·Original Article·

Minocycline reduces astrocytic reactivation and neuroinflammation in the hippocampus of a vascular cognitive impairment rat model

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Abstract: Objective   To study the neuroprotective mechanism of minocycline against vascular cognitive impairment after cerebral ischemia. **Methods**   The rat model with vascular cognitive impairment was established by permanent bilateral common carotid artery occlusion (BCCAO). The observing time-points were determined at 4, 8 and 16 weeks after BCCAO. Animals were randomly divided into sham-operated group ($n = 6$), model group (subdivided into 3 groups: 4 weeks after BCCAO, $n = 6$; 8 weeks after BCCAO, $n = 6$; and 16 weeks after BCCAO, $n = 6$), and minocycline group (subdivided into 3 groups: 4 weeks after BCCAO, $n = 6$; 8 weeks after BCCAO, $n = 6$; and 16 weeks after BCCAO, $n = 6$). Minocycline was administered by douche via stomach after BCCAO until sacrifice. Glial fibrillary acidic protein (GFAP) was examined by Western blotting and immunohistochemistry. Levels of cyclooxygenase-2 (COX-2) and nuclear factor-kappaB (NF-κB) were measured by immunohistochemistry. IL-1β and TNF-α levels were tested with ELISA method. **Results**   Levels of GFAP, COX-2, NF-κB, IL-1β and TNF-α were all up-regulated after permanent BCCAO, which could be significantly inhibited by minocycline. **Conclusion**   Minocycline could ameliorate the inflammation and oxidative stress in the hippocampus of the vascular cognitive impairment rat model.

Keywords: vascular cognitive impairment; minocycline; inflammation; astrocyte

1   Introduction

Chronic cerebral hypoperfusion (CCH) contributes to cognitive impairment[1,2], and the reduction of cerebral blood flow correlates with the severity of cognitive impairment[3]. CCH also plays a critical role in the pathogenesis of vascular cognitive impairment[4,5]. In addition, the secondary inflammation is involved in brain ischemia, and significantly contributes to the outcome after ischemic insult[6,7]. Since the inflammatory response is a delayed process, its related molecules are potential targets for the disease treatment in human. These molecules include cyclooxygenase-2 (COX-2)[8,9], nuclear factor-kappaB (NF-κB)[10-12], tumor necrosis factor alphfa (TNF-α), and interleukin 1beta (IL-1β), a pro-inflammatory cytokine released by reactive astrocytes[13,14]. Various mechanisms of neuronal injury in CCH have been proposed, including formation of free radicals, oxidative stress, mitochondrial dysfunction, inflammatory processes,

genetic factors, environmental impact factors, apoptosis[15-20], *etc.* These factors may interact with each other to amplify the toxicity, leading to neuronal dysfunction, and finally cell death.

Minocycline is a derivative of tetracycline. It can inhibit inflammation, oxidative stress, and apoptosis, and protect against cerebral ischemia[21-23]. Previously, we have found that minocycline attenuates cognitive impairment and restrains oxidative stress in the hippocampus of rats with CCH[22]. In the present study, the rat model of vascular cognitive impairment was established by permanent bilateral occlusion of both common carotid arteries. Rats were then administered with minocycline. The expression levels of glial fibrillary acidic protein (GFAP, a marker of astrocyte activation), COX-2, NF-κB, IL-1β and TNF-α in rat brain were detected, to further study the mechanism of the neuroprotective effect of minocycline against vascular cognitive impairment.

2   Materials and methods

2.1 Animals and drug application Wistar rats (female, 10 weeks old, weighting 200-250 g) were obtained from the Field Zoology Research Institute of Third Military Medical University of China, and housed separately in metal hanging cages at the constant room temperature of 25 ºC under a 12: 12 h day/night cycle with light off at 18:00. All the rats were habituated to the hanging cage and the handling at least 5 d before the experiments. Animals were fed with common food and water. The animal model of vascular dementia was established with permanent bilateral common carotid artery occlusion (BCCAO) for chronic bilateral common carotid artery occlusion[1,22,24]. The observing time-points were determined at 4, 8 and 16 weeks after BCCAO (average lifespan was 16 weeks after the establishment of CCH model). Animals were randomly divided into sham-operated (S) group (contour i.p. injection of saline, *n*=6), model (M) groups (subdivided into M4, 4 weeks after BCCAO, *n*=6; M8, 8 weeks after BCCAO, *n*=6; and M16, 16 weeks after BCCAO, *n*=6) and minocycline treatment (MT) group (subdivided into MT4, 4-week minocycline administration after BCCAO, *n*=6; MT8, 8-week minocycline administration after BCCAO, *n*=6; and MT16, 16-week minocycline administration after BCCAO, *n*=6).

Minocycline was administered by douche via stomach until sacrifice after permanent BCCAO. Rats were anesthetized with 10% chloral hydrate (350 mg/kg, i.p.) and allowed to breathe spontaneously throughout the surgical procedure. Both common carotid arteries were exposed via a midline cervical incision and were doubly-ligated with silk suture. Animals in sham group were treated in the same manner, except that the common arteries were not occluded. All the experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996), following the international ethical standards, and were approved by the research ethics committee of Chongqing Medical University, China.

Minocycline (100 mg/capsule, Huishi Pharmaceutical Limited Company, China) was diluted to 0.5 mg/mL by normal saline. Animals in S and M groups were given the same volume of normal saline through douche via stomach. Rats in MT groups received minocycline through douche via stomach at the dosage of 50 mg/kg per day, according to the previous reports[25,26].

2.2 Immunohistochemical assay Tissue samples were collected after surgery and immediately frozen in liquid nitrogen. Prior to immunohistochemistry assay, frozen sections were prepared with a cryostat (FACS caliber, Becton Dickinson, USA) at -20 ºC, dried at room temperature, and fixed with acetone. The ABC immunohistochemical assay was carried out according to the protocols described before[22]. Anti-GFAP, anti-COX-2 and anti-NF-κB antibodies were all from Sigma, USA. Biotin-labeled goat anti-mouse IgG was purchased from Vector Co., USA. A total number of 200 cells were counted and the staining intensity of each cell was adjusted. Five grades were employed to express the degrees of staining, which represented 5 reaction coefficient. The 5 products of every coefficient and the corresponding cell number were added up, which resulted in the value of a positive score. All slides were measured in duplicate. Samples with a positive score over 10 or frequency over 5% were considered as positive.

2.3 Western blotting Western blotting assay was performed according to the previous descriptions[27,28]. Tissues were

dissected and homogenized in T-PER buffer containing protease inhibitors. After homogenization, the lysates were centrifuged at 100 000 g, and the supernatant was collected for Western blotting assay, Ciphergen (Biosource International, Inc., USA). Equal amounts of lysates were resolved by SDS-PAGE (Tris–glycine mini gel, 1:2 500; Biosource International, Inc., USA) for Western blot analysis using antibodies specific for GFAP (1:2 500; Biosource International, Inc., USA), COX-2 (1:1 000; BioSource International, Inc., USA), NF-κB (1:1 000; Sigma, USA), and β-actin (1:5 000; Biosource International, Inc., USA). The optical densities of the specific bands were scanned and measured by image analysis software (HPIAS 2000, Tongji Qianping Company, Wuhan, China).

2.4 ELISA assay Rat tissues were dissected and homogenized in T-PER buffer (Biosource International, Inc., USA) containing protease inhibitors (Biosource International, Inc., USA). After homogenization, the lysates were centrifuged at 100 000 g, and supernatant was collected for ELISA analysis. Besides, protein inhibitors and AEBSF (Sigma) were added to the supernatant to prevent degradation of IL-1β and TNF-α. The concentrations of IL-1β and TNF-α were measured using the Colorimetric ELISA kit (Biosynthysis Company, China), according to previous descriptions[29].

2.5 Statistical analysis Data were expressed as mean±SD. Student's t test was employed to examine the significance of variation between groups using SPSS software for Windows 8.0 (SPSS, Inc., Chicago, IL, USA). For statistical evaluation, one-way analysis of variance (ANOVA) was employed. Student Newman Keuls test was performed when variance was equal, and Games-Howell test was performed when variance was not equal. $P < 0.05$ was considered as statistically significant.

3   Results

3.1 Minocycline reduced astrocytic reactivation Our previous studies using Morris water maze test and open-field task have shown that minocycline could ameliorate the cognitive impairment caused by permanent bilateral occlusion of both common carotid arteries[22]. To further discover the underlying mechanism, the expression of GFAP (a marker of astro-

cyte activation) in the hippocampus was examined by immunohistochemistry and Western blotting. The results showed that expression level of GFAP in MT group was significantly lower than that in time-matched M group. Expression levels of GFAP in M groups were significantly increased, compared to that in S group ($P < 0.05$), whereas minocycline treatment could inhibit this increase ($P < 0.05$).The number of reactive astrocytes was decreased after minocycline treatment (Fig. 1A). Moreover, there was no significant difference in GFAP level among either M groups or MT groups (Fig. 1A, B).

3.2 Minocycline down-regulated expression levels of COX-2 and NF-κB To further explain the mechanism by which minocycline attenuates the behavioral deficits, the expressions of COX-2 and NF-κB, the intermediate elements of inflammation and oxidative stress, were measured by immunohistochemistry. As shown in Fig. 2, the hippocampal expression levels of COX-2 and NF-κB in BCCAO rats were significantly increased, compared to those in control rats ($P < 0.01$). Moreover, minocycline treatment could significantly inhibit these increases induced by BCCAO ($P < 0.01$). Besides, there was no difference in either COX-2 or NF-κB expression level among the 3 time-points in each group.

3.3 Minocycline down-regulated IL-1β and TNF-α levels To further explore the neuroprotective mechanism of minocycline against chronic cerebral ischemia, the hippocampal levels of IL-1β and TNF-α, pro-inflammtary cytokines released by reactive astrocytes, were detected by ELISA. Results showed that the IL-1β level was significantly elevated from (16.12±2.06) pg/mg in S group to (34.41±2.82) pg/mg (the maximal level) in M groups ($P < 0.01$), the latter of which was then significantly decreasd in MT groups ($P < 0.01$) (Fig. 3A). Similarly, TNF-α was significantly up-regulated from (21.53±3.16) pg/mg in S group to (39.48±3.62) pg/mg (the maximal level) in M group ($P < 0.01$), the latter of which was then significantly decreased in MT groups ($P < 0.01$) (Fig. 3B). Besides, there was no significant difference among either M groups or MT groups.

4   Discussion

Minocycline is a semi-synthetic tetracycline antibiotic that could effectively cross the blood-brain barrier. Minocycline
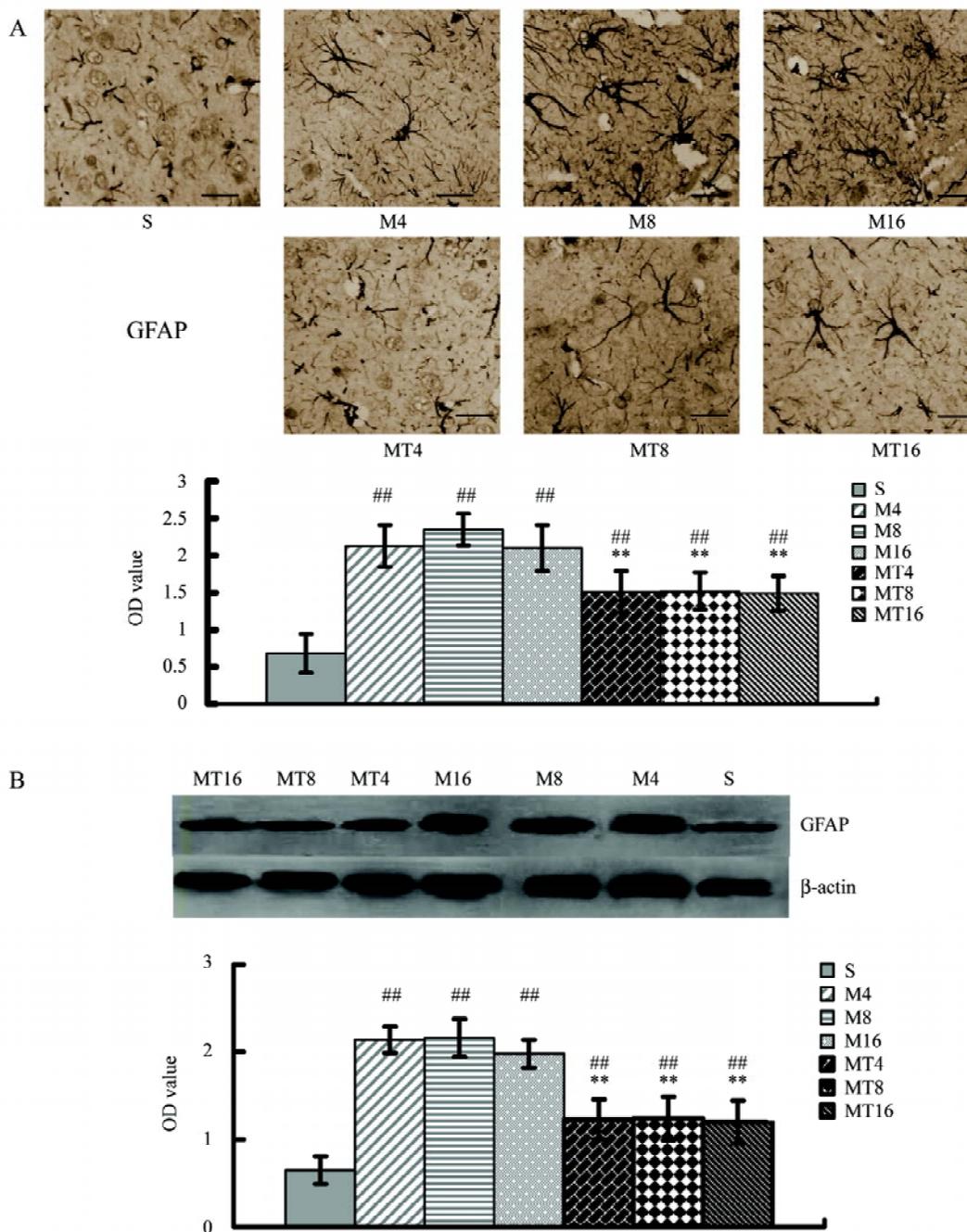
Fig. 1 The expression of GFAP in the hippocampus was measured by immunohistochemistry and Western blotting. A: Immunohistochemistry showed that minocycline treatment significantly inhibited BCCAO-induced increase in GFAP expression level. Optical density values in M and MT groups were both significantly higher than that in S group ($^{\#\#}P < 0.01$). Besides, the density value in MT group was significantly lower than that in time-matched M group ($^{**}P < 0.01$). Scale bar: 25 μm. B: Western blotting analysis of GFAP expression. The relative amount of GFAP was expressed by the densitometry ratio of GFAP to β-actin (mean±SD). The expression level of GFAP in MT group was significantly lower than that in time-matched M group ($^{**}P < 0.01$). Besides, optical density values in M and MT groups were both significantly higher than that of S group ($^{\#\#}P < 0.01$).
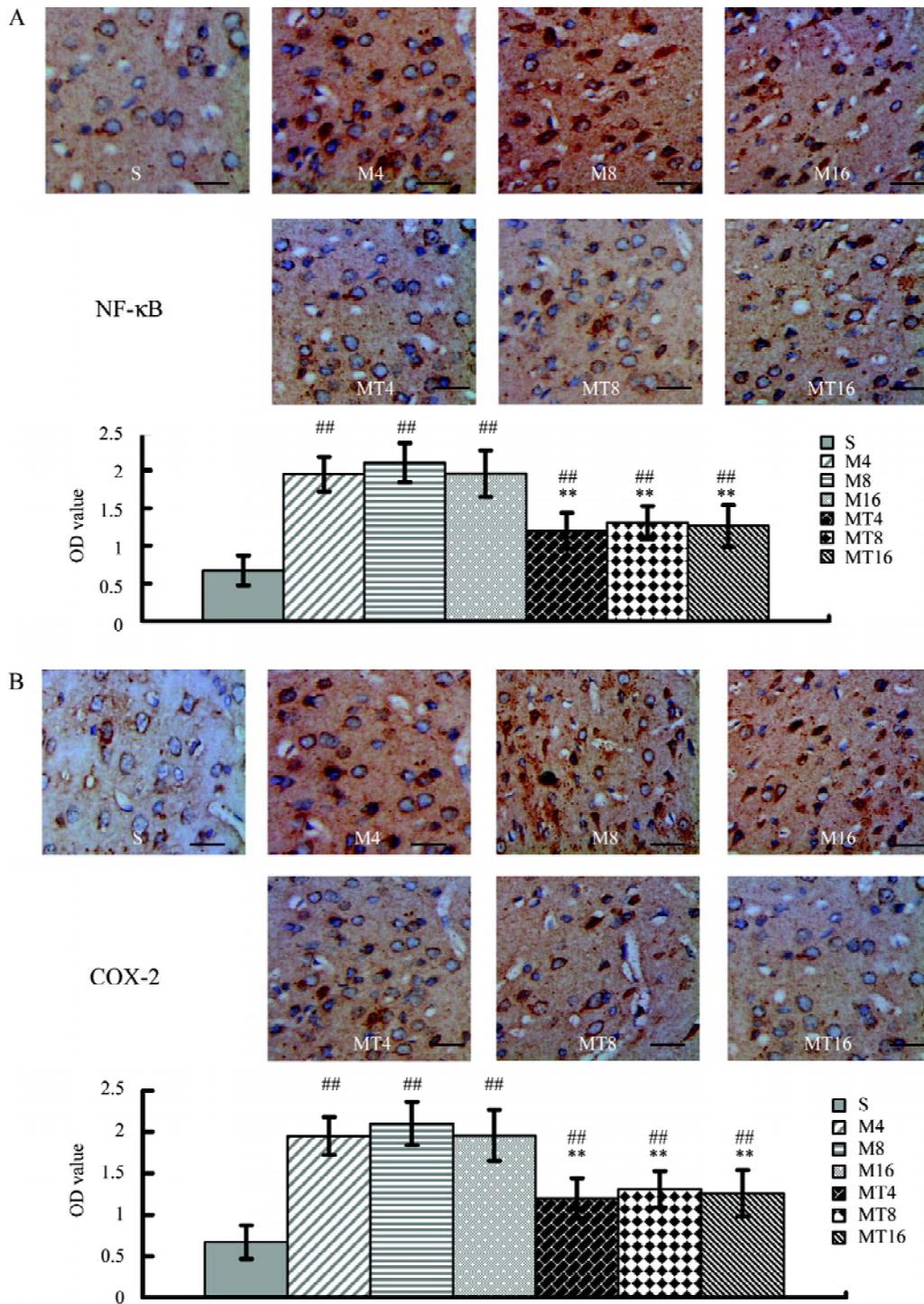
Fig. 2 The expressions of NF-κB and COX-2 in the hippocampus were measured by immunohistochemistry. A: The expression of NF-κB was significantly increased after BACCO, as compared to that in S group. However, minocycline could significantly inhibit this increase. Besides, optical density values in M and MT groups were both significantly higher than that in S group (##$P < 0.01$). B: Minocycline could significantly inhibit the BACCO-induced increase in COX-2 expression. Optical density values in M and MT groups were both significantly higher than that in S group (##$P < 0.01$). Scale bar: 25 μm.
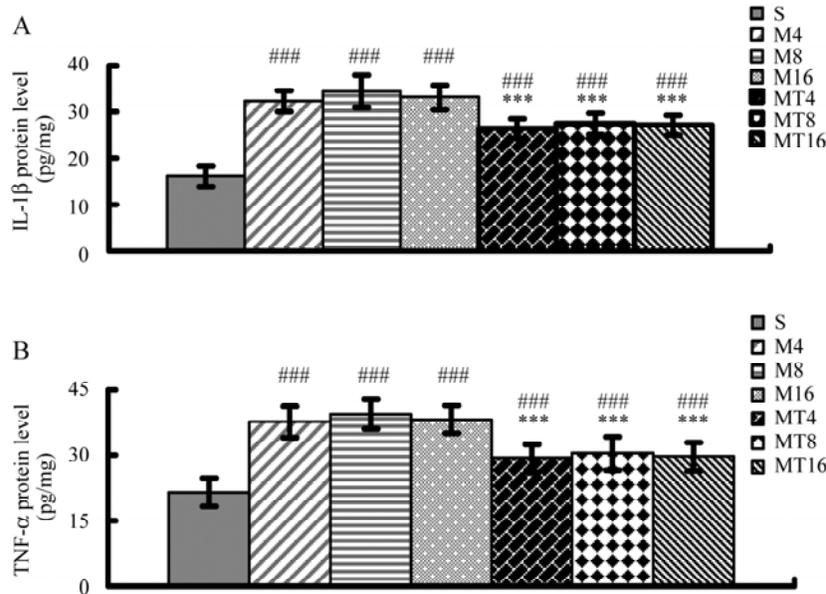
Fig. 3 ELISA analysis of IL-1β (A) and TNF-α (B) protein levels. Protein levels of IL-1β and TNF-α in M and MT groups were both significantly higher than those in S group (###$P < 0.001$), whereas expression levels of IL-1β and TNF-α in MT groups were significantly lower than those in time-matched M groups (***$P < 0.001$).

has been reported to exert significant neuroprotective effects in cerebral ischemia[30-32], amyotrophic lateral sclerosis[33], Alzheimer's disease[34], Huntington's[35,36] and Parkinson's diseases[37]. Besides, minocycline can inhibit post-ischemic brain inflammation[38], astrocyte reactivation, microglia activation[39], oxidative stress, apoptosis, extracellular matrix degradation[40], *etc*. One common pathophysiological mechanism of brain ischemic damage includes reactive oxygen reactive nitrogen, oxidative stress and neuroinflammation. In the present study, we established the vacsular cognitive impairment model by permanent bilateral occlusion of both common carotid arteries. Our previous studies using Morris water maze test and open-field task have shown that cognitive impairment occurs in the CCH model. Besides, cognitive impairment could be attenuated by minocycline treatment[22]. Here we find that minocycline down-regulated GFAP expression in the hippocampus and decreased the number of reactive astrocytes. Moreover, minocycline could restrain neuroinflammation and oxidative stress in the central nervous system during cerebral ischemia.

Astrocytes constitute the main population of glial cells in the brain and represent over 50% of the total cells in the cerebral cortex and 20%-30% of the total cell volume. Astrocytes normally perform many functions that are essential for neuronal viability. Reactive astrocytes can release proinflammatory factors, reactive oxygen species and reactive nitrogen species, leading to abnormal breakdown of molecules[41-43]. GFAP is the hallmark of astrocyte activation, revealing star-shaped morphology of astrocytes[44]. In the present study, minocycline could down-regulate GFAP expression in the hippocampus and decrease the number of reactive astrocytes, which indicates the suppression of astroglial cell activation by minocycline in rats with permanent bilateral occlusion of both common carotid arteries. We therefore propose that minocycline could not only delay the appearance of the activation of astrocytes, but also decrease the level of astroglial activation.

To further explain the mechanism by which minocycline ameliorates behavioral deficits, the levels of intermediate inflammation- and oxidative stress-related reagents, including

COX-2 and NF-κB, were measured. Minocycline could reduce the levels of COX-2 and NF-κB in the course of CCH. The decrease of astrocytic activation might result from minocycline-induced suppression of cerebral inflammation and oxidative stress. Affirmatively, minocycline improves the recovery of brain energy metabolism, and restrains neuroinflammation and oxidative stress after ischemia in rats.

The specific mechanism of inflammation that follows the activation of astrocytes is partially established. Recent investigations have shown that reactive astrocytes are important sources of pro-inflammatory cytokines especially following excitotoxic/ischemic damage or traumatic injury of the central nervous system[6,45]. TNF-α and IL-1β are common factors of inflammation. Minocycline inhibits the release of TNF-α and IL-1β in chronic cerebral hypoperfusion models, which may counteract the reaction of astrocytes.

In conclusion, this study evaluates the influences of minocycline on astrocytic activation, inflammation and oxidative stress in the course of CCH. The secretion of cytokines and levels of oxidative tissue damage markers in the course of CCH were analyzed. Clinically, the abilities to modulate inflammatory reaction and inhibit oxidative stress may be important criteria in the selection of neuroprotective drugs. Also, there are several additional factors contributing to the minocycline-induced inhibition of astrocytic reactivity. Further studies exploring the mechanisms underlying the effect of minocycline may lead to a better understanding of the role of minocycline in the treatment of the disease.

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美满霉素抑制血管性认知功能损伤大鼠海马星型胶质细胞激活和神经炎症

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摘要：目的　观察美满霉素(minocycline)对血管性认知功能损伤大鼠海马组织 GFAP、COX-2、NF-κB、IL-1β 和 TNF-α表达的影响，探讨美满霉素对血管性认知功能损伤脑保护作用的机制。**方法**　Wistar大鼠随机分为假手术组 (S组)、血管性认知功能损伤模型组(M组)和美满霉素治疗组(MT组)。免疫组织化学法检测大鼠海马组织COX-2和 NF-κB的表达，蛋白质印迹和免疫组织化学法检测大鼠海马组织GFAP的表达，ELISA法检测大鼠海马组织IL-1β和 TNF-α 的表达。**结果**　MT 组 GFAP、COX-2、NF-κB、IL-1β 和 TNF-α 表达较 M 组均降低(P<0.01)；MT 和 M 组 GFAP、COX-2、NF-κB、IL-1β 和 TNF-α 表达均显著高于 S 组(P<0.01)。**结论**　美满霉素能降低血管性认知功能损伤大鼠海马组织中 GFAP、COX-2、NF-κB、IL-1β 和 TNF-α 的表达，抑制血管性认知功能损伤大鼠海马星型胶质细胞活化和神经炎症，发挥脑保护作用。
关键词：血管性认知功能损伤；美满霉素；炎症；星型胶质细胞