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Improvement effects of tanshinone on the impaired cognition and motor function in MCAO mice with ischemic penumbra

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Abstract: This study aims to investigate the effects of tanshinone on improving the impaired cognition and motor function in MCAO model mice with ischemic penumbra. MWM test was carried out to evaluate the spatial learning and memory performance and the cognitive function of mice. The area of cerebral infarction was analyzed by immunohistochemistry. The TUNEL apoptosis detection kit was used to detect neuronal apoptosis. On the 25th day, the induction model group had lower body weight than the control group and the tanshinone treatment group; the induction model group had decreased walking deficiency and correct area escape times than the other two groups; while, tanshinone treatment group had higher movement distance, movement speed, periphery entry frequency, grooming rate, decreased center entry frequency, infarction area, apoptotic neuron number, latent escape time than induction model group; additionally, the control group had increased periphery and corner entry frequency, but decreased center entry frequency and latent escape time than the other two groups. Tanshinone can reduce neuronal damage in the ischemic penumbra after stroke, improve the integrity of white and gray matter, and restore connectivity in motor and cognitive functions, thereby supporting recovery from ischemic stroke.

Keywords: Tanshinone, neuron; middle cerebral artery occlusion; motor cognition; ischemic penumbra.

INTRODUCTION

In ischemic stroke treatment, most treatments show post-stroke disability, in fact only a few treatments show good results (Wang *et al*., 2017a). So far, thrombolysis and thrombectomy are common treatments for ischemic stroke, but no many patients get the required benefit from such treatments due to administration complexity (Ding *et al*., 2017). Therefore, restorative interventions are urgently needed (Xin *et al*., 2017). Studies have shown that damaged brain networks are the cause of impossible recovery and even progressive disease. In fact, ischemic stroke accounts for the majority of all strokes are ischemic, so treatments should focus on reduction of cerebral blood flow damage. Tanshinone is a typical example in which leading compound derived from traditional Chinese medicine provides neuroprotection in middle cerebral artery occlusion (MCAO) model animals (Zhao *et al*., 2018). Tanshinone is a lipophilic chemical substance found in Salvia miltiorrhiza, which is an important Chinese medicine component of dried roots or rhizomes to treat cerebrovascular diseases (Wu *et al*., 2017), tanshinone has been found to effectively influence cell lipid peroxidation, methylguanine methyltransferase activity, cell apoptosis and anti-inflammatory effects. Therefore, this study established a mouse model of cerebral infarction, and evaluated the effects of pretreated tanshinone on; Cerebral infarction volume, neurological deficit, cell apoptosis and motor and cognitive function responses of such examined animal model.

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MATERIALS AND METHODS

Experimental preparation

All the sixty C57BL/6J mice used in the experiment (3–4-month-old internally bred C57BL/6J mice, with average weight of 220~250g) were housed in 10 cages, each containing 6 animals, under temperature of 21°C. During the feeding period, the mice were provided with food and water until the day before the experimental operation.

The mice were anesthetized with 1.5% isoflurane mixture with 2:1 air to oxygen ratio. The right common carotid artery was prepared by inserting 7-0 mono-filament into the common carotid artery and placing it where the middle cerebral artery (MCA) branched out. Such filament was occluded for 30 minutes and then withdrawn to allow reperfusion. Blood flow reduction was monitored during the operation via a laser Doppler probe attached to the mouse skull. From the laser Doppler blood flow measurement, it was believed that ischemia induction is effective when the regional cerebral blood flow (CBF) decreases to 80% above the baseline value during filament insertion in the occlusion process (inclusion criteria). During the process, the mice were kept anesthetized under the effect of 1.5% isoflurane mixture with 2:1 air to oxygen ratio. Mice with decreased exercise capacity (tested baseline value of each behavior minus the baseline measurement value <50%) or extreme weight loss were excluded from this study.

Experimental grouping

The experimental mice were randomly divided into three groups: control group (n=20), model induction group (n=20) and tanshinone treatment group (n=20). Control

group: normal mice were treated with Sham operation; Model induction group: mice in this group were treated with MCAO operation; Tanshinone treatment group: on the basis of MCAO model group, tanshinone (Tanshinone II A injection was purchased from Shanghai No.1 Biochemical & Pharmaceutical Co., Ltd., SFDA approval number: H31022558) treatment was given every day. Tanshinone was diluted with 10% dimethyl sulfoxide (DMSO) solution to the desired concentration, then tanshinone IIA at a dose of 10 mg/kg was injected into the abdominal cavity of mice.

Experimental method

Exercise and exploratory behavior were assessed in the wilderness for 10 minutes before MCAO, that was carried out at the 3rd day and the 23rd day after the stroke. In addition, the investigation items (walking, sitting, leaning against the wall, jumping, raising, and grooming) were scored manually and analyzed according to the previous 5. The mice in each group were weighed using an electronic scale on the 7^{th} and 25^{th} days of the experiment.

Morris water maze (MWM) test was carried out to test the spatial learning and memory performance of mice. To be specific, the mice were required to find a hidden platform below the water surface of a circular pool. On the 21st day after treatment, the mice were trained in MWM for six trials every day. When the experiment started, the mice were placed in a pool without containing a platform. If the mouse failed to escape, it should be left for more 5 seconds. A probe test was performed 26 days after the operation checking the spatial reference memory. If the mice failed to find the hidden platform, they were placed on the platform to avoid fatigue. Image tracking software was used to record and analyze the performance. The software was also used to calculate the time required to discover the platform (delay time). The Y-shaped maze consisted of three identical arms, which were arranged at 120° intervals around a central connection area with a charged grid. There was a signal light at the end of each arm. The mice were placed in the random arm of the Y-shaped maze and left to explore for 3 minutes before the signal light turned on. Once illuminated in the non-safe area, the mice had 10 seconds to escape to the safe area. If the mice failed to escape within 10 seconds, there would be a short electric shock. On the 21^{st} day after the operation, the mice were tested 20 times a day for 5 consecutive days. On the 26^{th} day after the operation, the test was repeated.

Immunofluorescence staining of ischemic penumbra

All stained sections were derived from the ischemic penumbra tissue. Before labeling Iba1, the sections were incubated in Tris Buffered Saline (TBS) solution containing 20μg/ml proteinase K for 10 minutes and then in distilled water containing 3% H2O2 for 10 minutes to perform antigen retrieval on the tissue. The sections were pre-incubated in Phosphate Buffer Saline (PBS) containing 0.1% Triton X-100 and 2% normal goat serum, and then transferred to PBS containing rabbit polyclonal anti-Iba1 antibody.

Assessment of cerebral infarction area

After the behavioral experiment, the mice were quickly executed. The brain was removed from the mouse skull and the brain matrix was cut into coronal sections, then stained with 2,3,5-Triphenyltetrazolium chloride (TTC) for 30 minutes in the dark at 37°C and fixed with 10% formalin. Image J software was used to scan and analyze the sections.

TUNEL detection of neuronal cell apoptosis

According to the manufacturer's instructions, one-step TUNEL apoptosis detection kit was used for TUNEL staining. After the behavioral experiment, the mice were quickly executed. The brain was removed from the mouse skull and the brain matrix was cut into 2 mm thick coronal sections. The sections were fixed with 4% paraformaldehyde and then incubated with TUNEL staining solution in the dark at 37°C for 1 hour. The fluorescein-labeled TUNEL positive cells were imaged by fluorescence microscope under excitation at 550nm and emission at 570nm. The three regions of the substantia nigra of each TUNEL stained section were imaged using digital microscope objective lens, with no overlapping fields in the microscope and camera.

Ethical approval

Research experiments conducted in this article with animals were approved by the Ethical Committee and responsible authorities of our research organization(s) following all guidelines, regulations, legal, and ethical standards as required for animals.

STATISTICAL ANALYSIS

The data in this study were all processed by SPSS20.0 statistical analysis software (IBM, USA); measurement data were expressed as "mean ± standard deviation" ($\bar{x} \pm s$); the count data was expressed as percentage (%). The different was considered significant when $p<0.05$.

RESULTS

Inhibitory effect of Tanshinone on weight loss in MCAO mice

The mice in different experimental groups were weighed and compared on the 7th and 25th days. The control group had higher body weight than the induction model group and the tanshinone treatment group on the 7th day ($p<0.05$), the induction model group had lower body weight than the control group on the 25th day ($p<0.05$). table 1 shows that tanshinone can inhibit MCAO damage to mice after long-term treatment (Shown in table 1).

Table 1: Comparison of body weight of mice in different groups

Group	7th day	25th day
Control group*	22.14±2.06	23.23±2.38
Induction model group*	18.37±1.41	16.34±1.82
Tanshinone treatment group*	18.39±1.55	21.77±2.45
F value	21.438	19.238
p value	0.016	0.025

Table 2: Comparison of mouse exercise capacity

Group	Movement distance×10^3(cm)	Movement speed (cm/s)
Control group*	11.26±2.47	11.21±1.63
Induction model group*	5.35±1.68	4.69±0.84
Tanshinone treatment group*	10.26±1.88	7.71±1.29
F value	26.739	15.672
p value	0.00	0.027

Table 3: Comparison of frequency of mice entry into open field of view

Group	Periphery	Center	Corner
Control group*	176.61±25.19	58.14±9.05	136.27±28.19
Induction model group*	84.68±9.22	132.42±26.51	65.74±5.22
Tanshinone treatment group*	138.29±20.11	95.76±13.05	73.18±6.08
F value	42.039	31.472	30.672
p value	0.005	0.019	0.008

Table 4: Comparison of mouse action frequency

Group	wall leaning	sitting	grooming	walking
Control group*	56.37±11.06	64.74±5.22	24.28±5.15	82.65±12.39
Induction model group*	28.16±5.02	58.36±6.41	9.83±1.07	59.47±6.28
Tanshinone treatment group*	46.28±9.44	58.66±7.05	17.16±3.85	74.81±9.05
F value	16.574	19.268	35.672	31.068
p value	0.018	0.014	0.016	0.011

Table 5: Comparison of cognitive ability of mice

Group	Latent escape time (s)	correct area escape times
Control group*	21.36±2.47	24.28±5.15
Induction model group*	38.95±5.22	9.83±1.07
Tanshinone treatment group*	29.58±3.67	17.16±3.85
F value	9.327	10.672
p value	0.022	0.036

Table 6: Comparison of cerebral infarction area and neuronal apoptosis in mice

Group	Infarction area (mm3)	Apoptotic cells
Control group*	0.15±0.001	5.12±1.35
Induction model group*	186.47±25.16	46.84±12.68
Tanshinone treatment group*	73.95±13.05	27.14±8.15
F value	10.525	9.323
p value	0.006	0.014

*Data expressed as ($\bar{x}$±s, n=20), $F<0.05$, $p<0.05$

Improvement effect of Tanshinone on exercise capacity of MCAO mice

On the 25th day, the exercise capacity of the mice in each group was tested. The induction model group had lower movement distance than the control group ($p<0.05$), while, the tanshinone treatment group had higher movement speed than the induction model group ($p<0.05$) (Shown in table 2).

Comparative evaluation of frequency of mice entry into open field of view

The control group exhibited higher periphery entry frequency than the other two groups ($p<0.05$), the tanshinone treatment group had higher periphery entry frequency than the induction model group ($p<0.05$), otherwise, the control group divulged lower center entry frequency than the induction model group and the tanshinone treatment group ($p<0.05$) (Shown in table 3).

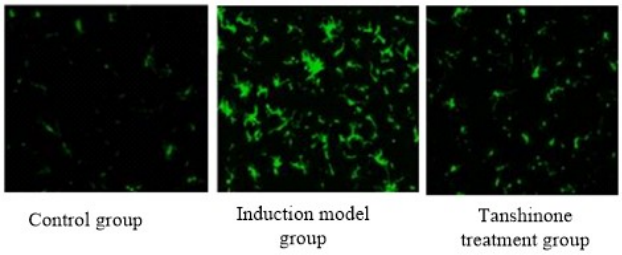

Fig. 1: Microglia activation in ischemic penumbra ($p<0.05$).

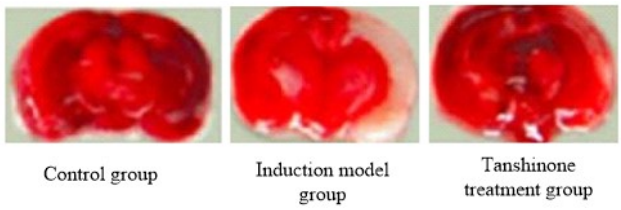

Fig. 2: Comparison of cerebral infarct area in mice ($p<0.05$).

Comparative evaluation of mouse action frequency

Observations and statistics of the movements of mice in each group showed that the induction model group had lower wall leaning rate than the other two groups ($p<0.05$). The induction model group had lower grooming rate than the control group ($p<0.05$), in contrast, the tanshinone treatment group had higher grooming rate than the induction model group ($p<0.05$). The induction model group revealed lower walking rate than the control group and the tanshinone treatment group ($p<0.05$) (Shown in table 4).

Improvement effect of Tanshinone on the cognitive ability of MCAO mice

The cognitive ability of mice was tested by a maze test. The control group showed shorter latent escape time than the other two groups ($p<0.05$). However, the model induction group had lower correct area escape times than the tanshinone treatment group and the control group ($p<0.05$) (Shown in table 5).

Fluorescence staining of mouse ischemic penumbra

Microglia was identified in the sections of CD11b-labeled normal brain or cerebral ischemic penumbra of mice in each group. CD11b positive microglia presented hypertrophy, showing characteristic clumping cell branches widely distributed in the ischemic penumbra. The induction model group had increased the fluorescence staining intensity of CD11b-positive microglia than the control group ($p<0.05$), on the other hand, the tanshinone treatment group had decreased the fluorescence staining intensity of CD11b-positive microglia than the induction model group ($p<0.05$) (Shown in fig. 1).

Reduction effect of Tanshinone on the infarction and neuronal apoptosis caused by MCAO

The induction model group had larger cerebral infarction area and more TUNEL positive cells than the control group ($p<0.05$), dissimilarly, the tanshinone treatment group revealed smaller cerebral infarction area and less TUNEL positive cells than the induction model group ($p<0.05$) (Shown in fig. 2, fig. 3 and table 6).

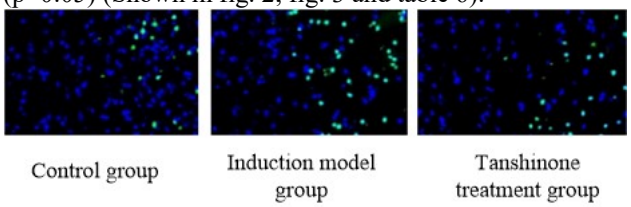

Fig. 3: TUNEL staining of mouse brain sections ($p<0.05$).

DISCUSSION

Stroke is a major cause of disability (El-Tawil *et al*., 2017; Zhang *et al*., 2017a). Over the past decades, great progress has been made in research of pathophysiology of cerebral ischemia. However, treatment for acute ischemic stroke is still limited. Hypoxia caused by ischemia and subsequent bioenergy failure are the main factors of neuronal damage (Tymianski, 2017; Komnig *et al*., 2018). The lack of ATP causes interference with the stable membrane potential, leading to depolarization and more energy around the infarct (Bok *et al*., 2017; Seiler *et al*., 2019a). Failure causes depolarization of the terminal ischemic cell membrane. Stroke is caused by partial or complete occlusion of cerebral arteries. Cerebral ischemia triggers an inflammatory response in the brain and surrounding areas. Cerebral ischemia usually causes movement disorders. Due to movement disorders, stroke survivors often suffer long-term disability and severe illness. At present, the MCAO model has been widely used in stroke research (Du *et al*., 2017; Wang *et al*., 2017b). Clinical practice shows that most monotherapy fails to achieve the expected results due to the lack of drug efficacy in the chronic phase. The current clinical

treatment of stroke has some shortcomings, such as narrow treatment window and bleeding risk (Zhang *et al*., 2017b; Sun *et al*., 2017). Therefore, development of more effective therapeutic drugs is needed.

In this study, it was found that the body weight of mice in the 25-day induced model group was lower than that in the control group ($p<0.05$), the body weight of mice in tanshinone treatment group was higher than that in induced model group ($p<0.05$); Compared with the control group, the movement distance, movement speed and grooming rate of the induced model group were lower ($p<0.05$), the size of cerebral infarction and the number of neuronal apoptosis increased ($p<0.05$). This suggests that tanshinone alleviates MCA-induced cognitive impairment, infarction, neuronal apoptosis and inflammatory response. There is growing evidence that MCAO may cause cognitive deficits (Cognitive abilities include memory, attention, learning ability and mental motor speed.). In our study, we used MWM test and Y test to evaluate the changes in cognitive ability of experimental mice, and the results showed that tanshinone treatment alleviated the cognitive impairment caused by MCAO. Previous studies have confirmed that cognitive dysfunction is associated with severe acute cerebral infarction, and that cognitive impairment and acute cerebral infarction are positively correlated with the severity of middle cerebral artery stenosis in patients (Caranfa *et al*., 2018). In addition, there is increasing evidence that apoptosis is crucial to the pathological process (Seiler *et al*., 2019b; Liu *et al*., 2017). Therefore, we further investigated the effect of tanshinone on MCAO-induced infarction and neuronal apoptosis, and the results showed that MCAO led to the production of cerebral infarct volume and increased the number of apoptotic neurons. Meanwhile, tanshinone treatment decreased these changes, suggesting that the protective effect of tanshinone on cognitive impairment may be related to infarct size and neuronal apoptosis (Tuo *et al*., 2017; Wang *et al*., 2021; Zhang *et al*., 2021).

Moreover, it was found that the walking rate and the number of avoiding the correct area were lower in the induction model group than in the control group and the tanshinone treatment group. Compared with the induction model group, the movement distance, motion speed, peripheral frequency and grooming rate were increased in tanshinone treatment group, while the frequency of entering the center, number of neuronal apoptosis and latency escape time were decreased. Compared with the induction model group and tanshinone treatment group, the frequency of entry into the periphery and corner was increased, the frequency of entry into the center was decreased, and the latency escape time was decreased in the control group. It was worth noting that on 23rd day after the stroke, all the mice in the tanshinone treatment group walked more frequently than those in the model induction group, and also walked more frequently compared with 3rd day after the stroke, which indicates that the exercise capacity was improved. These results demonstrate that tanshinone treatment has a beneficial effect on exercise activities.

CONCLUSION

In this study, we investigated the effect of tanshinone on stroke mice, and the results revealed that tanshinone alleviated stroke-induced cognitive impairment and reduced MCAO-induced infarction. Our results reveal the potential therapeutic effect of tanshinone on stroke and provide a theoretical basis for the clinical treatment of stroke.

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