·Original Article·

Minocycline attenuates cognitive impairment and restrains oxidative stress in the hippocampus of rats with chronic cerebral hypoperfusion

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Abstract: Objective Nitric oxide (NO) was speculated to play an important role in the pathophysiology of cerebral ischemia. Minocycline, a tetracycline derivative, reduced inflammation and protected against cerebral ischemia. To study the neuroprotection mechanism of minocycline for vascular dementia, the influences of minocycline on expressions of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) were observed in the brains of Wistar rats. **Methods** The vascular dementia rat model was established by permanent bilateral common carotid arteries occlusion (BCCAO). Wistar rats were divideded into 3 groups randomly: sham-operation group (S group), vascular dementia model group (M group), and minocycline treatment group (MT group). The behaviour was tested with Morris water maze and open-field task. Expressions of iNOS and eNOS were measured by immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR). The optical density value was measured by imaging analysis. Percentage of positive cells with iNOS and eNOS expression was analyzed with optical microscope. **Results** Minocycline attenuated cognitive impairment. Inducible NOS was significantly down-regulated in MT group, compared with that in M group ($P < 0.01$), while eNOS was significantly up-regulated, compared with that in M group ($P < 0.01$). The expressions of iNOS and eNOS in M and MT groups were higher than those in S group ($P < 0.01$). **Conclusion** Minocycline can down-regulate the expression of iNOS and up-regulate the expression of eNOS in vascular dementia, which restrains apoptosis and oxidative stress to protect neural function.

Keywords: vascular dementia; minocycline; nitric oxide synthase

1 Introduction

Nitric oxide (NO) was speculated to play an important role in the pathophysiology of cerebral ischemia. NO is produced endogenously by the conversion of *L*-arginine to *L*-citrulline by NO synthase (NOS). It is known that at the early stage of cerebral ischemia, endothelial NOS (eNOS) and inducible NOS (iNOS) are extremely active and produce large amounts of NO. There are increasing evidences that iNOS-derived NO is an important mediator in ischemic brain injury. Cerebral ischemia is associated with the expressions of iNOS and eNOS enzymes whose reaction products contribute to the evolution of ischemic brain injury. Ischemia increases the expression of eNOS in cerebral blood vessels. Endothelial NOS can attenuate ischemic brain injury. Reactive nitrogen species is closely related with pathogenesis of cerebral ischemia.

Oxidative stress describes the state level of oxidative damage caused by reactive oxygen species and reactive nitrogen species in a cell, tissue or organ. Cerebral ischemia induces oxidative stress in the brain resulting in an imbalance between oxidants and anti-oxidants. Increased reactive oxy-

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gen species and reactive nitrogen species (*i.e.*, NOS) production lead to abnormal breakdown of fat molecules (*i.e.*, lipid peroxidation). This complex process results in the formation of toxic compounds and may contribute to the pathogenesis of cerebral ischemia disease—vascular dementia.

Minocycline, a tetracycline derivative, reduces inflammation and oxidative stress apoptosis, and decreases neural impairment against cerebral ischemia[1-3]. Previously, we have found that minocycline inhibited the expression of MMP-2 and MMP-9 in rats model with cerebral ischemia-reperfusion and attenuated injury induced by cerebral ischemia-reperfusion[4]. In this study, we observed the influence of minocycline on the expressions of iNOS and eNOS in brains of Wistar rats with vascular dementia to study the neuroprotection mechanism of minocycline.

2 Materials and methods

2.1 Animals and agents Wistar rats (10 weeks old, female, weighing 200-250 g, purchased from the Field Zoology Research Institute of Third Military Medical University) were randomly assigned into three groups: sham-operation group (S group): a mean survival time of 16 weeks; chronic cerebral hypoperfusion model group (M group): established by permanent bilateral common carotid arteries occlusion (BCCAO); and minocycline treatment group (MT group): animals in MT groups, after having been established vascular dementia model, were given minocycline by douche via stomach. M and MT groups were subdivided into 4, 8 and 16 weeks old subgroups, respectively. Each subgroup had six animals. 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 double-ligated with silk suture. Sham-operation animals were treated with the same manner, except that the common arteries were not occluded.

Minocycline (100 mg/capsule, Huishi Pharmaceutical Limited Company, China) was diluted to 0.5 mg/mL concentration by normal saline. S group was not given any treatment. M groups were established by BCCAO and were given the same volume saline through douche via stomach. According to the references[5,6], MT groups, after having been established vascular dementia model, were given minocycline through douche via stomach by 50 mg/kg, per day.

2.2 Behaviour testing

2.2.1 Morris water maze task Five days before decapitation, mice were pretrained to climb and stay on a submerged platform (10×10 cm) placed in the center of a small pool (diameter, 45 cm). A mouse was placed in the water (face the platform) and allowed to swim, then was trained to climb on the platform and stay there for 10 s. Then it was picked up by its tail, dried with a paper tissue, and returned to a warm dry waiting cage for 7-8 min (an average duration of intertrial interval). Before testing with classic Morris water maze task, all mice were familiarized with procedural aspects of the task. The Morris water maze task was conducted in the pool (diameter, 100 cm) filled with opaque water [(22±2) ºC] and surrounded by a set of distal and proximal spatial cues. Five daily sessions included 10 platform trials, in which the platform was submerged but accessible to the mouse, as well as 2 probe trials (before and after the block of platform trials). During the probe trials, the platform was collapsed at the bottom of the tank for variable intervals (30-40 s). At the end of the probe trial, the collapsed platform was returned to its raised position, and the mouse was allowed to escape onto the platform. Distance (path from the start location to the platform, in centimeters) and swimming speed (average speed during a trial, in centimeters per second) were measured during the platform trials. In probe trials, swimming speed, time spent and escape latency in different quadrants were recorded and measured.

2.2.2 Open-field task The round white open-field arena had a diameter of 100 cm and 55-cm-high sidewalls. The same illumination as in other tasks was used, consisting of indirect diffuse room light. Each subject was released near the wall and was observed for 5 min. As in all other tasks, performance in the open-field task was recorded by a computer-based video tracking system (Chinese Academy of Medical Sciences, China). Activity measures included distance traveled, percentage of time spent, and speed of movement during active exploration. To analyze anxiety levels, the activity measures were broken down into two zones. A 20-cm-wide wall zone constituted the most preferred peripheral zone, whereas the rest of the open field was defined as a central zone, comprising 67% of the arena surface, and was most aversive for mice. The number of entries to the central zone of the open field was also recorded.

2.3 Immunohistochemical assay Tissue samples were collected after surgery and immediately frozen in liquid nitrogen.

Prior to immunohistochemistry assay, frozen sections were prepared with cryostat (FACS caliber, Becton Dickinson, USA) at –20ºC, then dried at room temperature, and fixed with acetone. The peripheral blood mononuclear cells (PBMC) were routinely isolated and the slides were prepared with a cytospinner. The avidin biotin complex (ABC) immunohistochemical assay was carried out according to the protocols. Anti-iNOS and anti-eNOS were offered by Santa Cruz Co., USA. Goat anti-mouse IgG labeled with biotin, was purchased from Vector Co., USA. Two hundred cells were counted and the intensity of staining for each cell was adjusted. Five grades were employed to express the degrees of staining, which represent 5 reaction coefficient, respectively. The corresponding cell numbers were added up, which resulted in the value of a positive score. All slides were measured in duplicate. Samples with score over 10 or frequency over 5% were considered as positive.

2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR) Animals were sacrificed at corresponding time points and total RNA in the treated sections was extracted with the total RNA extracting kit. Four microgram total RNA was heated at 70 ºC for 5min, then chilled on ice. A solution was added consisting of 10 mmol/L dNTP, 0.5 g/L oligo (dT), 40 U reverse transcriptase (m-mulv), 5× RT buffer (pH 8.3; in mmol/L: Tris-HCl 250, KCl 250, $MgCl_2$ 20, DTT 50), and deionized water. Total sample volume was 20 μL. Samples were incubated at 37 ºC for 1 h and the reaction was stopped by heating at 70 ºC for 10 min. Specific primers were designed for PCR: iNOS, sense: 5'-GTG TTC CAC CAG GAG ATG TTG-3', antisense: 5'-CTC CTG CCC ACT GAG TTC GTC-3'; eNOS, sense: 5'-CTG CTG CCC GAG ATA TCT TC-3', antisense: 5'-AAG TAA GTG AGA GCC TGG CGC A-3'; β-actin, sense: 5'-GTT CGC CAT GGA TGA CGA TAT C-3', antisense: 5'-GCC AGA TCT TCT CCA TGT CGT C-3'. The expected sizes of amplification was 576 bp for iNOS, 433 bp for eNOS and 665 bp for β-actin. PCR was performed using 2 μL cDNA, 2 mmol/L dNTP, 20 pmol specific pair of primers, 2 U DNA polymerase, 5× PCR buffer and deionized water. The total volume was 25 μL. Amplification was performed for 32 cycles. Each cycle consisted of denaturation at 93 ºC for 45 s, annealing at 58 ºC for 45 s, and extensing at 72 ºC for 1 min. There was a preheating at 94 ºC for 2 min before the first cycle and the extension time of the last cycle was 11 min. The PCR products were separated by electrophoresis using a 1.5% (w/v) agarose gel containing 0.5 mg/L of ethidium bromide. Single band corresponding to the predicted size of the amplified product for eNOS, iNOS and β-actin were identified under an ultraviolet transilluminator and transferred to a nylon filter membrane, and hybridized with 10 mL ECL-labeled probe (3'-oligolabelling and detection systems, Amersham). The bands corresponded in size to the ethidium bromide stained gels, thereby confirming that the amplified PCR products consisted of correct eNOS and iNOS sequences. The band densities were scanned with a densitometer (Bio-rad Instrument Ltd., USA). The relative amount of mRNA in each sample was calculated from the densitometry ratio of NOS optical density (OD) value/β-actin OD value.

2.5 Statistical analysis Data were expressed as mean±SD and analysed statistically with SPSS software for Windows 8.0 (SPSS, Inc., Chicago, IL, USA). NOS were calculated as: (positive cells/ staining cells) ×100%. For statistical evaluation, one-way analysis of variance (ANOVA) and Student's *t* test were employed. Pearson correlation analysis was also performed to some index. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Morris water maze and open-field task performance Chronic cerebral hypoperfusion (CCH) was induced in 10-week-old Wistar rats by BCCAO as described previously. At the end of BCCAO treatment, rats were subjected to Morris water maze. Escape latancy decreased significantly after one day training. On day 3, 4 and 5, animals in S group immediately swam toward the platforms in the water maze, whereas BCCAO rats swam longer distance before finding the platform. In general, escape latancy gradually decreased with BCCAO duration [$F(3,12) = 21.36$, $P < 0.01$; Fig. 1A]. After minocycline treatment, BCCAO rats swam shorter distance than modal animals before finding the platform [$F(5,20) = 28.36$, $P < 0.01$; Fig. 1A]. There was no significant difference of the swimming speed between each group, which excluded the possibility that the group difference in escape latency was due to difference in swimming ability (Fig.1B). BCCAO rats exhibited a reduced speed of swimming compared with littermate controls (Fig. 1B) in the Morris water maze task, suggesting that anxiety levels in these rats might be reduced because swimming speed in rodents may reflect a stress/anxiety reaction to the placement in cold water. In the open-field task,

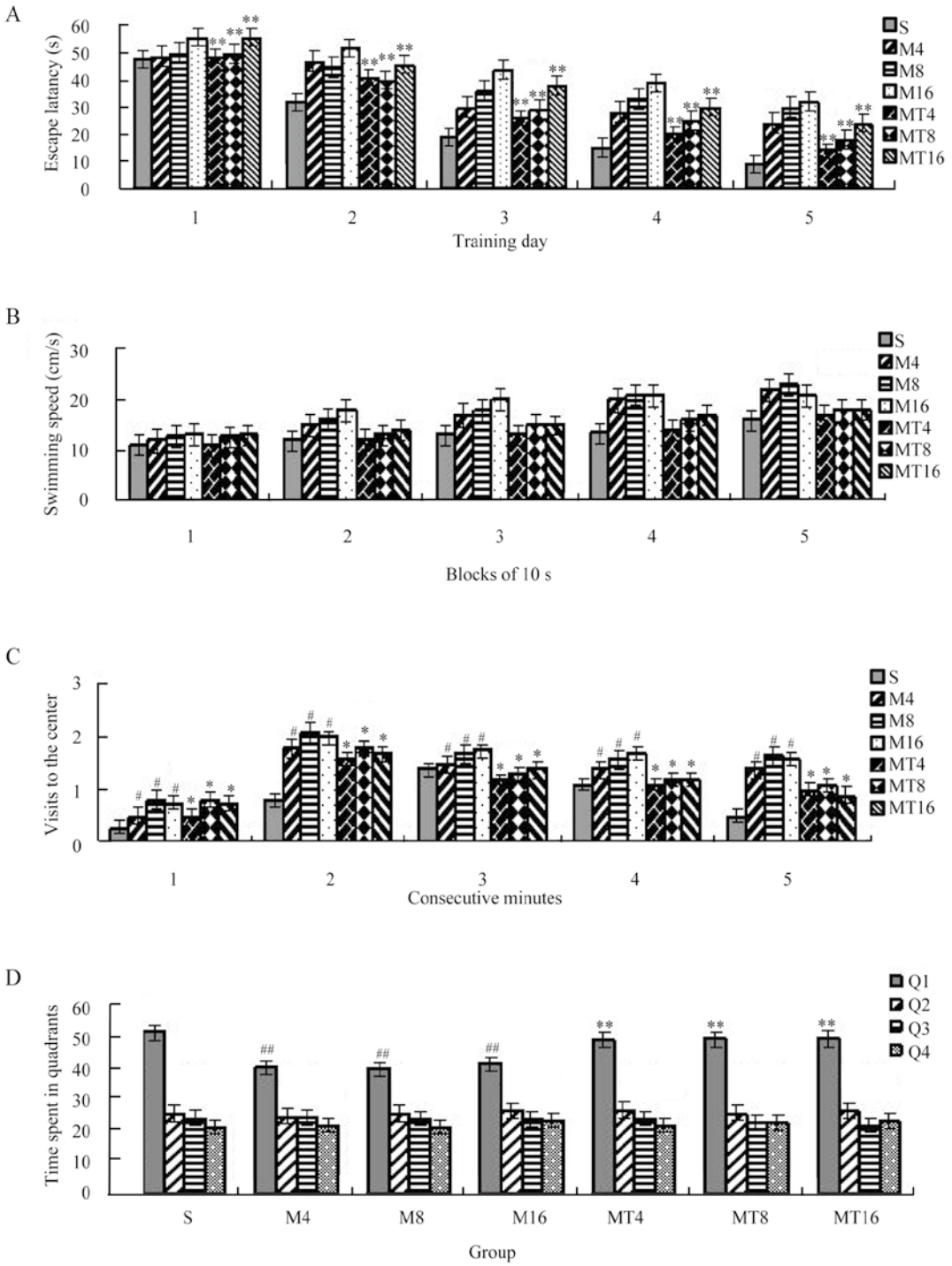

Fig. 1 **A: Escape latancy gradually decreased with BCCAO duration. After minocycline treatment, rats in MT group spent less time than rats in M group before finding the platform; B: Swimming speed showed no difference between groups; C: In the open-field task, visits to the center parts for BCCAO rats increased significantly, compared with rats in S group. After minocycline treatment, visits to the center parts for rats in MT group decreased significantly, compared with rats in M group. D: In the probe trials, time spent in different quadrants of the water maze during the last probe trial for S group rats was significantly longer than that for M group rats groups in quadrant 1—the platform location, but not in quadrant 2, 3 and 4 ($P > 0.05$). Rats in S group showed significantly stronger preference for the platform location (quadrant 1) compared with rats in M groups. After minocycline treatment, rats in MT groups spent significantly longer time in different quadrants of the water maze during the last probe trial than rats in M groups in quadrant 1—the platform location, but not in quadrant 2, 3 and 4 ($P > 0.05$). $^{*}P < 0.05$, $^{**}P < 0.01$, *vs* M group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs* S group. S, sham-operation group; M4, BCCAO 4 weeks group; M8, BCCAO 8 weeks group; M16, BCCAO 16 weeks group; MT4, minocycline treatment 4 weeks group; MT8, minocycline treatment 8 weeks group; MT16, minocycline treatment 16 weeks group; Q, quadrant.**

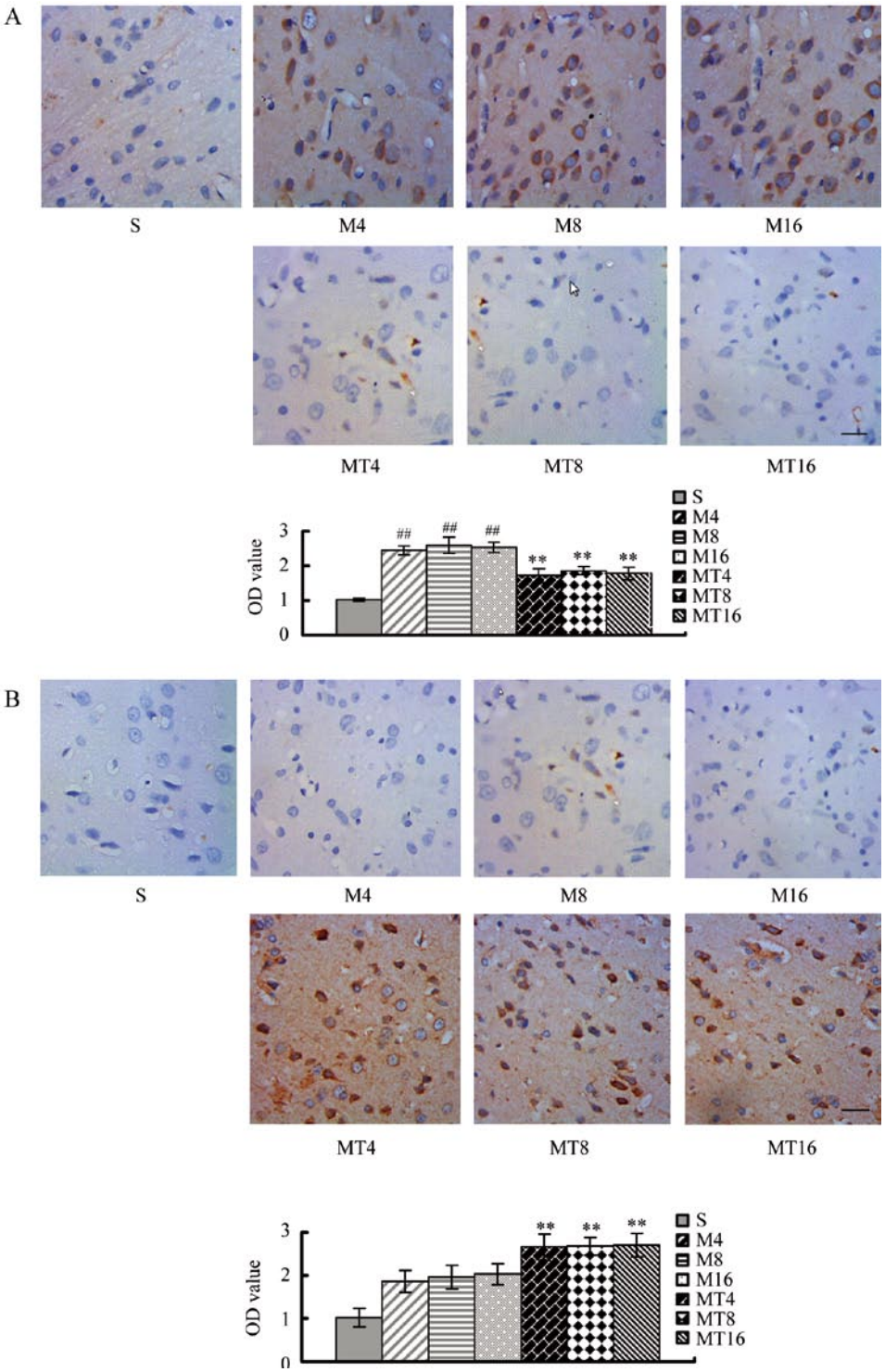

Fig. 2 The expressions of iNOS and eNOS in the hippocampus were measured by immunohistochemistry method. A: The expressions of iNOS in M groups significantly increased compared with those in S group, whereas the expressions of iNOS in MT groups significantly decreased compared with those in M groups; OD value of iNOS in MT group was significantly lower than that in M group at the same viewing time; B: The expressions of eNOS in MT groups significantly increased compared with those in M groups. OD value of eNOS in MT group was significantly higher than that in M group at the same viewing time. $^{\#\#}P < 0.01$, *vs* S group; $^{**}P < 0.01$, *vs* M group. S, sham-operation group; M4, BCCAO 4 weeks group; M8, BCCAO 8 weeks group; M16, BCCAO 16 weeks group; MT4, minocycline treatment 4 weeks group; MT8, minocycline treatment 8 weeks group; MT16, minocycline treatment 16 weeks group. Scale bar, 20 μm.

visits to the center parts for BCCAO rats increased significantly, compared with animals in S group [$F(5,20) = 7.36$, $P < 0.05$; Fig. 1C]. Collectively, our findings are consistent with the view that BCCAO rats exhibit a lower level of anxiety compared with S group rats in emotion. After minocycline treatment, visits to the center part for rats in MT groups decreased significantly, compared with rats in M groups [$F(5,20) = 16.36$, $P < 0.05$; Fig. 1C]. In the probe trials, time spent in different quadrants of the water maze during the last probe trial for S group rats was significantly longer than M group rats in quadrant 1—the platform location [$F(3,12) = 28.36$, $P < 0.01$], but not in quadrant 2, 3 and 4 [$F(3,12) = 24.51$, $P > 0.05$; Fig. 1D]. Rats in S group showed significantly stronger preference for the platform location (quadrant 1) compared with M group rats. After minocycline treatment, animals in MT group spent significantly longer time in different quadrants of the water maze during the last probe trial than M group rats in quadrant 1—the platform location [$F(5,20) = 21.36$, $P < 0.01$], but not in quadrant 2, 3 and 4 [$F(5,20) = 20.51$, $P > 0.05$; Fig. 1D]. In the probe trials, the swimming speed of rats in S group was significantly lower than those found in M and MT groups. Importantly, the swimming speed increased over the course of the probe trials, indicating that the low swimming speed was not simply a result of fatigue (which would be observed a reduced swimming speed as the probe trials progressed). Despite having similar swimming speeds, the spatial preferences between S group and M group were significantly different for the correct quadrant. Together, our results indicated that, in the observed range of changes, the swimming speeds did not interfere with the spatial performance

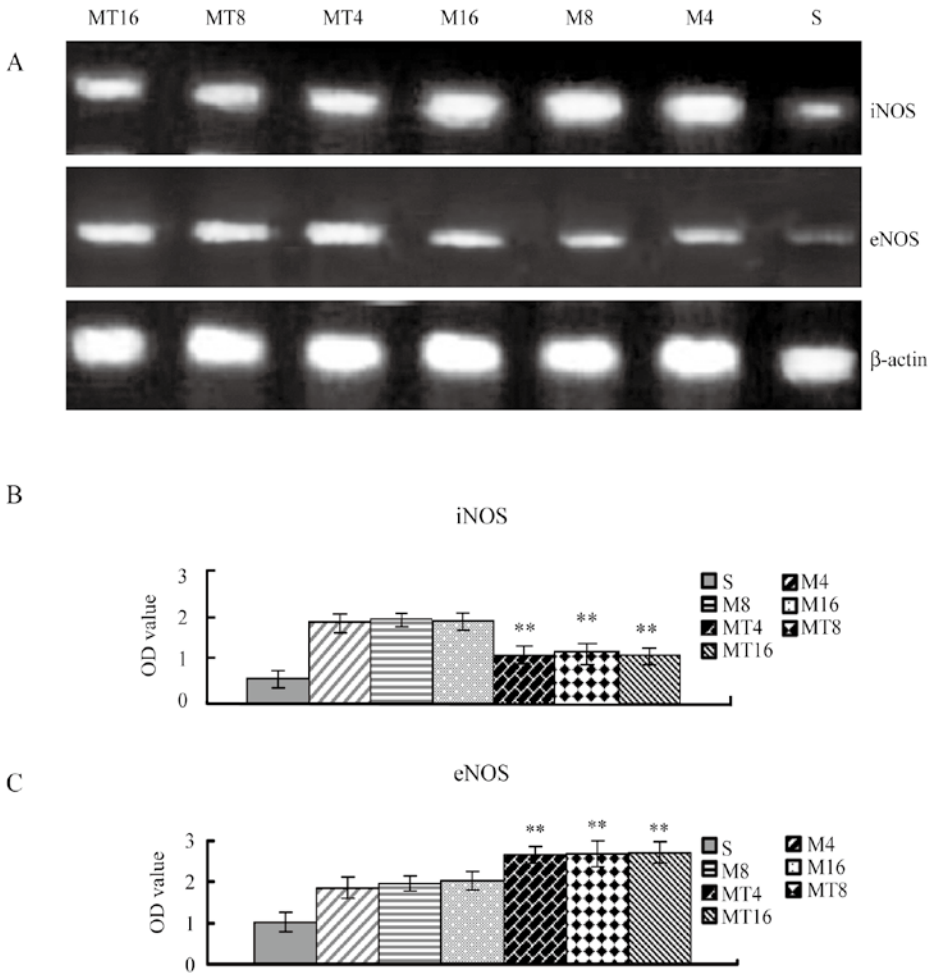

Fig. 3 Assay of mRNA level of NOS in rats with vascular dementia. Relative amount of NOS gene transcripts was expressed by the densitometry ratio of NOS to that of β-actin (Mean±SD). A: Assay of mRNA level of NOS in rats of different groups. B: MT group had a significantly lower level of iNOS mRNA than M group. C: MT group had a significantly higher level of eNOS mRNA than M group. **$P < 0.01$ *vs* M group. S, sham-operation group; M4, BCCAO 4 weeks group; M8, BCCAO 8 weeks group; M16, BCCAO 16 weeks group; MT4, minocycline treatment 4 weeks group; MT8, minocycline treatment 8 weeks group; MT16, minocycline treatment 16 weeks group.

during probe trials.

3.2 Immunohistochemistry analysis The expressions of iNOS and eNOS in the hippocampus were measured with immunohistochemistry method. OD value in M and MT groups was significantly higher than that in S group at the same viewing time ($P < 0.01$). OD value of eNOS in MT group was significantly higher than that in M group at the same viewing time($P < 0.01$), whereas iNOS in MT group was significantly lower than that in M group ($P < 0.01$). Expressions of iNOS and eNOS in M groups were significantly increased compared with those in S group [$F(5,20) = 16.31$, $P < 0.01$; $F(5,20) = 17.24$, $P < 0.01$], whereas expression of iNOS in MT group was significantly decreased compared with that in M group [$F(5,20) = 15.31$, $P < 0.01$; $F(5,20) = 15.28$, $P < 0.01$] and expression of eNOS in MT group significantly increased compared with that in M group [$F(5,20) = 12.34$, $P < 0.01$; $F(5,20) = 13.23$, $P < 0.01$]. (Fig. 2A, B).

3.3 RT-PCR analysis To further determine the expressions of iNOS and eNOS, RT-PCR analysis was carried out in the hippocampus. Inducible NOS at RNA level in MT group was significantly lower than that in M group at the same viewing time ($P < 0.01$), but expression in M group was significantly increased compared with that in control group (S group) [$F(3,12) = 16.12$, $P < 0.01$]. Up-regulations of iNOS and eNOS gene transcription in CCH condition matched with the protein levels of iNOS and eNOS that was significantly increased. Expression of iNOS in MT groups significantly decreased, compared with that in M groups [$F(5,20) = 16.01$, $P < 0.01$; $F(5,20) = 19.32$, $P < 0.01$], while the expression of eNOS in MT groups significantly increased compared with that in M groups [($F(3,20) = 21.43$, $P < 0.01$; $F(3,20) = 19.65$, $P < 0.01$; Fig. 3)].

4 Discussion

NO is a highly-activated gaseous molecule, almost ubiquitous in the body and involved in many physiologic and pathologic activities. It was reported that NO, one of free radicals and cell signaling molecules, could mediate the death of neurons as a toxic factor[7,8]. Inducible NOS and neuronal NOS (nNOS) can catalyze *L*-arginine and synthesize NO. There are three isozymes of NOS: nNOS, iNOS and eNOS. Activities of nNOS and eNOS (together called constructive NOS, cNOS) depend on Ca^{2+} and calmodulin. Since exocytotic Ca^{2+} can activate cNOS, and tissue injury usually accompanies a rapid rise of exocytotic Ca^{2+}, these characteristics suggest that cNOS may respond quickly to tissue injury. As a whole, cNOS is thought to have a protective role in injured tissue by increasing the level of cGMP and further modulating the tone of vascular smooth muscles to modify local blood flow, but there are still great differences between the options of nNOS and eNOS[9]. Endothelial NOS can protect disruption of the blood-brain barrier after ischemia[10]. Inducible NOS is usually induced by inflammatory factors and its activity is independent of Ca^{2+} and calmodulin. Inducible NOS has a tendency towards over-expression and is usually considered toxic for producing too much NO, which may provide negative feedback and be the fundamental cause of the toxicity of iNOS. More reseach evidence showed that iNOS and eNOS participate in the progression of cerebral ischemia injury[11]. In order to determine the clinical relevance of these findings for vascular dementia treatment, we will further investigate if the reactive nitrogen inhibition by minocycline in vascular dementia model will result in the expression of relevant NOS. In this present study, we found that iNOS and eNOS participated in the process of vascular dementia brain injury and that minocycline could down-regulate the expression of iNOS, up-regulate the expression of eNOS and attenuate cognitive impairment.

Minocycline is a semi-synthetic tetracycline antibiotic and can effectively cross the blood-brain barrier. It has been reported that minocycline had significant neuroprotective effects on cerebral ischemia[12-14], amyotrophic lateral sclerosis[15], Alzheimer's disease[16], Huntington's disease[17,18] and Parkinson's disease[19]. Furthermore, minocycline can inhibit post-ischemic brain inflammation[20], microglia activation[21], oxidative stress, apoptosis, extracellular matrix degradation[22], and so on. One common pathophysiological mechanism for models of brain ischemic damage is reactive nitrogen, oxidative stress and neuroinflammation. In this present study, we established the vascular dementia model by permanent bilateral common carotid arteries occlusion. Results from Morris water maze test and open-field task showed that cognitive impairment occurred with the vascular dementia models, whereas cognitive impairment of modal animals had been attenuated after minocycline treatment. The expression of iNOS in the hippocampus was down-regulated and eNOS was up-regulated after minocycline treatment. Therefore, reactive nitrogen[23,24] and oxidative stress[25] contributed to

pathogenesis of vascular dementia and minocycline could restrain oxidative stress in the process of vascular dementia. Interference of oxidative stress cascade by minocycline might be the central pathway for these neurovascular protective properties of decreasing tissue injury and also providing functional recovery. In this present study, we found that minocycline could improve the cognitive impair in vascular dementia rats, which might be due to its effect on inhibition of reactive nitrogen and oxidative stress. In conclusion, minocycline is a promising neuroprotective agent for vascular dementia.

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美满霉素改善血管性痴呆大鼠认知功能损伤并抑制氧化应激

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摘要: 目的 观察美满霉素(minocycline)对血管性痴呆大鼠学习记忆功能和脑组织内皮型一氧化氮合酶 (endothelial nitric oxide synthase, eNOS)、诱导型一氧化氮合酶 (inducible nitric oxide synthase, iNOS)表达的影响，探讨美满霉素对血管性痴呆的脑保护作用的机制。**方法** Wistar 大鼠随机分为假手术组 (S 组)、痴呆模型组 (M 组)、美满霉素治疗组 (MT 组)。RT-PCR 和免疫组织化学法检测大鼠脑组织 eNOS、iNOS 的表达，行为学检测大鼠学习记忆功能的改变。**结果** M 组与 S 组行为学检查显示，M 组大鼠有显著学习记忆障碍($P < 0.01$)，MT 组与 M 组比较行为学检测结果显示，MT 组大鼠学习记忆障碍有显著改善($P < 0.01$)。MT 组 iNOS 表达较 M 组降低($P < 0.01$), MT 组 eNOS 表达较 M 组增高($P < 0.05$)；MT 组 eNOS、iNOS 表达较 S 组增高($P < 0.01$)；M 组 eNOS、iNOS 表达较 S 组显著增高($P < 0.01$)。**结论** 美满霉素能降低血管性痴呆大鼠脑组织 iNOS 表达，增强 eNOS 表达，抑制氧化应激反应，发挥脑保护作用。

关键词：血管性痴呆；美满霉素；一氧化氮合酶