The Effect of Thymoquinone on the Characteristics of the Brain Extracellular Space in Transient Middle Cerebral Artery Occlusion Rats

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The extracellular space (ECS) is the space between the neurons and the capillaries in the brain. The volume fraction (α) and the tortuosity (λ) are the main parameters used to describe its characteristics. Thymoquinone has been proved to possess anti-oxidant and anti-inflammatory activity. In this study, we used a gadolinium-diethylenetriaminepentaacetate (Gd-DTPA)-enhanced magnetic resonance imaging (MRI) system to determine the effects of thymoquinone on ECS parameters in transient middle cerebral artery occlusion rats (tMCAO) to prove the neuroprotective effect of thymoquinone on brain tissue damage caused by ischemic stroke. Neurological examinations, 2,3,5-triphenyltetrazolium chloride (TTC) staining, hematoxylin–eosin (H&E) staining and assaying of ECS parameters using MRI were performed 24 h after surgery. We found that thymoquinone could improve the behavioural performance by neurological examinations. TTC staining indicated that thymoquinone significantly decreased the percentage of hemi-cerebral infarction. Also, H&E staining showed that thymoquinone could inhibit the neuron necrosis in the hippocampal CA1 region. We found that thymoquinone treatment could inhibit the changes in ECS diffusion parameters, which might prove that thymoquinone might protect brain tissue damage caused by ischemic stroke. Thymoquinone can protect the brain against cerebral ischemia–reperfusion injury, effectively ameliorate abnormalities in characteristics of ECS and decrease cerebral infarction in tMCAO rats.

Key words thymoquinone; ischemia–reperfusion injury; magnetic resonance imaging (MRI); brain extracellular space (ECS); volume fraction; tortuosity

INTRODUCTION

Stroke is a global disease and one of the leading causes of death and disability worldwide. 1) Ischemic stroke, which is caused by the obstruction of blood vessel in the brain and leads to neuronal ischemia and hypoxia, accounts for approximately 87% of all strokes. 2) Currently, the only drug approved by the U.S. Food and Drug Administration (FDA) for the treatment of ischemic stroke is tissue plasminogen factor (tPA), which has a narrow therapeutic time window. 3) Determining an effective treatment for ischemic stroke is urgently needed.

The brain consists of three main components: nerve cells, vascular system, and extracellular space (ECS). The nerve cells occupy approximately 70–80% of the total brain volume. The vascular system occupies approximately 3–5% of the total brain volume, while the ECS of the brain occupies 15–20% of that volume. 4) In recent years, a growing body of research shows that the ECS of the brain plays an important role in brain function as well as the development of brain diseases. 4, 5)

The brain ECS is the space that exists between neurons and brain capillaries, which, together with the intracerebral vascular system, form the brain microenvironment. It is the space in which neurons live in and plays an important role in the exchange of neuronal substances and energy. 5) The main parameters used to describe its physical properties are the volume fraction (α) and the tortuosity (λ). The volume fraction (α) refers to the percentage of total brain capacity occupied by the ECS, and the tortuosity (λ) reflects the diffusion of substances in the brain. 6) These parameters can be indirectly reflected by the diffusion of molecules in ECS. The diffusion of molecules in ECS are affecting by the geometry of ECS, dead-space microdomain, extracellular matrix molecules, binding sites for the diffusing molecule and fixed negative charges on the extracellular matrix, 6) and all of factors are changed with the development of diseases.

After the occurrence of cerebral ischemia, there is an ischemic penumbra in the hypoperfusion state between the irreversible region of necrosis at the ischemic centre and normal brain tissue. Although the ischemic penumbra is unable to perform its normal function due to decreased perfusion, no neuronal necrosis occurs. After the timely recovery of blood perfusion, the ischemic penumbra is likely to return to normal brain tissue. 7)

However, the process of restoring perfusion is often accompanied by oxidative stress, the accumulation of excitatory amino acids in the ECS of the brain, and inflammation. 8–10) Both the inhibition of oxidative stress on biofilms or mitochondrial function and the excitotoxicity caused by excitatory amino acid accumulation lead to calcium overload; a large number of calcium ions enter the neuron, resulting in a depolarized state, a large amount of extracellular ions (Na⁺ and Cl⁻) enter the cell, and the water in the brain enters the neurons to maintain the balance of osmotic pressure.

And then, the neurons appear swollen or even undergo lytic death, causing changes in the structure of the ECS of the
brain. At the same time, immune cells such as microglia, astrocytes and leukocytes located in the central nervous system are activated by ischemia–reperfusion injury, and the activated immune cells release inflammatory factors, proteins and other substances into the ECS, causing changes in the composition of ECS. Changes in the geometry of ECS, dead-space microdomain, and extracellular matrix molecules of the ECS caused by cerebral ischemia–reperfusion injury decrease the volume fraction \(a\) and increase the tortuosity \(\lambda\). Although the changes in the ECS diffusion parameter were proved in the cerebral ischemia, research has neglected the ECS changes after stroke was treated. Therefore, we used a magnetic resonance imaging (MRI) method to determine ECS diffusion parameter for our pharmacodynamics study.

Brain MRI with gadolinium-diethylenetriaminepentacetate (Gd-DTPA) as a tracer is a detection technique. The diffusion of Gd-DTPA in the ECS is affected by the physical properties of the ECS. Based on this, on the concentration of Gd-DTPA in the ECS can be combined with the diffusion equation, to calculate brain ECS parameters.\(^{4,12,13}\)

Thymoquinone (Fig. 1) is the main active ingredient of \textit{Nigella sativa}, commonly known as black seed or black cumin. Black cumin is a traditional Uighur medicine that is mainly distributed in Xinjiang and the Middle East and has been used for a very long time to cure various disorders, including asthma, hypertension, diabetes, inflammation, headache, fever, and bronchitis.\(^{14}\) Previous studies have shown that thymoquinone has anti-oxidant, anti-inflammatory, anti-cerebral ischemia, immunomodulatory activity.\(^{15,16}\)

Edaravone is an antioxidant with free radical scavenging activity. It was marketed for improving the neurologic recovery following acute brain infarction.\(^{17}\) Therefore, Edaravone was chosen as the positive control drug.

Here, we investigated the effects of thymoquinone on behavioural performance, 2,3,5-triphenyltetrazolium chloride (TTC) staining and hematoxylin–eosin (H&E) staining in transient cerebral middle cerebral artery occlusion (tMCAO) rats; furthermore, we detected the variation in ECS parameters using the Gd-DTPA-enhanced MRI system in tMCAO rats.

**METHODS AND MATERIALS**

**Chemicals and Reagents** Thymoquinone was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Edaravone was purchased from Nanjing Xiansheng Dongyuan Pharmaceutical Co., Ltd. (Nanjing, China). TTC was purchased from Sigma-Aldrich. Gd-DTPA (Magnevist) (4.69 g/10 mL) was purchased from Bayer-Schering (Berlin, Germany).

**Animals** The animals used in this experiment were adult male Sprague-Dawley rats weighing 280–300g and were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The animal certificate number was SCXK (Beijing) 2016–0010. The rats were housed 3–4 per cage in an animal colony room (12-h light–12-h dark cycle) at a controlled temperature (22 ± 1°C) and humidity (50 ± 10%). Before and after surgery, the rats were allowed to eat and drink freely, and the rats were fasted 12h before surgery. After the operation, food was placed in the cage to facilitate consumption. All experiments were conducted according to the “Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research.” The experimental procedures were approved by the local Committee of Beijing on Animal Care and Use.

**Surgical Procedures of tMCAO** The modelling procedure is described below.\(^{18}\)

(a) Except for the sham operation group, all other groups were subjected to tMCAO surgery. Twelve hours before surgery, the rats were fasted but were given access to water. Before the surgery, the rats were anaesthetized with 10% chloral hydrate at a dose of 3.5 mL/kg and fixed in the supine position on a board, and the hair was removed from the neck. The skin was cut along the trachea, and the right common carotid artery, the internal carotid artery and the external carotid artery were bluntly separated with forceps. Then, sutures were placed on the common carotid artery and the external carotid artery.

(b) An arterial clip was used to clamp the common carotid artery and internal carotid artery, and a small cut was made in the external carotid artery to insert the occlusion line. Subsequently, the external carotid artery was cut off so that the external carotid artery formed an obtuse angle with the internal carotid artery, and then the filament was gently pushed into the internal carotid artery until there was slight resistance or until the mark reached the vascular bifurcation of the external carotid artery and the internal carotid artery.

(c) Two hours after the filament was inserted, the filament was pulled out to restore blood perfusion. At the same time, the external carotid artery stump was ligated, and the incision on the neck of the rat was sutured. In the sham operation, only the external carotid artery was ligated; A small amount of penicillin was applied to the incision (Fig. 2).

**Grouping and Administration** Before the formal experiments, we conducted two pre-experiments. During the fist pre-experiment, based on recommended dose in previous study,\(^{19}\) we set up three different doses of thymoquinone. Thirty rats were performed tMCAO model and 15 of them were treated with different doses of thymoquinone (2.5, 5, and 10 mg/kg, 5 rats per group) for the mortality study. The death of rats in the high-dose administration group was severe (3 in 5), and the rats in the medium-dose administration group did not die. During the second pre-experiment, 40 rats were performed tMCAO model and 30 of them were treated with different doses of thymoquinone (2.5, 5, and 10 mg/kg, 10 rats per group) to determine the effect of thymoquinone on neurological examinations and TTC staining experiment. Both the medium-dose (5 mg/kg) and high-dose (10 mg/kg) are effective in the two assay. Therefore, considering mortality, in
the formal experiments, the middle dose was selected as the administration dose.

In the formal experiments, male Sprague-Dawley rats weighing 280–300 g were randomly divided into the sham operation group, tMCAO model group, edaravone treated group (positive drug), and thymoquinone treated group. The total numbers of each group were 10, 15, 14, and 14, respectively, while the survival numbers before sacrifice were 10, 9, 9, and 10, for the sham, model, edaravone, and thymoquinone group, respectively.

To calculate the parameters of the ECS more accurately, after the injection of the contrast agent Gd-DTPA into the target area of the rat, continuous observation was required until the contrast agent was completely eliminated. This process often takes approximately 4 h. Constrained by this, we conducted a total of 3 batches of experiments at different times, each time one rat was randomly selected from four groups for MRI scanning, respectively. And the remaining rats were sacrificed for the neurological examinations, TTC staining, and H&E staining.

In the sham operation and model groups, 1% Tween-80/saline was intraperitoneally injected as a control. The positive control drug group was given 6 mg/kg edaravone intraperitoneally, while the thymoquinone group was given an intraperitoneal injection of 5 mg/kg thymoquinone dissolved in 1% Tween-80/saline at 0 and 12 h after surgery, respectively.

Neurological Examinations A total of two neurological examinations were performed after surgery. The rats were first screened after they awoke, and rats with a behavioural score between 2–4 were used for subsequent experiments. The second neurological examination was performed approximately 24 h after surgery as one of the indicators of the efficacy of thymoquinone. The neurological examinations were based on the Longa scale:

- 0 point: no nerve damage
- 1 point: neither eyes closed, curled front paw
- 2 points: fixed leaning to the right
- 3 points: large circling to the right
- 4 points: small circling to the right and tail biting
- 5 points: paralysis despite the presence of reflexes

6 points: no reflexes or death

**TTC Staining** TTC staining and H&E staining were performed in another batch of rats that underwent the same procedures. The process of TTC staining is described below.

Twenty-four hours after surgery, anaesthetized rats were sacrificed, and the brains were removed. The brain tissues were frozen at −4°C for 30 min. After freezing, a blade was used to cut an average of 6 pieces along the coronal axis, with each piece having a thickness of approximately 2 mm. Then, the tissue pieces were placed in a small dish with 4–5 mL of 1% TTC solution in PBS, dyed in a water bath at 37°C for approximately 40 min, and turned over once.

After staining the brain tissues, they were fixed with 4% paraformaldehyde for 12 h and then photographed with a camera. The photos were analyzed with ImageJ software. The percentage of hemi-cerebral infarction was calculated according to the following formula:

\[
\text{Percentage of hemi-cerebral infarction} (%) = \frac{-\text{Total area of right hemisphere red area}}{\text{Total area of left hemisphere}} \times 100\%
\]

The mean percentage of hemi-cerebral infarction on both sides was taken as the percentage of hemi-cerebral infarction in the brain tissue.

**H&E Staining** Twenty-four hours after surgery, anaesthetized rats were perfused with saline followed by 4% paraformaldehyde. Then, the brains were removed quickly and further fixed with 4% paraformaldehyde at 4°C overnight. All brains were dehydrated with different concentrations of ethanol, embedded in paraffin blocks, and then cut into 6 serial slices of 10 μm. The coronal sections were selected from the dorsal hippocampus to use for H&E staining. The sections with H&E staining were scanned under 20× magnification with an automated microscope (Motic BA600) and photographed using Motic DSAssistant Lite software 1.0.

**The Measurement of ECS Diffusion Parameters with the MRI Tracer-Based Method** ECS diffusion parameters were determined with the MRI tracer-based method.\(^\text{15}\) Approxima-
mately 24 h after surgery, three rats from each group were randomly selected for MRI scanning. Each rat was required to have a pre-scan before Gd-DTPA injection. The anaesthetized rat was placed in the prone position and scanned with a T1-weighted three-dimensional magnetization prepared-rapid acquisition gradient echo (T1 3D MP-RAGE) sequence in a 3.0T MRI system (Magnetom Trio, Siemens Medical Solutions, Erlangen, Germany).

After the final administration and the pre-scan, using a stereotactic locator, 2 µL of 10 mM Gd-DTPA was injected into the right caudate nucleus of the rat for 10 min. The injection was made 1 mm anterior to bregma, 3.5 mm to the right, and 4.0 mm deep. Scans were performed 15, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min after injection. Each scan required 5 min.

A software package based on MATLAB was developed to process MRI images. A subtracted image was obtained by subtracting the pre-injected images from the post-injected images, and the signal intensity on the subtracted image was the amount of change in signal intensity (ΔSI) associated with the concentration of Gd-DTPA. Within a certain concentration range, ΔSI is proportional to the concentration of Gd-DTPA in the ECS of the brain, so we were able to obtain the concentration of Gd-DTPA in the ECS at each time point. Furthermore, brain ECS parameters were calculated by fitting the values to the concentration–time curve.

Statistical Analysis The data are expressed as the mean ± standard deviation (S.D.). The significances of the differences between groups were evaluated by one-way ANOVA using the Student–Newman–Keuls post hoc test. A value of p < 0.05 was considered statistically significant.

RESULTS

Mortality in Different Groups after Administration of Thymoquinone In the dose-dependent pharmacodynamic experiments of thymoquinone, we set low, medium, and high doses of thymoquinone at 2.5, 5, and 10 mg/kg. After excluding the death of rats before administration (preoperative or intraoperative death), the mortality of each group was counted. The death of rats in the high-dose administration group was severe (3 in 5), and the rats in the middle-dose administration group did not die. Therefore, in the subsequent experiments, the middle dose was selected as the administration dose.

Neurologic Deficit Scores Rats underwent neurological examinations 24 h after surgery. The neurological deficit scores were significantly higher in the model group than that of sham group (p < 0.01). Rats treated with thymoquinone (p < 0.05) or edaravone (p < 0.05) exhibited better behavioural performance than model group (Fig. 4).

TTC Staining There was obvious infarction in the tMCAO model group, and the first four slices had obvious white areas. After the intraperitoneal administration of 6 mg/kg edaravone, the infarct area was reduced, and infarction in the first brain slice was not obvious. The infarct area was further decreased after the intraperitoneal administration of 5 mg/kg thymoquinone (Fig. 5).

Percentage of Hemi-Cerebral Infarction In the rats, surgery caused neuronal necrosis that appeared as a white infarct area upon TTC staining. The statistical results showed that the percentage of hemi-cerebral infarction in the model group was 22.92 (± 4.31)%, which was significantly different from that in the sham operation group (p < 0.001). The intraperitoneal injection of 6 mg/kg edaravone was effective in reducing the percentage of hemi-cerebral infarction to 7.76 (± 3.96)%, which was significantly different from that in the model group (p < 0.01). The intraperitoneal injection of 5 mg/kg thymoquinone reduced the percentage of hemi-cerebral infarction to 6.92 (± 5.03)%, which was statistically different from that in the model group (p < 0.01) (Fig. 6).

H&E Staining The neurons in the hippocampal CA1 region of the model group showed necrosis and a scarce distribution, and there were many missing in some areas (Fig. 7b). This was significantly different from what was observed in the sham operation group (Fig. 7a). Five milligrams/kilogram thymoquinone treatment resulted in a morphology and number of neurons in this region that was more similar to in the hippocampal CA1 region of normal brain tissue (Fig. 7).
Injection Site and Diffusion of the Contrast Agent

According to our previous experiments, the damage caused by tMCAO model mainly occurred from the caudate nucleus to the cortical area. Therefore, Gd-DTPA should also be injected into this area during MRI scanning. The highlighted area indicated by the red arrow in Fig. 8A is the area in which Gd-DTPA was injected. The image shows that the contrast agent was mainly distributed from the caudate nucleus to the cortical area in the right hemisphere of the brain after injection. This is consistent with the ischemic injury caused by tMCAO, indicating that the injection site was appropriate (Fig. 8A).

After the injection of Gd-DTPA, the distribution and concentration of Gd-DTPA changed over time (Fig. 8B). The Gd-DTPA concentration is closely related to the brightness in MRI images. It can be clearly observed from the images that was gradually eliminated over time (the brightness of the region, as indicated by the red arrow, gradually decreased) after the injection of Gd-DTPA. The signal was no longer obvious at 210 min after injection. Therefore, the expected scanning time for this experiment is 240 min (Fig. 8B).

Since the linear relationship between ΔSI and the concentration of Gd-DTPA is strong only when the concentration of Gd-DTPA is 0–1 mM, the 15-, 30- and 45-min images were generally discarded for curve fitting. In order to get the parameters of ECS, we performed a total of 12 curve fittings, and Fig. 8C shows only one of the fitting results (Fig. 8C).

Coronal MRI Images of Gd-DTPA Diffusion and ECS Parameters in the Brains of tMCAO Rats after TQ Treatment

The right hemisphere of the rats that underwent tMCAO surgery showed a significant shaded area before the injection of Gd-DTPA. After the pre-scan, 2 µL of 10 mM Gd-DTPA was injected into the right caudate nucleus of the rats for 10 min. Then, the anaesthetized rats were placed in the prone position and scanned on a 3.0 Tesla MRI system. For each subject, repeated scans with a 3D MP-RAGE T1W sequence were performed every 15 min in the first hour post-injection and every hour thereafter. Figure 9A showed coronal MRI images of Gd-DTPA diffusion in the brains of tMCAO rats after thymoquinone treatment (Fig. 9A).

During our calculations, the image at \( t_0 \) is used as the baseline, and the data obtained by subtracting the image at \( t_0 \) from the subsequent image at a certain time point \( t \) is what we use for subsequent calculations. In our experiment, we set 10 time points for image scanning except \( t_0 \) which means that \( t = 15, 30, 45, 60, 90, 120, 150, 180, 240 \) min. After subtracting the image at \( t_0 \) from the images at these ten time points, fitting is performed, that is, as shown in Fig. 8C, to obtain the corresponding data. Because the \( t_0 \) of each group of data in Fig. 9A is different, it cannot be directly compared. This figure is only
a schematic diagram of the diffusion of the contrast agent.

The tortuosity of the ECS in the model group increased and was significantly different from that of the sham operation group \((p < 0.01)\). Thymoquinone or edaravone treatment were able to decrease the tortuosity to normal level, which were significantly different from that of the model group \((p < 0.01)\). This result indicated that thymoquinone could reduce the abnormal high tortuosity of ECS caused by ischemia–reperfusion injury to normal levels (Fig. 9B).

The volume fraction of the ECS in the model group was decreased and was significantly different from that of the sham operation group \((p < 0.01)\). Intraperitoneal injections of both thymoquinone and edaravone increased the volume fraction to normal level, which were significantly different from those of the model group \((p < 0.01)\). It was concluded that thymoquinone had the effect of protecting brain ECS from the damage caused by ischemia–reperfusion injury (Fig. 9C).

Fig. 7. Representative Images of the Brain Morphology Revealed by H&E Staining

H&E staining in the hippocampal CA1 area of the cerebral ischemic side of the rat brains after 2 h of ischaemia and 24 h of reperfusion are shown above. The hippocampus was scanned at 20× and is shown on the right. Scale bar = 2 mm for the full coronal section. Scale bar = 80 \(\mu\)m for microscopic observation. (a) Sham operation group, (b) tMCAO model group, (c) Thymoquinone treated group. \(n = 3\). (Color figure can be accessed in the online version.)

DISCUSSION

The tMCAO Model Establishing and the Choice of the Thymoquinone Dose

During the process of model building, anesthesia and surgical procedures are an invasive damage for rats. In the tMCAO model, such endovascular approaches have been associated with high rates of vessel perforation. Additionally, when large strokes are generated by endovascular approaches, significant brain edema will ensure, which will be associated with high mortality because of the increased intracranial pressure. The previous report the mortality of tMCAO model was from 25 to 60%.\(^{18,20}\)

So after models were established, rats with a behavioural score between 2–4 were used for subsequent experiments.

In the previous pharmacodynamics experiment of thymoquinone, the doses of 2.5, 5, and 10 mg/kg were used. The effects of the doses of 5 and 10 mg/kg were robust in our study. For the intraperitoneal injection of thymoquinone, the median lethal dose for rats is 57.5 mg/kg,\(^{16}\) and this is why the 5 mg/kg dose was used for subsequent experiments.

Effect of Thymoquinone on the Neuroethology and Infarction Area of in tMCAO Model Rats

Cerebral ischemia–reperfusion in rats is accompanied by neurobehavioural damage, which is manifested as motor functional deficits, and the necrosis of cells in the ischemic region, which is manifested as significant changes in cell morphology and the infarcted area upon staining. Our experimental results showed that thymoquinone can effectively ameliorate neurological deficit scores, decrease cerebral infarction and ameliorate cell morphology and quantity in the infarcted areas.

The key to the treatment of stroke is the timely restoration of the blood supply to the ischemic area, whether mechanically or with medical treatment. However, the recovery of the blood supply is accompanied by ischemia–reperfusion injury. During this period, oxidative stress, mitochondrial dysfunction, excitotoxicity, and inflammation lead to the necrosis of neurons in the ischemic area, aggravating brain tissue damage.\(^{8–10}\) The antioxidant and anti-inflammatory effects of thymoquinone\(^{16}\) may affect multiple key processes during ischemia–reperfusion injury. In the previous study, Silachev \textit{et al.} reported that thymoquinone could accumulate in mitochondria and play an antioxidant role in a rat model of brain ischemia–reperfusion injury.\(^{21}\) In our study, we also confirmed that thymoquinone had the therapeutic effects on brain ischemia–reperfusion injury. In the future, we will determine some anti-inflammatory and antioxidant indexes to explore the...
mechanism of action of this molecule.

**Effect of Thymoquinone on the ECS Parameters of tMCAO Model Rats** Cerebral ischemia–reperfusion is accompanied by the redistribution of ions and water in the ECS and neurons. A large number of ions and water molecules are transported into neurons, causing swelling or even the lytic death of the neurons. At the same time, immune cells located in the central nervous system are activated by the damage, and they release inflammatory factors, proteins and other substances into the ECS. Changes in the morphology of neurons and changes in the composition of the liquid in the ECS both result in changes in the ECS parameters, manifested...
as a decrease in the volume fraction ($\alpha$) and an increase in the tortuosity ($\lambda$) in Cerebral ischemia–reperfusion.

Treatment with 5 mg/kg thymoquinone (intraperitoneally (i.p.)) can protect ECS against ischemia–reperfusion injury by inhibiting oxidative stress and inflammation during cerebral ischemia–reperfusion in rats. The abnormal brain ECS volume fraction (16.43 ($\pm$ 0.21)%) and tortuosity (1.423 ($\pm$ 0.05)) were restored to normal levels, which were 17.61 ($\pm$ 0.29) % ($p < 0.01$) and 1.181 ($\pm$ 0.05) ($p < 0.01$), respectively. This indicates that thymoquinone can protect brain tissue against ischemia–reperfusion injury by maintaining the normal structure and composition of the ECS, and it is expected to become a new neuroprotective drug. The mechanism of its specific protective effects still requires further research.

This study was the first to evaluate the effect of thymoquinone on ECS parameters in tMCAO rats with Gd-DTPA-enhanced MRI. In previous studies on stroke, most of the attention was placed on the vascular system in the ischemic region. Given that ECS plays an important role in the process of material and information exchange between neurons, therefore, ensuring the normality of ECS is important for the treatment of brain diseases. In our study, we demonstrated treatment with 5 mg/kg thymoquinone (i.p.) could reduce brain injury areas in rats, improve behavior scores, and improve survival rate of rats, and all those were closely related to the improvement of ECS parameters. As we all known, Alzheimer’s disease is caused by the deposition of $\beta$-amyloid in the brain, these injuries could manifest as abnormalities in brain ECS parameters. Improving ECS parameters of the brain may be helpful for the treatment. Focusing on the ECS can provide new ideas for the diagnose and treatment of brain diseases.

In summary, thymoquinone, the effective ingredient of *Nigella sativa*, has effects against ischemia–reperfusion injury. The ECS parameters of tMCAO rats, specifically the volume fraction and tortuosity of the ECS, were significantly improved after thymoquinone treatment. Further studies are needed to elucidate the specific mechanisms of thymoquinone’s protective effects on ECS in the context of cerebral ischemia–reperfusion injury.
fraction $\alpha$ and the tortuosity $\delta$, were restored after treatment with thymoquinone. Using a MRI tracer-based method, we found that thymoquinone treatment could inhibit the changes of ECS diffusion parameters, which might provide some information about neuronal loss and astrogliosis activation and might also help to prove that thymoquinone exerted a neuroprotective role in tMCAO model.

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Author Contributions Chaoxin Fan organized and performed the experiments and wrote the manuscript. Fang Tian performed partially the tMCAO experiments. Xin Zhao, Yi Sun and Xiaogai Yang helped organize the experiment and provided operational guidance. Hongbin Han provided technical guidance for MRI. Xiaoping Pu helped organize the experiment and performed partially the tMCAO experiments. Xin Zhao, Yi Sun and Xiaogai Yang helped organize the experiment.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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