Effects of minocycline on learning and memory of mice following ischemic-hypoxic cerebral injuries*

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Abstract
An ischemic-hypoxic animal model was established using right common carotid artery occlusions and inhalation of low concentrations of oxygen in mice. At 10 days after the ischemic-hypoxic injuries, saline-treated mice exhibited significantly prolonged escape latencies in water-maze tests and significantly shorter memory latencies and more mistakes in step-down tests. In contrast, mice treated with 5 mg/kg minocycline exhibited significant reversals of each of these effects compared with the saline-treated control mice. Moreover, we found that minocycline can relieve brain water content and morphological changes in mice following ischemic-hypoxic cerebral injuries. Accordingly, our findings indicate that minocycline provides some protections against the deleterious effects of these injuries in mice.

Key Words: minocycline; ischemic-hypoxic cerebral injury; learning and memory; cognition

INTRODUCTION
It is generally acknowledged that ischemic cerebrovascular disease is triggered by a number of factors and pathways. Moreover, its deleterious effects are related to the production of oxygen free radicals, the toxic effects of excitatory amino acids, intracellular calcium overload, energy metabolism disturbances, and the release of inflammatory mediators[1-3]. A large number of in vivo and in vitro animal experiments have found neuroprotective agents that can reduce these deleterious effects to varying degrees. Unfortunately, these promising preclinical studies have not led to any clinically effective neuroprotective agents[4-5]. One of the reasons for this clinical ineffectiveness is that most neuroprotective agents that have been developed do not readily cross the blood-brain barrier because they are water-soluble or their molecular weight is greater than 500 kD. As such, these drugs often need to be given prior to the onset of ischemic cerebral injuries or immediately after ischemia occurs[6].

Minocycline hydrochloride is a semi-synthetic tetracycline derivative. It has been reported that minocycline can readily cross the blood-brain barrier. Moreover, in addition to anti-microbial effects, it appears to exhibit anti-inflammatory effects, reduce NMDA toxicity, inhibit the activation of microglial cells, and suppress the release of inflammatory cytokines[7]. However, there is no behavioral evidence available regarding its neuroprotective effects.

In summary, in the present study, we sought to further elucidate the neuroprotective effects of minocycline in mice after ischemic-hypoxic cerebral injuries. To this end, we examined these mice using the water-maze and step-down tests and determinations of brain edema and brain tissue morphology.

RESULTS
Quantitative analysis of experimental animals
Fifty mice that exhibited similar baseline memory performances as determined by the water-maze and step-down tests, were randomly divided into a normal control group (normal feeding), a sham operation group (separation of the common carotid artery but no ligation), an ischemia + hypoxia group (right common carotid artery ligation and hypoxia inhalation), a minocycline group (right common carotid artery ligation, hypoxia inhalation and minocycline hydrochloride treatment), and a saline group (right common carotid artery ligation, hypoxia inhalation and saline treatment). Each group contained 10 mice. In total, 50 mice were involved in the final analysis.
Effects of minocycline on learning and memory in mice following hypoxic-ischemic brain injuries

Learning and memory in mice were determined using the water-maze and step-down tests. There was no statistically significant differences in the water-maze escape latencies, the step-down latencies, and the number of mistakes across the groups of mice before the ischemia/hypoxia injuries were conducted (P > 0.05; Tables 1, 2).

**Table 1** Results of the water-maze test

| Group                  | Escape latency (second) | Before administration 10 days after administration |
|------------------------|-------------------------|----------------------------------------------------|
| Normal control         | 64.4±18.2               | 67.4±15.3                                          |
| Sham operation         | 64.3±18.5               | 69.4±14.8                                          |
| Ischemia + hypoxia     | 62.5±15.1               | 113.8±6.2<sup>a</sup>                              |
| Minocycline            | 66.5±14.1               | 95.5±11.4<sup>abc</sup>                            |
| Saline                 | 63.2±16.2               | 115.5±5.9<sup>a</sup>                              |

Data are expressed as mean ± SD of seven rats in each group (three rats were used for the slices); <sup>a</sup>P < 0.05, vs. normal control group; <sup>b</sup>P < 0.05, vs. ischemia + hypoxia group; <sup>c</sup>P < 0.05, vs. saline group (one-way analysis of variance test followed by Student Newman-Keuls-q test).

**Table 2** Results of step-down test

| Group                  | Baseline (square) | 10 days after administration (square) |
|------------------------|-------------------|---------------------------------------|
|                        | Learning period (second) | Number of mistake (5 min) | Memory latency (second) | Number of mistake (5 min) |
| Normal control         | 39.1±2.4          | 4.4±0.9                               | 176.7±5.1              | 4.1±0.7                  |
| Sham operation         | 40.8±2.1          | 4.3±1.0                               | 176.3±6.4              | 3.9±0.5                  |
| Ischemia + hypoxia     | 39.8±2.7          | 4.1±1.1                               | 99.0±5.8<sup>a</sup>   | 5.8±0.5<sup>a</sup>      |
| Minocycline            | 40.5±2.7          | 4.1±1.1                               | 139.8±1.5<sup>abc</sup>| 4.7±0.7<sup>abc</sup>    |
| Saline                 | 39.5±3.4          | 3.8±0.9                               | 102.5±6.4<sup>a</sup>  | 5.4±0.6<sup>abc</sup>    |

Data are expressed as mean ± SD of seven rats in each group (three rats were used for the slices); <sup>a</sup>P < 0.05, vs. normal control group; <sup>b</sup>P < 0.05, vs. ischemia + hypoxia group; <sup>c</sup>P < 0.05, vs. saline group (one-way analysis of variance test followed by Student Newman-Keuls-q test).

**Water-maze test results**

In the water-maze test, longer escape latencies represent worse learning and memory performance[8]. The results of this study showed that 10 days after the surgeries, escape latencies were not significantly different between the sham-operated mice and the normal control mice, whereas escape latencies were significantly prolonged in mice in the ischemia + hypoxia, minocycline, and saline groups compared with the normal control group (P < 0.05). In addition, escape latencies were significantly shorter in the mice in the minocycline group compared with the ischemia + hypoxia group (P < 0.05). Escape latencies were not significant different between the saline group and the ischemia + hypoxia group (Table 1).

**Step-down test results**

In the step-down test, shorter memory latencies and more mistakes indicate impaired learning and memory in mice[9]. The results of this study showed that 10 days after the surgeries, memory latencies and the number of mistakes were not significantly different between the normal control mice and the sham-operated mice (P > 0.05). In addition, memory latencies were significantly shorter and the numbers of mistakes were significantly greater in the mice in the ischemia + hypoxia group, minocycline group, and saline group compared with normal control group (P < 0.05). Compared with the ischemia + hypoxia group, memory latencies were prolonged and the numbers of mistakes reduced in the minocycline group (P < 0.05), whereas the mice in the saline group showed no significant differences on these measures compared with the mice in the ischemia + hypoxia group (P > 0.05; Table 2).

**Effect of minocycline on brain water content following hypoxic-ischemic brain injuries**

Brain water content was significantly higher in mice in the ischemia + hypoxia and saline groups compared with those in the normal control group (P < 0.05). The minocycline-treated mice had significantly lower brain water contents than the ischemia + hypoxia mice (P < 0.05; Table 3).

**Table 3** Results of brain tissue water content (% assessments)

| Group                  | Brain tissue water content |
|------------------------|----------------------------|
| Normal control         | 78.36±0.99                 |
| Sham operation         | 78.18±0.96                 |
| Ischemia + hypoxia     | 87.60±3.19<sup>a</sup>     |
| Minocycline            | 82.43±0.82<sup>b</sup>     |
| Saline                 | 86.84±1.25<sup>c</sup>     |

Data are expressed as mean ± SD of seven rats in each group (three rats were used for the slices); <sup>a</sup>P < 0.05, vs. normal control group; <sup>b</sup>P < 0.05, vs. ischemia + hypoxia group; <sup>c</sup>P < 0.05, vs. saline group (one-way analysis of variance test followed by Student Newman-Keuls-q test).

Effect of minocycline on cerebral cortex morphology following hypoxic-ischemic brain injuries

Hematoxylin-eosin staining showed that the cortical neurons of mice in the normal control and sham-operated groups were oval-shaped with complete and full cell bodies, good refraction, abundant cytoplasm, and clear nuclear membranes and nucleoli. In the subjects in the ischemia + hypoxia and saline groups, cortical interstitial tissues were loose, there was apparent edema, there were necrotic neurons, and occasionally we could not observe the nuclei. The lesions that resulted from the injuries in the minocycline group were significantly improved compared with those in the ischemia + hypoxia and saline groups (Figure 1).

**DISCUSSION**

A direct consequence of ischemic brain injuries is local or large blood supply disturbances in brain tissue that induces neuronal damage or death, thus resulting in the dysfunction of the nervous system[10]. In this study, we
produced a hypoxic-ischemic brain injury model through unilateral (right side) ligation of the common carotid artery and inhalation of air with a low concentration of oxygen. The water-maze and step-down tests and morphological examinations of brain tissue showed apparent behavioral and cerebral dysfunctions in the mice. This suggests that our approach is a valid means of assessing hypoxic-ischemic brain injuries in mice.

The water-maze and step-down tests are commonly used to observe behavioral changes in mice because of their simplicity\[11\]. At the beginning of the experiment, the experimental mice were examined using the water-maze and step-down tests. This allowed the mice to habituate to these procedures and showed that there were no significant baseline differences between the groups. Moreover, we suggest that these baseline determinations did not overly influence the subsequent test sessions because the natural amnesia time of mice has been described to be 4 days\[12\], and we conducted this at 10 days, so the memory obtained by test trainings prior to the experiment had no impact on the final results. Recent studies found that minocycline played a positive role on patients with acute cerebral ischemia\[13\]. Although a number of studies have found significant neuroprotective effects of minocycline, there are few reports available regarding whether it protects learning and memory following neurological insults. In the present study, minocycline significantly protected the learning and memory abilities of mice following brain injuries as assessed by the water-maze and step-down tests, which is consistent with previous studies\[14\]. In addition, minocycline was shown to significantly reduce brain edemas. Accordingly, these behavioral and histological data collectively indicate that minocycline protect the brain against the untoward effects of hypoxic-ischemic brain injuries.

**MATERIALS AND METHODS**

**Design**
A randomized controlled animal experiment.

**Time and setting**
Experiments were performed in a laboratory at the Zhuhai Campus of Zunyi Medical College, China from August 2010 to December 2010.

**Materials**
Fifty adult male Kunming Species mice that were 2 months old, weighed 22.6 ± 2.3 g, and of clean grade were provided by the Animal Center of Sun Yat-sen University, China (license No. 2006A055). The conduct of the animal experiments was in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China\[15\].

**Methods**
**Establishment of animal models of hypoxia-ischemia and intervention**
Mice were housed in cages and allowed free access to food. According to previously described methods\[16\], mice were intraperitoneally anesthetized with 4% chloral hydrate at 5 mL/100 g and fixed in the supine position. The median neck was disinfected and the right common carotid artery was separated and ligated. The skin incision was sutured, and the mice were placed in a closed chamber containing 92% nitrogen and 8% oxygen for 2 hours.

![Figure 1](image1.png)

Figure 1  Morphological observations in cortices of representative mice in the normal control group (A), the sham operation group (B), the ischemia + hypoxia group (C), the minocycline group (D), and the saline group (E) (hematoxylin-eosin staining, × 200).
According to previously described methods\textsuperscript{[17]}, minocycline (100 mg per tablet; China Wyeth Pharmaceuticals Co., Ltd., Suzhou, Jiangsu, China) was diluted with saline to 1 mg/mL. From the second day after the surgeries, for 10 days, the minocycline-treated mice were fed daily 5 mg/kg minocycline (equal to the clinical dose), and the saline group was given saline. The mice in the normal control group, sham operation group, ischemia + hypoxia group received normal feedings over these 10 days. The behavioral observation was conducted 1 day after drug discontinuation.

**Water-maze test results**

A DB001-type water-maze (Zhishu Duobao Beijing Biotech Co., Ltd., China) device is shown in Figure 2.

![Image](image_url)

It is a circular pool with an 80 cm diameter and a 30 cm height, divided into four quadrants (N, S, W, E). An escape platform (diameter 5 cm) was placed between the N and E quadrants, 0.5 cm higher than the horizontal plane. Water in the pool was dyed dark with carbon ink, and the N and E quadrants, 0.5 cm higher than the horizontal escape platform (diameter 5 cm) was placed between them. Water in the pool was dyed dark with carbon ink, and the N and E quadrants, 0.5 cm higher than the horizontal escape platform (diameter 5 cm) was placed between them. Water in the pool was dyed dark with carbon ink, and the N and E quadrants, 0.5 cm higher than the horizontal escape platform (diameter 5 cm) was placed between them. Water in the pool was dyed dark with carbon ink, and the N and E quadrants, 0.5 cm higher than the horizontal escape platform (diameter 5 cm) was placed between them. Water in the pool was dyed dark with carbon ink, and the N and E quadrants, 0.5 cm higher than the horizontal escape platform (diameter 5 cm) was placed between them.

The results were recorded.

**Step-down test results**

The step-down test was conducted with a STT-2 device (Institute of Materia Medica, Chinese Academy of Medical Sciences, China) according to methods previously described\textsuperscript{[18]}. Mice were randomly put into a step-down device at a 36 V voltage. They were allowed 5 minutes to adapt to the environment and 5 minutes for switching. Mice subjected to electric shocks must find an insulated platform to discontinue the electric shock. The time from the beginning of the electric shocks to the first jump onto the insulated platform was recorded during training and the number of mistakes made when stepping down from the insulated platform within 5 minutes was also recorded. Mice were placed directly onto the step-down platform 24 hours later to record determine whether there were any changes in their latencies and number of mistakes. If a mouse stayed on the insulated platform for more than 5 minutes, 5 minutes was recorded as that mouse’s latency.

**Determination of brain tissue water content in mice**

After the water-maze and step-down tests were completed, seven mice in each group were decapitated, their right brain hemispheres were removed, and its wet weight was measured. Brain tissue was dried at 120°C in a drying box for 24 hours, and its dry weight was measured. The brain water content was calculated according to the following formula: (wet weight of the brain – dry weight of the brain) / wet weight of the brain × 100%\textsuperscript{[19]}. 

**Determination of brain cortex morphological changes in mice as determined by hematoxylin-eosin staining**

10 days after the surgeries, the remaining three mice in each group underwent a thoracotomy under deep anesthesia with 4% chloral hydrate (Wuhan Boster, China) to expose the heart. Specimens were rinsed first with saline and perfused with 4% paraformaldehyde after the blood was removed. The right cerebral cortex was fixed with 4% paraformaldehyde\textsuperscript{[19]}. After dehydration, embedding in paraffin, slicing, and hematoxylin-eosin staining, pathological changes in the cerebral cortex were observed under an optical microscope (Olympus, Tokyo, Japan).

**Statistical analysis**

Data are expressed as mean ± SD. They were analyzed with SPSS 11.5 statistical package (SPSS, Chicago, IL, USA) using one-way analysis of variance test followed by Student Newman-keuls-q test. A \( P < 0.05 \) was considered to represent a significant difference.

**Author contributions:** Quanzhong Chang was responsible for the study design and supervision. All authors participated in the implementation of the experiment. Hongling Fan analyzed and integrated data, and wrote the manuscript. Quanzhong Chang was responsible for funding.

**Conflicts of interest:** None declared.

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