into a micro homogenizer, added with 0.1mL of water-free ethanol and triturated, then added with 0.9mL of physiological saline and fully triturated into homogenate. The homogenate was centrifuged at 4°C 4500 xg (centrifugal force) for 15min, then the supernatant was taken. The hydroxylamine colorimetric

method was applied to detect the activity of AchE; all the operations strictly followed the instructions of the reagent kit.

The TUNEL staining was applied. The right brain was made into paraffin sections, routine dewaxed, hydrated with ethanol, rinsed with PBS buffer, digested by protease K working solution, and rinsed again with PBS buffer, then the sections were sealed in the 30% H_2O_2 blocking solutions at room temperature and rinsed with PBS buffer. The absorbent papers were applied to drain the water around the samples. Afterward, the samples were added with terminal deoxynucleotidyl transferase (TdT) reaction solution dropwise, rinsed with PBS buffer, dripped with fluorescein working fluid, rinsed with PBS buffer, dripped with DAPI colourant, rinsed with PBS buffer, and detected under a fluorescence microscope. Staining results gave the following results: the nucleus was stained blue by DAPI, the apoptotic cells were stained green by TUNEL, the apoptosis rate calculation equation=number of TUNEL staining cells/number of DAPI staining cells * 100%.

The SPSS 20.0 statistics software was applied to analyze the data; all the measurement data were expressed as ($\chi \pm s$), the group comparisons were analyzed by One-way ANOVA, $P<0.05$ was statistically significant.

Results

Mice Blood Lead Level Results

As shown in Figure-1, the blood lead content of mice in the model group was 231.42±12.53μg/L, which was significantly higher than in the control group, indicating statistical significance. However, no significant differences in blood lead content between the intervention group and the model group were found.

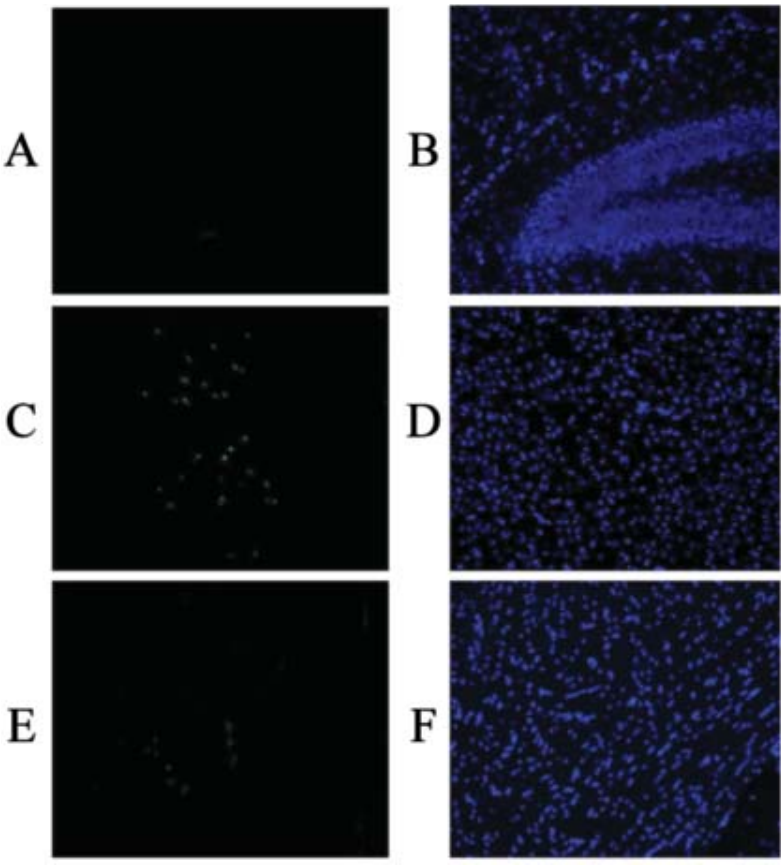
Morris Water Maze Test Results of Mice

As shown in Table-2, in the orientation navigation experiment, compared with the control group, as the time duration of training increased, since the 4th day, the escape latency of mice in the model group increased significantly, $P<0.05$, it was of statistical significance; besides, after scalp acupuncture treatment, since the 3rd day, compared with the model group, the escape latency of mice in the intervention group decreased significantly, indicating statistical significance. As shown in Table-3, in the space exploration experiment, the distance and time of mice in the model group in terms of passing through the platform was longer than that in the control group, after scalp acupuncture treatment, the distance and time of mice in the intervention group in terms of passing through the platform was shorter than

Table-1: The blood lead level results of mice in the 3 experimental groups (μg/L, $\chi \pm s$).

Group	Number of animals	Blood lead level
The control group	15	20.43±4.62
The model group	15	231.42±12.53##
The intervention group	15	228.12±5.21##

Note: Compared with the control group, # $P<0.05$ indicated statistical significance, # $P<0.01$ indicated remarkable statistical significance. Compared with the model group, & $P<0.05$ indicated statistical significance, && $P<0.01$ indicated remarkable statistical significance.



Note: A: the TUNEL staining of the control group (×200); B: the DAPI staining of the control group (×200); C: the TUNEL staining of the model group (×200); D: the DAPI staining of the model group (×200); E: the TUNEL staining of the intervention group (×200); F: the DAPI staining of the intervention group (×200).

Figure-1: The staining results of hippocampus apoptotic cell of mice in the 3 experimental groups.

Table-2: The results of escape latency in orientation navigation experiment of mice in the 3 experimental groups (s, $\chi \pm s$).

Group	Number of animals	Day 2	Day 3	Day 4	Day 5
The control group	15	13.42±2.78	16.33±3.76	13.64±2.93	11.79±2.24
The model group	15	13.43±2.34	19.95±3.52	22.2±4.10#	19.73±2.88#
The intervention group	15	13.64±1.62	13.52±9.18	15.63±9.62&	12.54±2.55&

Note: Compared with the control group, # P<0.05 indicated statistical significance, ## P<0.01 indicated remarkable statistical significance. Compared with the model group, & P<0.05 indicated statistical significance, && P<0.01 indicated remarkable statistical significance.

Table-3: The results of space exploration experiment of mice in the 3 experimental groups ($\chi \pm s$).

Group	Number of animals	Distance (m)	Time (s)
The control group	15	0.73±0.44	3.24±2.24
The model group	15	1.57±0.49#	15.54±3.72#
The intervention group	15	0.41±0.28&&	3.0±1.93&&

Note: Compared with the control group, # P<0.05 indicated statistical significance, ## P<0.01 indicated remarkable statistical significance. Compared with the model group, & P<0.05 indicated statistical significance, && P<0.01 indicated remarkable statistical significance.

Table-4: The AchE expressions of mice in the 3 experimental groups ($\chi \pm s$).

Group	Number of animals	AchE activity (U/ug)
The control group	15	0.23±0.04
The model group	15	0.5±0.13#
The intervention group	15	0.43±0.06

Note: Compared with the control group, # P<0.05 indicated statistical significance, ## P<0.01 indicated remarkable statistical significance. Compared with the model group, & P<0.05 indicated statistical significance, && P<0.01 indicated remarkable statistical significance.

Table-5: The differences of hippocampus apoptosis rate of mice in the 3 experimental groups ($\chi \pm s$).

Group	Number of animals	Apoptosis rate (%)
The control group	15	3.56±0.44
The model group	15	8.79±0.37#
The intervention group	15	4.36±0.12&

Note: Compared with the control group, # P<0.05 indicated statistical significance, ## P<0.01 indicated remarkable statistical significance. Compared with the model group, & P<0.05 indicated statistical significance, && P<0.01 indicated remarkable statistical significance.

that in the model group, P<0.01, the result was of statistical significance.

Measurement Results of Ache Activity

As shown in Table-4, the AchE expressions of mice in the model group increased significantly compared with the control group; however, after scalp acupuncture treatment, the AchE expressions of mice in the intervention group had no significant difference with that of mince in the model group.

Results of TUNEL Staining

As shown in Table-5 and Figure-1, the hippocampus apoptosis rate of mice in the model group increased significantly compared with the control group, the results are of statistical significance. However, after the scalp acupuncture treatment, the hippocampus apoptosis rate of mice in the intervention group decreased significantly compared with the model group.

Discussion

Lead is neurotropic and acts on the central neurotrophin system by passing through the blood brain barrier, leading to learning and memory dysfunction.[5] Although the effect of lead on developing neurotrophin systems is more obvious, research has proved that lead could also affect the neurotrophin systems of adults, leading to learning and memory dysfunction.[6,7]

Some studies have found that compared with the healthy volunteers, the delayed recall, attention, and the Montreal Cognitive Assessment (MOCA) scores of lead-affected people whose blood lead level are >200ug/L are obviously lower, indicating statistical significance.[8] Besides, the effects of lead on cognitive function would persist after years of separation from occupational exposure.[9] Schwartz BS et al. traced the lead-affected people who had been moved from occupational exposure for 16 years; their research found that the memories, the visual constructing abilities, and the learning and cognitive functions of the lead-affected people were obviously lower than that of their peer groups; besides, the reason of such damages were related to the high tibia lead levels.[10] In this experimental study, the blood lead levels of adult mice in the model group were significantly higher than the control group; besides, in the Morris water maze test, the average escape latency and the distances and time spans of passing through the platform of adult mice in the model group were significantly longer than the control group. Therefore, it is considered that the lead poisoning damaged the learning and memory abilities of adult mice.

Current studies have proved that the possible mechanisms of lead poisoning causing learning and memory dysfunction include the following: 1. Lead poisoning would enhance the Bax gene expression and reduce the Bcl-2 gene expression of hippocampus neuron, causing the apoptosis of hippocampus neurons.[11,12] Hippocampus is the important anatomical basis and nerve center of learning and memory; any form of damages to its neurons would lead to learning and memory dysfunction.[2] Lead poisoning would reduce the releases of multiple nerve transmitters including acetylcholine, dopamine, and amino acid in the central neurotrophin system, thereby affecting the learning and memory function. This experiment also showed that the AchE expression and the apoptosis rate of hippocampus neurons of mice in the model group were increased as compared to the control group.[13,14] Clinically, the commonly applied lead poisoning therapy is chelator treatment; chelator would excrete the excessive lead in organisms; however, it is incapable of effectively repairing the damage to the neurotrophin system caused by lead poisoning. Therefore, safe and effective treatment for damaged nerves is the focus of current clinical research.[15-20]

According to Chinese Traditional Medicine, scalp acupuncture could stimulate the head related meridians and acupoints, regulate the function of organs and blood circulation, thereby treat central neurotrophin system disorders, which has been confirmed in various neurological cognitive dysfunction diseases. Acupuncture has been widely used in China. Acupuncture has a potential role in improving learning and memory ability. It can be used as a treatment for memory loss, lead poisoning brain injury and other related diseases. Thus, the paper selected Baihui and Dazhui acupoints for scalp acupuncture stimulation to observe the improvement of learning and memory function of mice with lead poisoning. The results have shown that in the Morris water maze test, compared with the model group, the escape latency and the distances and time spans of passing through the platform of mice in the intervention group significantly decreased (P values were all <0.05). Meanwhile, the mice hippocampus apoptotic cells of the intervention group were obviously reduced, $P<0.01$, it was of statistical significance. The results indicated that the scalp acupuncture treatment would improve the learning and memory functions of mice with lead poisoning. Its mechanism could relate to the reduction of lead poisoning hippocampus apoptotic cells caused by the stimulation of Baihui and Dazhui acupoints that the scalp acupuncture brings. In addition, the research has discovered that compared with the model group, the AChE activity of mice in the intervention group was of no significant differences, indicating that the acetylcholine transmitters may not be involved in the improvement of learning and memory abilities of lead poisoning mice through scalp acupuncture.[21-25]

There are also some related studies to explain the mechanism of acupuncture. It is found that acupuncture treatment can significantly promote growth and development, improve neurobehavioral function, and improve learning and memory ability. It can reduce the apoptosis of hippocampal cells and up regulate the levels of GDNF and BDNF. It may play a neuroprotective role by inhibiting the apoptosis of hippocampal cells and increasing the expression of GDNF and BDNF in hi rats.[26] In addition, researches have been carried out on acupuncture to change the cognitive state. It is found that acupuncture shortens the escape latency of the anterior platform quadrant and prolongs the stay time of the anterior platform quadrant in mice. After acupuncture, the number of apical and basal dendrites and the total length of apical and basal dendrites increased significantly. The research shows that acupuncture improves the ability of spatial learning and memory of mice by improving the dendritic structure.[27] Acupuncture not only has a good effect on the improvement of simple cognitive impairment, but also has a good effect on patients with complex diseases. For example, some studies have found that the cognitive ability of rats suffering from diabetes and cerebral infarction is significantly reduced, and the brain tissue and nerves are obviously damaged. Acupuncture has a good effect on the changes of behaviour and brain morphology, which is helpful to improve the learning and memory ability.[28]

Conclusion

In summary, scalp acupuncture reduces the hippocampus apoptotic cells in mice with lead poisoning, which could be one of the mechanisms for improving the learning and memory abilities of mice. The research has provided an evidence for clinical scalp acupuncture treatment of lead poisoning in mice.

Disclaimer: I hereby declare that this research paper is my own and autonomous work. All sources and aids used have been indicated as such. All texts either quoted directly or paraphrased have been indicated by in-text citations. Full bibliographic details are given in the reference list which also contains internet sources. This work has not been submitted to any other journal for consideration.

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