

Effects of Voluntary Wheel Running Exercise on Learning and Memory Function of Young Mice and Related Mechanisms

Sun Wei¹, WU Qiao¹

¹ Department of Psychiatry, the Third People's Hospital of Mianyang, Mianyang, Sichuan, 621000, China

Corresponding author: WU Qiao, Email: wuqiao_self@163.com

Keywords: Learning and memory function, Voluntary wheel running exercise, Plasminogen activator inhibitor-1 (pai-1), Mirna (mir)-30b

Abstract: Objective: This study is to investigate the effects of appropriate exercise on learning and memory function of mice, and the related mechanisms involving PAI-1 and miRNA (miR)-30b. Methods: Mice were subjected to the voluntary wheel running exercise training for 8 m. Morris water maze test was performed to assess the animal learning and memory function. Quantitative real-time PCR was conducted to detect the mRNA expression levels, while Western blot analysis and ELISA were used to determine protein expression levels. Bioinformatics analysis and dual-luciferase reporter assay were used to predict and confirm the up-stream regulator of PAI-1. Results: Morris water maze test showed that, compared with the control group, the escape latency was significantly declined in the exercise group. The swimming distance was significantly declined, while the platform crossing number was significantly increased, in the exercise group. Quantitative real-time PCR and Western blot analysis showed that, compared with the control group, the mRNA and protein expression levels of PAI-1 in both the hippocampal and blood tissues were significantly declined in the exercise group. According to the bioinformatics analysis, miR-30b might be the up-stream regulator of PAI-1, which was confirmed by the dual-luciferase reporter assay. In addition, compared with the control group, the expression levels of miR-30b in both the hippocampal and blood tissue samples were significantly elevated for the exercise group. Conclusion: Appropriate amount of exercise could improve the mouse learning and memory function, which might involve the up-regulated miR-30b expression and down-regulated PAI-1 expression in the hippocampal and blood tissues.

1. Introduction

Human brain is a very complex organ, and the normal function of cerebral cortex provides the basis for cognition. Any factors associated with cerebral cortex functional and structural abnormalities could lead to learning and memory disorders. So far, investigation of learning and memory function has mainly focused on hippocampus, in which the neurons represent the research

hotspots in recent years [1].

Studies have shown that children's learning and memory ability could greatly benefit from appropriate amount of exercise [2]. Moreover, it has been demonstrated that prolonged active exercise can elevate the expression of BDNF mRNA in rat hippocampus, and enhance the learning and memory ability [3]. Furthermore, it has been shown that, in rat models, active training could significantly improve their performance in the maze test, indicating significantly increased learning and memory function [4]. In addition, running exercise has also been shown to be able to promote the rat hippocampus neural activity and synaptic activity, thereby promoting the learning and memory function [5]. These findings suggest that appropriate amount of exercise training could improve the body's learning and memory function. However, the specific underlying mechanisms are still unclear. Learning and memory ability is related to the brain blood flow [6]. Brain tissue ischemia, hypoxia, and nerve cell necrosis induced by head trauma, cerebral vascular inflammation, cerebral vascular stenosis, and cerebral embolism have been recognized as physiologically inducing factors for the pathogenesis of cognitive disorders [7]. Therefore, brain vascular lesions have been intensively studied for its involvement in the process of cognitive impairment in recent years.

Abnormal hemorheology is one of the causes of brain vascular disorders. Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor that inactivates plasminogen activator t-PA and u-PA, which inhibits intravascular fibrinolysis and causes hemorheological changes, finally aggravating ischemic injuries [8]. It has been reported that PAI-1 is a risk factor for the development of ischemic heart and brain diseases [9, 10]. Moreover, PAI-1 is the target gene of miRNA (miR)-30b in regulating the proliferation and apoptosis of gastric cancer cells [11]. However, the up-stream regulators of PAI-1 in the pathogenesis and development have not yet been reported.

In this study, the mechanisms through which appropriate exercise improved the learning and memory functions were investigated. Mouse models were subjected to the voluntary wheel running exercise, and their behavioral performance in the Morris water maze test was evaluated. Moreover, the expression levels of related factors in the hippocampal and blood tissues were detected with the quantitative real-time PCR, Western blot analysis, and ELISA.

2. Materials and Methods

2.1 Study Animal Grouping and Voluntary Wheel Running Exercise

Male C57BL/6J mice, weighing 18-22 g, were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). These animals were housed for one week before experiments, with free access to food and water. Animal experimental procedures were approved by the local animal care committee.

Totally 56 mice were finally subjected to the following experiments. These animals were randomly divided into the control and exercise groups ($n = 28$). Mice in the exercise group received the voluntary wheel running exercise training. Self-made running wheels were equipped with the electronic counter. These mice were forced to run for 1 h every day, from 5:00 PM to 6:00 PM, for totally 8 w.

2.2 Morris Water Maze Test

After the voluntary wheel running exercise training, the Morris water maze test was performed on the DMS2-Morris water maze system (Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China), to assess the animal behavioral performance. For the navigation test, the mice were trained for the first 4 d, four times each day, in

a 30-cm depth water pool at room temperature. The mouse was put into the water, facing the wall, in the quadrants except for the one including the platform. Training on each day lasted for 60 s. If the mouse found the escape platform within 60 s, the swimming time was recorded as the latency; if not, the mouse was guided to the platform, and the latency was recorded as 60 s. After swimming, these mice were allowed to stay on the platform for 15 s, and then the next training started. On day 5, the space exploration test was conducted, in which the platform was removed. The mouse was put in to the water to swim for 60 s. The number it swimming across the platform region and the swimming path were recorded.

2.3 Sample Preparation

After behavioral tests, the tissue samples were collected. These mice were anesthetized by abdominally injected with 10% chloral hydrate under 12-h fast condition. Blood sample was collected through the abdominal aorta puncture, from which serum was separated and stored at -80°C . Then the mice were sacrificed by decapitation, and the hippocampi were removed. Tissue was washed with 0.9% saline, and stored at -80°C .

2.4 Quantitative Real-Time Pcr

Total RNA was extracted with Trizol. Totally 1 μg RNA was used for the reverse transcription to obtain the cDNA template. Quantitative real-time PCR was performed with the miRcute miRNA kit (FP401; Tiangen, Beijing, China) on an iQ-5 PCR system (Bio-Rad, Reinach, Switzerland). The primer sequences were as follows: PAI-1, forward 5'-AGGGCTTCATGCCCACTTCTTCA-3' and reverse 5'-AGTAGAGGGCATTACCAGCACCA-3'; and GAPDH, forward 5'-CAAGGTCATCCATGACAACCTTTG-3' and reverse 5'-GTCCACCACCCTGTTGCTGTAG-3'. The 20- μg reaction system consisted of 10 μl qRT-PCR Mix, 0.5 μl primer each, 2 μl cDNA, and 7 μl ddH₂O. Reaction conditions were set as follows: 95°C for 2 min; followed by 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, for totally 40 cycles. For the detection of miR-30b expression level, the primer sequences were as follows: miR-30b, forward 5'-GCGCCTGTAAACATCCTACAC-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'; and U6, forward 5'-GCTTCGGCAGCACATATACTAA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'. Reaction conditions included 95°C for 30 s, followed by 95°C for 5 s, 60°C for 30 s, for totally 45 cycles. Expression levels of target genes were calculated with the $2^{-\Delta\Delta\text{Ct}}$ method. GAPDH and U6 were used as internal interferences.

2.5 Western Blot Analysis

Tissue was lysed with the lysis. Protein concentration was determined with the BCA method (RTP7102; Real-Times, Beijing, China). Totally 20 μg protein was separated by 10% SDS-PAGE, and then electronically transferred onto the membrane. After blocking with 5% non-fat milk at room temperature for 1 h, the blot was incubated with rabbit anti-mouse anti-PAI-1 (ab7205; 1:1000 dilution; Abcam, Cambridge, MA, USA) and rabbit anti-mouse anti- β -actin (1:5000 dilution; Abcam) primary antibodies, respectively, at 4°C overnight. Then the membrane was incubated with the secondary antibody (ab6721; 1:3000 dilution; Abcam) at room temperature for 1 h. Protein band development was performed with the ECL method (ab65623; Abcam). Images were acquired and analyzed with the Image Lab software. β -actin was used as internal interference.

2.6 Enzyme-Linked Immunosorbent Assay (Elisa)

Blood sample was centrifuged at 3000 rpm for 10 min to separate the serum and red blood cells. The expression levels of PAI-1 in the serum samples were assessed with an ELISA kit (ab157529; Abcam), according to the manufacturers' instructions. Briefly, 10 μ l sample and 40 μ l diluting solution were added into the plate wells. Then 100 μ l HRP-conjugated detection antibody was added. The plate was sealed and incubated at 37°C for 1 h. After washing, 50 μ l substrates A and B each were added, followed by incubation at 37°C for 15 min. After adding 50 μ l stop solution, the OD value at 450 nm was detected within 15 min. Concentrations of PAI-1 were calculated according to the standard curve.

2.7 Bioinformatics Analysis

Based on the literature retrieval concerning the up-stream regulating miRNAs of PAI-1 (PAI-1 has been shown to be regulated by miR-30b in gastric cancer [7]), bioinformatics prediction was performed with the following prediction software: miRanda (<http://www.microma.org/rnicroma/home.do>), TargetScan (www.targetscan.org), PiTa (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>), and PICTA (<http://pictar.mdc-berlin.de/>).

2.8 Dual-Luciferase Reporter Assay

The normal and mutant seed regions of PAI-1 3'-UTR targeted by miR-30b were synthesized (Sangon Biotech, Shanghai, China), and the digestion sites of Spe-1 and HindIII were added at both ends. These two kinds of DNA fragments were cloned into the PMIR-REPORT luciferase reporter plasmid. Mutant 3'-UTR seed region was used as control. Totally 0.8 μ g plasmids containing wild-type and mutant 3'-UTR sequences were transfected into the 293T cells with the liposome, which were then transfected with 100 nM agomiR-30b for 24 h. Then the cells were lysed, and the fluorescence was determined by the GloMax 20/20 luminometer. *Renilla* was used internal interference.

3. Statistical Analysis

Data were expressed as mean \pm SD. SPSS 18.0 software was used for statistical analysis. After the normality test, one-way ANOVA was performed for group comparison, with LSD and SNK methods for homogeneous variance, or with Tamhane's T2 or Dunnett's T3 method for non-homogeneous variance. $P < 0.05$ was considered statistically significant.

4. Results

4.1 Animal Behavioral Tests of Mice after Voluntary Wheel Running Exercise

To investigate the effects of voluntary wheel running exercise on the mouse learning and memory functions, animal behavior after training was assessed by the Morris water maze test. For the navigation test, our results showed that, on days 1-2, compared with the control group, no significant differences were observed in the escape latency for the exercise group ($P > 0.05$). On days 3-4, compared with the control, the escape latency was significantly declined in the exercise group ($P < 0.05$) (Table 1). On the other hand, results from the space exploration on day 5 showed that, compared with the control group, the total distance of the swimming path was significantly declined, while the numbers of crossing platform region were significantly increased, in the exercise group (both $P < 0.05$) (Table 2). Taken together, these results suggest that voluntary wheel

running exercise could improve the learning and memory functions in mice.

4.2 Expression Levels of Pai-1 in Hippocampal and Blood Tissues

To investigate the mRNA and protein expression levels of PAI-1 in the hippocampal and blood tissues, quantitative real-time PCR and Western blot analysis were performed, respectively. Our results from the quantitative real-time PCR showed that, compared with the control group, the mRNA expression levels of PAI-1 in both the hippocampal and blood tissues were significantly declined in the exercise group (both $P < 0.05$). Similar results were observed in the detection of protein expression of PAI-1 in hippocampal and blood tissues by Western blot analysis. Compared with the control group, the protein expression levels of PAI-1 in both the hippocampal and blood tissues were significantly declined in the exercise group (both $P < 0.05$) (Fig. 1). Taken together, these results suggest that voluntary wheel running exercise could decline the mRNA and protein expression levels of PAI-1 in mice, which might contribute to the enhancement of the mouse learning and memory function.

4.3 Prediction and Confirmation of Up-Stream Regulator of Pai-1

In order to investigate the up-stream regulator of PAI-1, bioinformatics analysis was first performed with the miRanda, TargetSean, PiTa, RNAhybrid, and PICTA software. Our results indicated that miR-30b might be the up-stream regulator of PAI-1. To further confirm this prediction, dual-luciferase reporter assay was conducted. Our results showed that, the co-transfection of agomiR-30b and pMIR-REPORT reporter plasmids significantly declined the fluorescence ($P < 0.05$), while no significant difference was observed in the fluorescence for the mutation group ($P > 0.05$) (Fig. 2). These results suggest that miR-30b could bind to the PAI-1 3'-UTR to regulate its expression.

4.4 Expression Levels of Mir-30b in Hippocampal and Blood Tissues

Quantitative real-time PCR was then performed to detect the expression levels of miR-30b in the hippocampal and blood tissue samples. Our results showed that, compared with the control group, the expression levels of miR-30b in both the hippocampal and blood tissue samples were significantly elevated for the exercise group (both $P < 0.01$) (Fig. 3). These results suggest that miR-30b might play a regulatory role in the learning and memory process of mice.

5. Discussion

In the present study, the behavioral performance of mice after 8-month voluntary wheel running exercise was investigated. The expression levels of miR-30b and its down-stream target PAI-1 in the hippocampal and blood tissues were detected. The mechanism through which miR-30b regulated PAI-1 to affect the learning and memory functions in mice were primarily explored.

It is generally believed that appropriate exercise training may promote the learning and memory function. Previous studies have shown that, long-term exercise could not only be able to accelerate the blood circulation in the body to ensure the brain can obtain adequate oxygen and nutrients, but also increase blood flow rate to increase the number of red blood cells and increase the hemoglobin content. Moreover, long-term exercise could improve the anti-acid/alkali ability and the anti-oxidase system function in the brain, to enhance the excitability, flexibility, balance, and coordination of the neural activity in cerebral cortex, which contributes to the improvement of body learning and memory function [12-14]. However, the exact mechanism through which exercise

training benefited the learning and memory ability is still not clear. Different conclusions have been addressed from different studies. In this study, our results showed that, compared with the control group, the escape latency was significantly shortened for the exercise group in the navigation test. Moreover, compared with the control group, the total distance of the swimming path was significantly declined, while the numbers of crossing platform region were significantly increased, in the exercise group. These results suggest that voluntary wheel running exercise could improve the learning and memory function in mice.

Studies have shown that vascular amyloid lesion of the cortical arteries and arterioles represent one of the pathologic features of learning and memory impairment. Moreover, it has also been shown PAI-1 is close associated with the vascular disorders. PAI-1 not only affects thrombosis, but also participates in extracellular matrix accumulation and proliferation of smooth muscle cells. PAI-1 induces the binding of low density lipoprotein and vascular smooth muscle cells, which might be deposited in the extracellular matrix and promote the formation of fatty streaks and atherosclerotic plaques. Furthermore, the vascular basement membrane would be thickened, resulting in vascular wall stiffness and thereby promoting vascular disease, especially for the occurrence and development of atherosclerosis [15-19]. Considering that some vascular lesions can cause abnormal blood flow, including cerebral ischemia, hypoxia, metabolite accumulation, and even cognitive disorders [20], it is of great importance to investigate the regulating mechanism of PAI-1 in the learning and memory process.

In theory, appropriately reduced PAI-1 expression could increase the amounts of plasminogen activator t-PA and u-PA in blood, maintaining the brain blood vessels, accelerating the blood circulation, increasing the oxygen supply in brain, and ultimately enhancing the learning and memory ability [21]. In the present stud, our results showed that, the mRNA and protein expression levels of PAI-1 were significantly declined in both the hippocampal and blood tissues in mice after voluntary wheel running exercise. These results suggest that PAI-1 might play a regulatory role in the enhanced learning and memory function of mice receiving voluntary wheel running exercise.

Regulation of mRNA transcription and expression is a complex process involving multiple factors. To study the up-stream regulators of PAI-1, we focused on a class of intracellular endogenous, small, non-coding miRNAs, which could cleave and inhibit the target mRNA to exert negative regulatory role [22, 23]. As important regulatory factors in physiological and pathological processes, many miRNAs have been recognized as biomarkers of various diseases [24, 25]. In this study, bioinformatics analysis was first performed to predict the up-stream regulator of PAI-1. Based on the literature mining, miR-30b might be one of the up-stream regulators of PAI-1 [11], and a previous study has shown that miR-30b might be associated with schizophrenia [26]. Our results showed that, the expression level of miR-30b was significantly elevated in the mice after voluntary wheel running exercise, which was opposite to the alteration of PAI-1 expression. In line with our findings, Zhu *et al.* [11] have shown that miR-30b negatively regulates the expression of PAI-1. Combined with the results from the animal behavioral tests, a close correlation among miR-30b, PAI-1, and learning and memory function in mice has been suggested.

In conclusion, our results showed that up-regulated miR-30b expression and down-regulated expression of PAI-1 might be the underlying mechanism through which voluntary wheel running exercise improve the learning and memory function in mice. Up-regulated miR-30b expression and down-regulated expression of PAI-1 might maintain the brain blood vessels, accelerate the blood circulation, and increase the oxygen supply in the brain. As a potent regulator of PAI-1, miRNA-30b may contribute to the investigation of learning and memory function enhancement, and provide evidence for the diagnosis, prevent, and treatment of cognitive impairment in the future.

6. Acknowledgements

Thank colleagues and family for their understanding and help.

References

- [1] Christie BR, Cameron HA. Neurogenesis in the adult hippocampus. *Hippocampus*.2006;16(3):199-207.
- [2] Hillman CH, Biggan JR. A Review of Childhood Physical Activity, Brain, and Cognition: Perspectives on the Future. *Pediatric exercise science*.2017;29(2):170-176.
- [3] Adlard PA, Perreau VM, Engesser-Cesar C, Cotman CW. The timecourse of induction of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus following voluntary exercise. *Neuroscience letters*.2004;363(1):43-48.
- [4] Anderson BJ, Rapp DN, Baek DH, McCloskey DP, Coburn-Litvak PS, Robinson JK. Exercise influences spatial learning in the radial arm maze. *Physiology & behavior*.2000;70(5):425-429.
- [5] van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proceedings of the National Academy of Sciences of the United States of America*.1999;96(23):13427-13431.
- [6] Ogoh S. Relationship between cognitive function and regulation of cerebral blood flow. *The journal of physiological sciences : JPS*. 2017;67(3):345-351.
- [7] de la Monte SM, Tong M. Brain metabolic dysfunction at the core of Alzheimer's disease. *Biochemical pharmacology*.2014;88(4):548-559.
- [8] Hua Y, Xi G, Keep RF, Wu J, Jiang Y, Hoff JT. Plasminogen activator inhibitor-1 induction after experimental intracerebral hemorrhage. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2002;22(1):55-61.
- [9] Eitzman DT, Westrick RJ, Xu Z, Tyson J, Ginsburg D. Plasminogen activator inhibitor-1 deficiency protects against atherosclerosis progression in the mouse carotid artery. *Blood*.2000;96(13):4212-4215.
- [10] Kohler HP, Grant PJ. Plasminogen-activator inhibitor type 1 and coronary artery disease. *The New England journal of medicine*.2000;342(24):1792-1801.
- [11] Zhu ED, Li N, Li BS, et al. miR-30b, down-regulated in gastric cancer, promotes apoptosis and suppresses tumor growth by targeting plasminogen activator inhibitor-1. *PloS one*.2014;9(8):e106049.
- [12] Frith E, Loprinzi PD. Physical activity and cognitive function among older adults with hypertension. *Journal of hypertension*.2017;35(6):1271-1275.
- [13] Monnier A, Garnier P, Quirie A, et al. Effect of short-term exercise training on brain-derived neurotrophic factor signaling in spontaneously hypertensive rats. *Journal of hypertension*.2017;35(2):279-290.
- [14] Chang YK, Alderman BL, Chu CH, Wang CC, Song TF, Chen FT. Acute exercise has a general facilitative effect on cognitive function: A combined ERP temporal dynamics and BDNF study. *Psychophysiology*.2017;54(2):289-300.
- [15] Denorme F, Wyseure T, Peeters M, et al. Inhibition of Thrombin-Activatable Fibrinolysis Inhibitor and Plasminogen Activator Inhibitor-1 Reduces Ischemic Brain Damage in Mice. *Stroke*.2016;47(9):2419-2422.
- [16] Seferovic MD, Gupta MB. Increased Umbilical Cord PAI-1 Levels in Placental Insufficiency Are Associated with Fetal Hypoxia and Angiogenesis. *Disease markers*.2016;2016:7124186.
- [17] Ji Y, Meng QH, Wang ZG. Changes in the coagulation and fibrinolytic system of patients with subarachnoid hemorrhage. *Neurologia medico-chirurgica*.2014;54(6):457-464.
- [18] Thogersen AM, Jansson JH, Boman K, et al. High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: evidence for the fibrinolytic system as an independent primary risk factor. *Circulation*.1998;98(21):2241-2247.
- [19] Hamsten A, de Faire U, Walldius G, et al. Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. *Lancet*.1987;2(8549):3-9.
- [20] Jiang H, Li X, Chen S, et al. Plasminogen Activator Inhibitor-1 in depression: Results from Animal and Clinical Studies. *Scientific reports*.2016;6:30464.
- [21] Oszejka K, Wronski K, Janiszewska G, Bienkiewicz M, Bartkowiak J, Szemraj J. The study of t-PA, u-PA and PAI-1 genes polymorphisms in patients with abdominal aortic aneurysm. *Molecular biology reports*.2014;41(5):2859-2864.
- [22] Inoue K. [MicroRNA function in animal development]. *Tanpakushitsukakusankoso. Protein, nucleic acid, enzyme*.2007;52(3):197-204.
- [23] Williams AE, Moschos SA, Perry MM, Barnes PJ, Lindsay MA. Maternally imprinted microRNAs are differentially expressed during mouse and human lung development. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2007;236(2):572-580.
- [24] Li X, Yu Z, Li Y, et al. The tumor suppressor miR-124 inhibits cell proliferation by targeting STAT3 and functions

- as a prognostic marker for postoperative NSCLC patients. *International journal of oncology*.2015;46(2):798-808.
- [25] Lv ZC, Fan YS, Chen HB, Zhao DW. Investigation of microRNA-155 as a serum diagnostic and prognostic biomarker for colorectal cancer. *Tumourbiology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2015;36(3):1619-1625.
- [26] Mellios N, Galdzicka M, Ginns E, et al. Gender-specific reduction of estrogen-sensitive small RNA, miR-30b, in subjects with schizophrenia. *Schizophrenia bulletin*.2012;38(3):433-443.
- [27] Figure legends
- [28] Fig. 1 Expression levels of PAI-1 in hippocampal and blood tissues.
- [29] (A-B) The mRNA expression levels of PAI-1 were detected with the quantitative real-time PCR in the hippocampal (A) and blood (B) tissues, respectively. (C-D) The protein expression levels of PAI-1 in the hippocampal (C) and blood (D) tissues were detected with the Western blot analysis and ELISA, respectively. Compared with the control group, * $P < 0.05$, ** $P < 0.01$.
- [30] Fig. 2 Dual-luciferase reporter assay.
- [31] Dual-luciferase reporter assay was performed to confirm the interaction between PAI-1 3'-UTR and miR-30b. Compared with the NC group, * $P < 0.05$, ** $P < 0.01$.
- [32] Fig. 3 Expression levels of miR-30b in hippocampal and blood tissues.
- [33] The expression levels of miR-30b were detected with the quantitative real-time PCR in the hippocampal (A) and blood (B) tissues, respectively. Compared with the control group, * $P < 0.05$, ** $P < 0.01$.