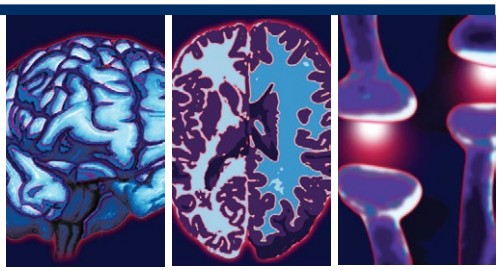

The Effects of Exercise Therapy on Motor Behaviors of Preterm Infant Rats Induced by Intrauterine Inflammation

Shu-ying Cai[1,†], Zhen-huan Liu[2], Gui-lan Peng[3], Rui-hua Tian[3], Pei Li[3]

Abstract

Objective:

The aim of this study was to investigate the effects of exercise therapy on motor behaviors of preterm infant rats induced by intrauterine inflammation.

Methods:

Lipopolysaccharide was intraperitoneally injected to pregnant rats to induce intrauterine inflammatory response, and then animal models of preterm infant rats were prepared. The preterm infant rats were randomly divided into two groups, including the exercise group (group A), and the model group (group B), while the normal infant rats without preterm birth produced by pregnant rats in the non-modeling group were used as control group (group C). Balance beam training, roller training, grasp training, and open-field test were performed to test the motor behaviors. HE and immunofluorescence staining of hippocampal tissues were performed. Image analysis was performed to observe the number of GFAP-positive astrocytes in hippocampus.

Results:

The test scores of motor behaviors in the control group were the highest; the model group showed the lowest, and no statistical significance was observed between the control and exercise groups. Compared with control group, the number of GFAP-positive astrocytes was increased in the model group with statistical significance ($P<0.05$). However, the number of GFAP-positive astrocytes was decreased in the exercise group after intervention of exercise therapy, which was statistically significant with the model group ($P<0.05$).

Conclusion:

Exercise therapy can improve motor behaviors in preterm rats caused by lipopolysaccharide-induced intrauterine inflammatory, and this might be due to the inhibition of reactive hyperplasia of astrocytes, which then plays a key role in repairing the damaged motor behaviors.

Keywords

Exercise therapy, Intrauterine inflammatory, Preterm birth, Motor behavior, Glial fibrillary acidic protein

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Introduction

Preterm infant, also known as premature infant, refers to live-born infant with a gestational age of less than 37 weeks. Prematurity is associated with elevated risks of brain injuries and in turn impaired quality of life. The two important factors that lead to brain injury in preterm infants are intrauterine infections and hypoxia-ischemia (mainly ischemia-reperfusion injury) [1]. Of these, intrauterine infection is the key factor that leads to preterm birth related pathological types such as brain injury [2], while utero-placental ischemia and the occurrence of preterm birth have an important association [3]. Furthermore, hypoxia-ischemia at perinatal and/or neonatal period also results in related encephalopathy, leading to long-term injury to the nervous system [4]. Studies have found that nervous system injury in preterm infants accounted for 23% at abroad, which was as high as 33.7% in China [5]. Therefore, the prevention and study of brain injury in preterm infants is of great importance to reduce the incidence of neurological sequelae as well as adverse prognosis in preterm infants.

Astrocytes are numerous and diverse neuroglial cells present in the central nervous system. These neuroglial cells play a significant role in regulating the function of blood-brain barrier, the formation of synapses, glutamate uptake, along with nutritional support to the surrounding neurons and neuroglia [6]. Glial fibrillary acidic protein (GFAP) is a protein that is composed of intermediate filaments in astrocyte, and is an important part of the cytoskeleton of astrocytes. Studies have revealed that GFAP is involved in the functions of astrocytes, such as nerve regeneration, synaptic plasticity, and proliferation of reactive glia [7]. The impact or role of astrocytes in brain injury of preterm infants differs. For example, a hypoxic environment in the perinatal period (chronic) would block the maturation of astrocytes, which stay in a more naive stage with reduced expression of GFAP and increased expression of nestin; while the expression of glutamate transporter would decrease, resulting in the damaged scavenging effect of excitatory toxins [8, 9]. However, on the other hand, in neuro-inflammatory responses caused by hypoxia-ischemia (rather than chronic hypoxia), astrocytes are activated to become reactive astrocytes, which are consecutively enriched in the cerebral white matter lesions, secrete hyaluronic acid, along with the inhibition of differentiation and maturation of oligodendrocytes and the formation of myelin [10]. Therefore, it is necessary to study the inhibition of hyperplasia of astrocytes which remains to be one of the key factors affecting neural repair after premature brain injury.

Hence, this study used infant rat models with preterm brain injury induced by intraventricular inflammation. According to the characteristics of pregnancy cycle of rats, the gestation period of normal rats are 19-23 days [11], while infant rats were delivered before day 19 of pregnancy and were considered as preterm rats in this study. We observed the impact of exercise therapy on motor behaviors as well as the number of GFAP-positive astrocytes in the hippocampus of preterm infant rats. Also, the effective methods and possible mechanisms for the prevention and treatment of neurological injury in preterm infants were investigated.

Materials and Methods

■ Materials and Subjects

A total of six healthy female specific pathogen-free (SPF) pregnant Wistar rats weighing between 275g and 305g were selected. The experimental animals were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. Production license of the experimental animals was SCXK (Jing) 2012-0001, and usage license was SYXK (Min) 2013-0007. Disposal method during the experiment has been reviewed by the Ethics Committee of the Experimental Animal Center of Xiamen University, which was in line with relevant ethical requirements.

■ Methods

***Model preparation*:** A total of six female rats were detected for pregnancy with vaginal plugs in the morning after male and female rats were co-caged. Two pregnant rats were selected by simple random method and were used in non-modeling group, and the other four were used in the model group. The preparation of animal models was referred to the method described in Bell, *et al.* study [12] which used intrauterine infection to induce preterm birth to obtain a similar animal model of perinatal brain injury. Model preparation was done as follows: pregnant rats were weighed at day 16 and day 17 of pregnancy. Lipopolysaccharide (LPS) was intraperitoneally injected at 350μg/kg in the ventral midline depending on the weight (Serotype was 055:B5, which was provided by

SIGMA, America), which was performed for two consecutive days. The newborn infant rats were fed in a cage with female rats. Nothing was done for the rats in the non-modeling group, and had undergone natural delivery. Water and feedstuff in the experiment were normally supplied to the pregnant rats. All experimental animals were kept in the barrier environment with humidity of 20-26°C, relative humidity of 40-70%, and air cleanliness of 5°C.

Grouping of experimental infant rats: Preterm infant rats those survived for 7 days after birth were randomly divided into two groups, including the exercise group (group A), and the model group (group B). The non-preterm normal infant rats were grouped in the non-modeling group and were considered as the control group (group C).

Interventions: All infant rats received interventions at 7 days of age. Specific methods were as follows:

Exercise training- Rats in the exercise group started exercise training at 10:00 am each morning. The rats will rest for 10 minutes after balance beam training, then taken a roller training. The specific operations were as follows:

Balance beam training-A 30cm long, 5 cm wide rectangular stick was laid flatly at 5 cm to the ground as a balance beam. Each rat was trained to walk on the balance beam once every day with five minutes each time and five times as a course. The run-in interval of each course was two days, and a total of three courses were carried out.

Roller training-Self-made drum-mesh training device with a diameter of 20 cm was used **(Figure 1)**. Each rat was trained to run on the drum-mesh training device once every day with five minutes each time and five times as a course. The interval of each course was two days, and a total of three courses were taken.

Only the model and control groups performed grasp training at 10:00 am each morning.

Test of motor behaviors: Test of motor behaviors was performed for all infant rats at 28 days of age. Data for the test of motor behaviors was collected using Panlab Harvard Apparatus Smart 3.0 software. The test items were as follows:

Open field test, also known as open box test, is used to evaluate anxiety, autonomic behaviors, as well as exploratory behaviors of rats in the new environment. The detection index can be reflected by the time and duration of some rat behaviors in the open field. Box of open-field response was 50cm in height, and the length of the bottom side was 50 cm. The interior zone of the open field was set to 4×4 squares, and the combination of the central four squares was the central area while the combination of the peripheral squares was the surrounding area **(Figure 2)**. The open field was placed directly below an incandescent lamp, and a camera was adjusted just above the open field. The rats were placed in the center of the box of open-field response with their heads towards one of the four corners (the place and direction of each rat was the same, as indicated by the arrow in **Figure 1**). Each rat was tested for 10 min, and the changes of their behaviors

Figure 1: Drum-type training device with mesh type.

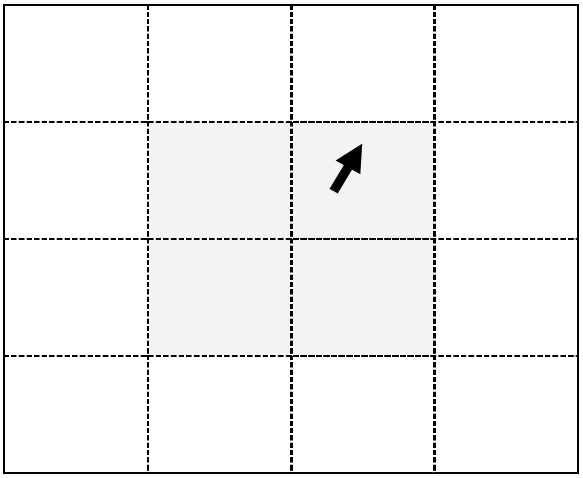

Figure 2: Open field test.

were recorded. After test of each rat, excrement in the open field was cleaned, and the open field was wiped by spraying 70% alcohol. The total distance of movement of rats in the open field and the percentage of time in the central area were counted.

The experiments were all conducted in a laboratory without noise, and with appropriate light intensity, temperature, and humidity, together with consistent behaviors. On the day of experiment, the rats were transferred to the behavioral laboratory 30 ± 5 minutes ahead of schedule, and the tests of motor behaviors begun after adapting to the environment.

Specimen collection: Specimens of hippocampal tissues were collected from 28-day-old infant rats after finishing the test of motor behaviors. After the rats were anesthetized with 10% chloral hydrate (0.36 ml/100 g), the right atrial appendage was cut through thoracotomy, and then a ballpoint needle was inserted into the aortic inlet of the left ventricle from a position slightly left to the apex, followed by rapid bolus injection with saline incubated at 37°C. When effluent liquid from the right heart was light red or nearly colorless, it was fixed using 4°C fixed solution (2.5% glutaraldehyde - 2% paraformaldehyde + 0.1M phosphate buffer, and PH7.4). The infusion rate was 5～10ml/min, which was fast at first and then was slowed down until the end of the tail was stiffened and became straight. After finishing of the injection, heads of rats were broken, and bilateral hippocampal tissues were taken out. A drop of cooled 4% neutral formaldehyde fixative was added, and CA1 area of the hippocampal tissues was cut into about 1 mm wide and 2~3mm long pieces using a new oil-free sharp double-sided blade. Subsequently, pieces with less injury were selected from them, which were then cut into smaller pieces of $1mm^3$. After fixation, the fixative solution was diluted for three times with PBS, followed by placing it in the freezer compartment to be fixed at low temperature (0~4℃).

***Histopathological changes and immunofluorescence staining of hippocampus in* rats:**

Optical microscopy sample preparation and observation of steps-After harvesting, the specimens were fixed with 4% neutral formaldehyde, then dehydrated conventionally, embedded in paraffin, sliced into pieces with a thickness of 3μm, and were HE stained. After which, the pathological changes of hippocampal neurons were observed under BX51-OLYMPUS optical microscope. One slice was selected from each rat, and similar parts in hippocampal CA1 area were taken out, then changes in neurons from the hippocampal CA1 area were observed under the same high magnification (600 times), as well as taken photos

Immunofluorescence staining: Tissues were taken, and after fixation as well as conventional embedding with paraffin, paraffin sections were obtained (5μm in thickness) before storing in a refrigerator at a temperature of 4°C. The sections were roasted in an incubator at a temperature of 60°C and then were stayed overnight. Then they were dewaxed with xylene, rehydrated with gradient ethanol, repaired with antigen, and eliminated nonspecific background staining if any. Then, GFAP rabbit monoclonal antibody was dropped (purchased from abcam Company) with a dilution of primary antibody of 1:100, followed by fluorescein-labeled goat anti-rabbit IgG secondary antibody (purchased from thermo). The sections were washed with PBS, and then DAPI staining was performed. Subsequently, the sections were sealed with glycerol buffer. One section was selected from each rat to be inspected under a high magnification (600 times). Fluorescence images of GFAP-positive astrocytes were harvested using Nikon ECLIPSE Ti-s, and Image J image processing software was used to analyze the average optical density of fluorescence.

Statistical analysis

Data were recorded in the excel table. Data of rotarod experiments and the mean optical density of fluorescence in GFAP-positive astrocytes were represented by mean ± standard deviation (SD). SPSS17.0 software was used to perform statistical analysis. Comparison between groups was done by one-way ANOVA. Homogeneity of variance was tested. If requirements for homogeneity of variance were satisfied, then pairwise comparison of the mean values in each group was conducted using Scheffe method. $P<0.05$ was considered to be statistically significant.

Results

■ General conditions of the delivery of pregnant rats as well as the infant rats

1) The two non-modeling pregnant rats had normal delivery, and a total of 23 infants with

full-term fetal age were delivered without stillbirth.

2) After intraperitoneal injection of LPS to the four modeling pregnant rats, three of them delivered ahead of schedule, and one had normal delivery. There were a total of 11 infant rats with full-term fetal age, and 17 preterm infant rats with a rate of preterm delivery of 60.7%. There was no death observed in all the preterm infant rats, and all of them entered the experimental intervention.

3) After grouping using simple random method, the number of preterm infant rats in each group was as follows: 8 cases in A group, with weight at birth of 5.212±0.635g; 9 cases in B group, with weight at birth of 5.000±0.678g. A total of 10 infant rats with full-term fetal age were selected by simple random method as the control group ((group C)), with weight at birth of 7.409±1.403g. There was a statistical significance in weight at birth among the three groups (P<0.05).

■ Results of the pathological examination of uterus and placenta

Results of pathological examination of uterine wall and placenta in the modeling rats that delivered ahead of schedule demonstrated significant vascular congestion and edema in the uterine wall and placenta, which was accompanied by a large number of neutrophil infiltrates. There was no inflammatory response in the uterus and placenta of non-modeling pregnant rats.

■ Results of behavioral test

In the open field experiment, the total distance of exercise in the model group (group B) was significantly reduced compared with that in the control group (group C). There was no statistical significance observed in the total distance of exercise between the exercise group (group A) and the control group (group C), which suggested that exercise training can improve the motor ability of preterm rats.

Compared with the control group, the percentage of time in the central area of the open field for rats in the model group was significantly decreased, which suggested that the exploratory ability of preterm rats was lower than that of the normal rats. However, it no statistical significance was observed between the exercise group and the control group, indicating that exercise training can improve the exploratory ability of preterm rats. The results were shown in Table 1.

■ Changes of neuron structure in hippocampus of rats

The pathological changes of hippocampal CA1 area were observed by HE staining, and the results revealed that:

The number of neuronal cell layers in the hippocampal CA1 area was more in the exercise group (group A), with 4-7 layers arranged neatly. However, the space in some areas was relatively sparse; color of cytoplasm was deepened, and was halo-shaped. Nucleus was round or oval, chromatin was rough, nuclei were visible in some nucleoli, and the number of astrocytes

Table 1: Comparison of the results of open-field experiments.

Grouping	Cases (n)	Total distance of exercise (cm)	Time in the central area (%)
The exercise group	8	3088.559 ± 949.953#	5.277 ± 1.141#
The model group	9	1901.438 ± 777.500*	0.998 ± 1.288*
The control group	10	3512.178 ± 240.479	6.083 ± 2.114
F value	12.942		23.164
P	<0.001		<.001

Note: compared with the control group, * means P<0.001, # represents P>0.05.

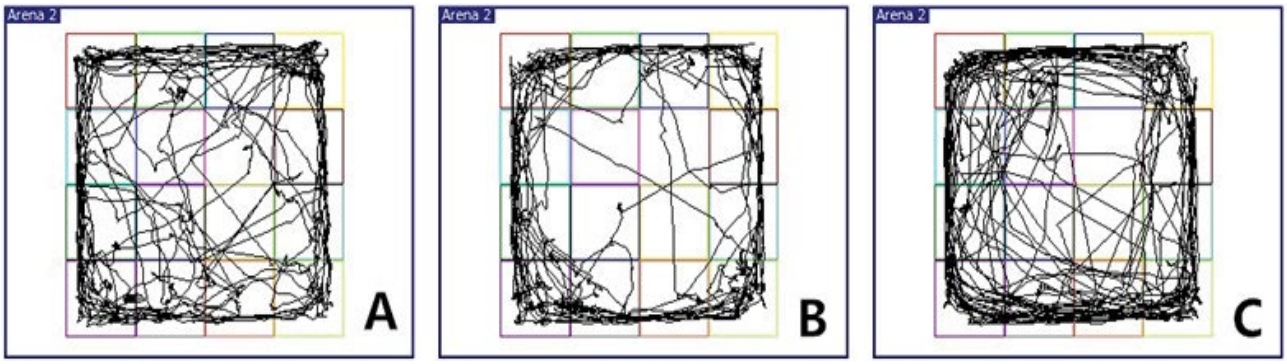

Figure 3: The path traveled by the rats.

surrounded was more. The results were shown in Figure 3.

The number of neuronal cell layers in the hippocampal CA1 area of the model group (group B) was less with 2-4 layers arranged sparsely. Cytoplasm was less, and cell membrane appeared blur with long slender axons. The volume of nuclei was small with polygonal and oval in shape. Chromatin was thickened, nucleolus was obvious, and multiple nucleoli were visible. The results were shown in Figure 4.

The number of neuronal cell layers in the hippocampal CA1 area was normal in the control group (group C). The neurons were arranged neatly and tightly with clear boundary, cytoplasm appeared to be transparent, nucleus was round or oval, chromatin was distributed uniformly, and nucleolus was clear with basically normal morphology. Results were shown in Figure 5.

Immunofluorescence staining and image analysis were performed after the intervention for infant rats in each group. The changes of GFAP showed that GFAP positive expression was labeled by green fluorescence. Distribution of GFAP signals was visible in the astrocytes in the exercise group, and the signals in the astrocytes were continuously increased (Figure 6). Astrocytes were still connected and the concentration area was mainly astrocytes. It was visible that signals were enhanced in the model group. Discontinuous and lightly stained GFAP signals were observed in the astrocyte area. Crab-like arrangement was faintly visible, and the intensity of fluorescence was significantly lower than that in the model group (Figures 7).

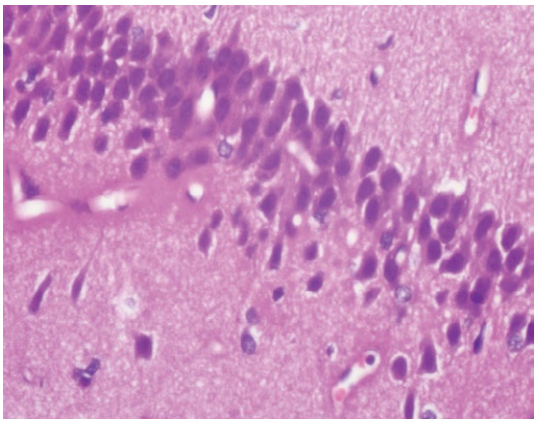

Figure 4: The number of neuronal cell layers in the hippocampal CA1 area was more in the control group, and was arranged sparsely but neatly. Space for some of them was relatively sparse. Color of the cytoplasm was deepened and halo-shaped. The chromatin was rough, and the number of surrounding astrocytes was more (HE ×600).

Compared with the control group, the mean optical density of GFAP in the model group was increased obviously with a statistical significance (P <0.05). This indicated that the number of astrocytes was increased during injury. Compared with the model group, the mean optical density of GFAP was decreased significantly after intervention in the exercise group with statistical significance (P<0.05), which suggested that the intervention therapy reduced the number of astrocytes in preterm rats. The difference between group A and the control group was statistically significant (P<0.05), which in turn demonstrated that exercise training can reduce the number of astrocytes, but cannot achieve the normal level (Table 2).

Discussion

Previous studies have demonstrated a close relation between intrauterine infection and preterm birth. Histological evidence of intrauterine infection demonstrated approximately 19% -74% of preterm births [13]. Since the nerves of the rats were very close nerve anatomy of the human [14], and hence experiment of this study used preterm rats as animal models caused by lipopolysaccharide-induced intrauterine inflammation according to the literature. There were a total of 19 preterm infant rats participated in the experiment with the rate of preterm delivery of 60.7%.

Intrauterine infection is the main cause of chorioamnionitis, infection of the amniotic cavity, placental inflammation, as well as preterm rupture of membranes, which are closely associated with white matter damage in preterm infants, and are the main causes, that leads to neurological sequelae [15]. The results of behavioral tests in the experiment demonstrated significant lower motor ability of infant rats in the model group than normal infant rats born at full-term fetal age, which suggested that preterm birth may impact the motor abilities of infant rats.

Intrauterine infection can significantly affect the perinatal brain development, leading to significant changes in brain structure and function [16]. Astrocytes are the most widely distributed and were abundant in the brain of mammals, maintaining the structure and function of nerve cells, local immune response, and the occurrence of neurological diseases. Few studies confirmed that GFAP is a specific marker

of astrocytes, and its expression can reflect the functional status of astrocytes. When brain injury occurred, astrocytes were increased in the cerebral cortex and correspondingly the GFAP level was also increased, indicating that GFAP could be used as a marker of severity of brain injury [17]. Results of this experiment showed that the mean optical density of GFAP-positive astrocytes in the hippocampus of preterm infants in the model group was significantly higher than the normal infant rats in the control group with statistical significance ($P<0.05$), revealing that the number of astrocytes were increased when preterm brain injury occurred.

Early intervention therapies such as exercise therapy, can promote the recovery of brain injury. Studies have shown that exercise training not only improves motor function of limbs, but also improves the attention and learning abilities [18]. Results of this study demonstrated that after exercise training, the number of GFAP-positive astrocytes in the hippocampus of preterm rats was decreased; suggesting that exercise therapy may play a role in repairing the impaired motor behaviors in preterm rats.

It is believed that the astrocytes can promote synapse formation, accelerate synaptic transmission, regulate synaptic activities, and together play an important role in synaptic plasticity, including synaptic morphology, structure, number, and electrophysiology of synapse, as well as synaptic immunological expression, synaptic vesicle cycle, and the release of neurotransmitters [19]. Synapses are specialized structures that transmit information between neurons, and also form the structural basis of neural activities. The possible mechanism of exercise therapy on the repair of preterm labor with impaired motor behaviors was considered to inhibit the reactive hyperplasia of astrocytes to a certain extent. Therefore, the number of GFAP-positive astrocytes was reduced, and more favorable conditions can be provided for the establishment of new contacts between neuronal synapses, which then rapidly repairs the injury. However, study of specific mechanism needs more confirmation through animal experiments, so that the results can further guide to the clinical treatment.

Conclusion

In conclusion, the exercise training can repair the impaired motor behaviors in preterm rats caused by lipopolysaccharide-induced intrauterine inflammatory, and this might be due to the

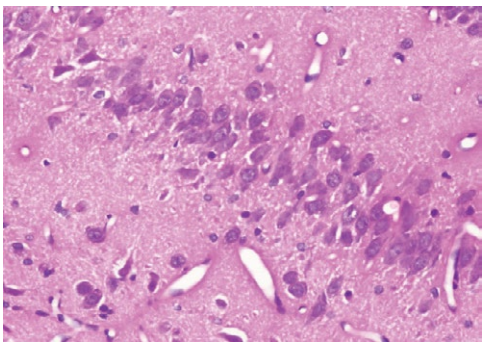

Figure 5: The number of neuronal cell layers in the hippocampal CA1 area was less in the model group, and was sparsely arranged. Cell membrane was blur, and the volume of nucleus was small. The chromatin was deepened in color, and multiple nucleoli were visible (HE ×600).

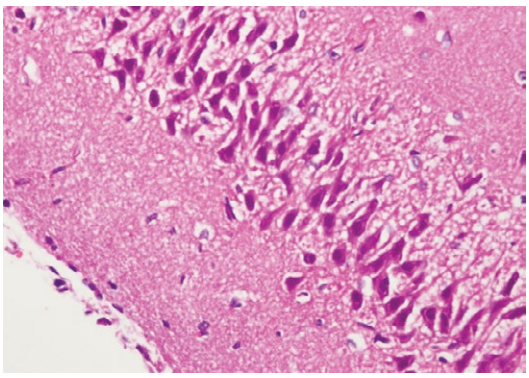

Figure 6: The number and morphology of hippocampal CA1 neurons in the control group were normal (HE ×600) 3.5 Results of immunofluorescence staining.

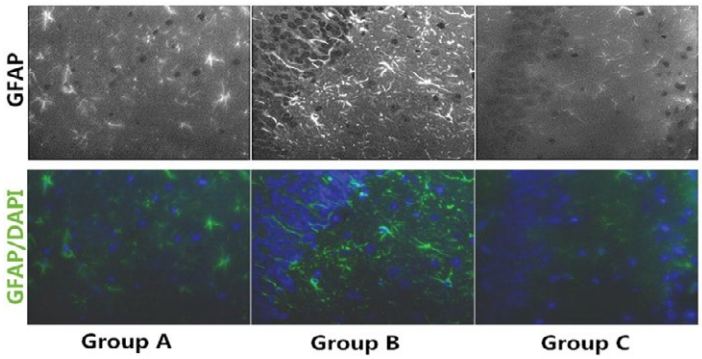

Figure 7: Expression of GFAP (immunofluorescence).

Table 2: Comparison of mean optical density of GFAP-positive astrocytes in different groups.

Groups	Cases (n)	Mean optical density
The exercise group	8	20.932 ± 2.788#
The model group	9	30.826 ± 3.819*
The control group	10	13.690 ± 2.240
F value	40.550	
P	<0.001	

Note: * means $P<0.05$ compared with the control group; # indicates $P<0.05$ compared with the model group, and represents $P<0.05$ compared with the control group

inhibition of reactive hyperplasia of astrocytes, the reduction of the expression of GFAP-positive astrocytes, have certain nerve repair ability, which can be nerve protective effect.

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