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Original Article

Tooth loss, cognitive impairment and chronic cerebral ischemia

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KEYWORDS
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Abstract *Background/purpose:* Vascular factor is an important risk factor in the process of cognitive impairment or dementia. Tooth loss could cause impairments of spatial learning and memory in mice, and nitric oxide (NO) and its synthase might be involved in the process. The objectives of this study were to investigate and compare the behavioral impairments between the Wistar rats with tooth loss and those with chronic ischemia and to determine the changes in nitric oxide (NO) and its synthases under those two conditions.
Materials and methods: The Morris water maze was used to test the spatial learning and memory abilities in the Wistar rats 8 weeks after the molar extraction procedure and the occlusion of 2 blood vessels to produce cerebral ischemia. The changes in NO and its synthases were evaluated using the Griess assay, Western blotting, and immunohistochemistry.
Results: Similar impairments in the spatial learning and memory of Wistar rats were found after tooth loss and the induction of cerebral ischemia. The levels of NO and iNOS in the rat hippocampus increased, and the levels of eNOS decreased. *Conclusion*: For Wistar rats, the results of cognitive impairments related to tooth loss and those that occur due to chronic cerebral ischemia were statistically not significant and that NO, iNOS and eNOS in the hippocampus are involved in both cases.

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Introduction

In recent decades, human teeth, tools used for mastication, have also been considered essential for the nutrition and health of the human body.[1] The human studies provided the evidence of correlation between tooth loss and cognitive impairment,[2,3] and the results from the animal studies further elucidated the cause—effect relationship.[4] With the world rapidly aging, the quality of life in the elderly is adversely affected as dementia becomes a serious public health problem.[5] Brain ischemia increases the risk of both ischemic dementia with Alzheimer's phenotype and Alzheimer's disease dementia.[6] In 2011, the American Heart Association/American Stroke Association claimed that there are "vascular factors contributing to cognitive impairment and dementia". Vascular factors are officially identified as important risk factors for dementia.[7]

Mastication correlates with an increase in cerebral blood circulation and affects postnatal brain development, aging and locomotor function; chewing leads to increased blood flow.[8] Dental occlusal changes (e.g., tooth loss) induce motor cortex neuroplasticity.[9] Altered dentitional states (including tooth loss and their restoration) are accompanied by widespread structural and functional brain changes in regions involved in processing and controlling sensory, motor, cognitive and emotional functions.[10,11]

Nitric oxide (NO) and its synthases play important dual roles in the pathophysiology of cerebral ischemia in animal models.[12] NO regulates cerebral blood flow and cell viability and protects nerve cells or fibers against pathogenic factors associated with cerebral ischemia, trauma, and hemorrhage.[13] Basal release of nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) plays a neuroprotective role that contributes to an increase in cerebral blood flow in various mammals,[14] preventing neuronal injury and inhibiting platelet and leukocyte adhesion. Excessive production of NO is clearly neurotoxic. Overproduction of NO by inducible nitric oxide (iNOS) has been implicated in various pathological processes, including tissue injury and cell apoptosis caused by ischemia and inflammation. In the early stage of cerebral ischemia, the amount of NO produced by endothelial cells is higher than that produced by neurons. The toxicity of NO is reduced by increasing collateral circulation, platelet aggregation, and microvascular blockage of leukocytes. Circulation counteracts the toxic effects of NO.[15]

However, with the prolongation of ischemia, the damage zone can be observed in the late ischemia and reperfusion period. A marked inflammatory response and invasion of leukocytes occurs, accompanied by a high level of mRNA expression of iNOS, resulting in a large amount of NO.[15] Molar extraction is associated with behavioral impairment, and changes in NO and iNOS in the hippocampus of KunMing mice were involved in behavioral changes after molar loss.[16]

Cerebrovascular risk factors are easy to be found and controlled. Lack of awareness of the dangers of tooth loss has aggravated the prevalence of long-term loss of occlusal support without dental inlay. Studies have confirmed that the blood flow stimulation produced by mastication after restoration and reconstruction of occlusal support function may delay the process of cognitive decline, which will greatly increase people's awareness of the importance of masticatory function and thus improve the quality of life of the elderly. Therefore, this topic has important social significance.

This study aimed to further investigate the effects of tooth loss on the development of spatial learning and memory in Wistar rats, comparing the results to those of chronic cerebral ischemia; in addition, this study aimed to determine the role of NO and its synthases in the process. If the degree of cognitive impairment caused by molar loss were proved similar to which caused by chronic cerebral ischemia, it would greatly increase the awareness of the importance of tooth loss. The hypothesis is that the difference in the behavioral impairments between tooth loss and chronic cerebral ischemia may statistically not significant and that the changes of NO, iNOS and eNOS in the hippocampus may be similar in both cases.

Materials and methods

Animals

Forty-eight male Wistar rats (3 months old) (Vital River Laboratory Animal Technology Co. Ltd) were used. All rats were habituated for at least 7 days before the experiments. Wistar rats weighing approximately 300 g were housed with five per standard cage in a light-controlled room (a 12:12-h cycle starting at 10:00 h) at room temperature (23 °C) and maintained on food and water provided ad libitum. The experiments complied with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The rats were randomly divided into 5 groups (16 rats each group) as follows: the chronic cerebral ischemia group (2-vo group), the chronic cerebral ischemia sham group (2-vo S group), the loss of occlusal support through molar extraction group (M group), the loss of occlusal support sham-operated group (MS group) and the control group (C group). The results of 2-vo S group and MS group will be provided as supplementary data. This study was approved by the animal care and use ethical committee of Beijing Stomatology Hospital (13-2-26).

Preparation of ischemia model and molarless model

2-vessel occlusion (2-vo)

Rats were anesthetized with 10% chloral hydrate (400 mg/kg), and the common carotid arteries were exposed by a ventral midline incision. For the 2-vessel occlusion procedure, the common carotid arteries were gently isolated from the adjacent vagus nerves and double ligated with silk

sutures. The neck wound was sutured. Sham-operated animals were treated the same way except that the common carotid arteries were not occluded. The skin incision was sutured closed with 3-0 Nylon Monofilament suture (Ethicon). Rats were allowed free access to normal pelleted diet and water after the procedure. The general condition and body weight of each rat were monitored during the entire process.

Tooth loss model

The rats were anesthetized via intraperitoneal (i.p.) injection of 10% chloral hydrate (400 mg/kg). In the tooth loss group, all bilateral maxillary molars were removed. If there was root fracture, all the tooth structure visible above the gum was removed to eliminate occlusal contacts. In the sham group, small amounts of bilateral maxillary alveolar bone were removed with rongeur from the toothless gap region between molars and canines in the superior alveolar ridge. After the procedure, the rats were allowed free access to normal pelleted diet and water. The general condition and body weight of each rat were monitored during the entire process.

Morris water maze (MWM)

The MWM test was performed according to the protocol in the original study.[17] The device consisted of a painted black circular pool (diameter 120 cm) filled with water ($22 \pm 1\,^{\circ}C$) in which a small escape platform is hidden. The maze was divided into four quadrants. In the target quadrant, a transparent escape platform (diameter 10 cm) was placed 1 cm beneath the water surface. The behavior of the rat in the pool was recorded by a video camera positioned over the pool. During the entire test, the lighting of the testing room indirectly illuminated the pool, and the environment (e.g., experimenter, work table, door, and pipes) was kept consistent.

Eight weeks after surgery, the acquisition training session was performed. On the first day, the rats were placed into the pool without the platform one by one, and each rat swam separately and freely for 60 s. A similar procedure was repeated in a pool with the platform that was fixed with a red flag and placed in the center of one of the four quadrants of the pool. In the procedure, each rat was placed into the water at a position opposite the platform, and they were found the flag within 60 s, thus eliminating the influence of impaired vision on the measurement.

Rats were trained to locate the hidden platform in two trials per day over four consecutive days. Temperature and adequate water depth were assessed prior to each day of testing. For each trial, a rat was placed in the maze at two points (south and west) as the starting positions and allowed to swim freely until it found and climbed onto the hidden platform (in the northeast quadrant of the pool). After successfully reaching the hidden platform, the rat was allowed to remain on the platform for 15 s. If unsuccessful, the trial ended after 90 s when the experimenter placed the rat on the platform for 15 s. At the end of day 5, the platform was removed, and a 60-s probe trial was conducted. The starting position was set opposite to the original platform position (southwest of the pool) with the platform removed. The time of the rats first passing the platform and the frequency of passing the platform were recorded.

The latencies to swim to the platform were monitored with a CCD video camera linked to a computer system.

Griess assay

Nitric oxide can be quantified using the Griess assay.[18] Immediately after finishing the MWM test, the production of NO was determined by an assay for nitrite. Six rats from each group were decapitated, and the whole brain was removed. Then, the hippocampal areas were rapidly separated on ice plates and weighed. The tissue was ground nine times with phosphate-buffered saline, and 0.3% Triton X-100 were added. Then, the samples were shocked for 3 s and bathed in water for 5 min. Next, the tissue was centrifuged (12,000 g, 5 min) at $4\,^{\circ}C$, and the culture of the supernatant was maintained. The procedure followed the instructions of the total nitric oxide assay kit (Biyotime Institute of Biotechnology, S0024). The optical density of the assay samples was measured spectrophotometrically at 540 nm.

Immunohistochemistry

Immediately after finishing the MWM test, five rats in each group were anesthetized with 10% chloral hydrate (400 mg/kg) and were rapidly washed with phosphate-buffered saline (PBS, pH = 7.4). The hippocampus of each rat was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, followed by postfixation for 24 h in the same buffer. The tissue was then embedded into paraffin. Sections with 4-μm thickness were prepared on a microslicer and then processed according to a standard immunohistochemical protocol. After rinsing with PBS, the sections were incubated with 30% H_2O_2 for 10 min to quench the endogenous peroxidase activity and then blocked with 5% BSA for 20 min at room temperature, followed by incubation for 2 days at $4\,^{\circ}C$ with rabbit anti-iNOS (Abcam, ab3525, UK) and mouse anti-eNOS antibodies (Abcam, ab50010, UK), diluted 1:100. The sections were rinsed in PBS 3 times for 2 min each time and incubated for 20 min at $37\,^{\circ}C$ with goat anti-rabbit IgG (TDY bio, S004, China) and goat anti-mouse IgG (TDY bio, S001, China). Then, the sections were incubated with ABC reagent in PBS before being treated for approximately 10 min at room temperature with diaminobenzidine (DAB). Each sample was tested with positive and negative control samples. After a final wash with ddH_2O_2, the resulting staining was assessed microscopically.

Western blot analysis

The hippocampi of 5 rats of each group were dissected, weighed, recorded and snap frozen in liquid nitrogen immediately after finishing the MWM test. Proteins were extracted with an Applygen total protein extraction kit. The protein concentration was normalized using Coomassie brilliant blue G-250 staining. Equal amounts of proteins were separated by SDS-PAGE on a 10% polyacrylamide gel, and the proteins were transferred to a PVDF membrane.

After blocking with 0.1% TBST containing 5% nonfat milk at room temperature for 2 h. Primary antibodies (iNOS, ab3525, 1:500 from Abcam, UK; eNOS, ab50010, 1:500 from Abcam, UK; β-actin, Immunoway, YM3028, 1:5000, USA) were incubated with the samples overnight. The membrane was rinsed with 0.1% TBST three times for 10 min each and incubated with secondary antibody (ZB2301, 1:5000, from Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 2 h. The color was developed using the ECL kit. Images were acquired using the Fuji Digital Science Imager and analyzed with Gelpro Analyzer (Version 4.0) to measure the integrated optimal density (IOD) values of specific bands.

Statistical analysis

The statistical analyses were performed using SPSS Statistics V17.0 software (SPSS Inc.). Treatment differences of 5 days in the escape latency in the water maze task were analyzed using a repeated measures analysis of variance (ANOVA). One-way ANOVA followed by LSD and the Tukey post hoc test was used for the probe test and other data. The significance level was 0.05. All data were expressed as the mean ± standard deviation.

Results

MWM performance

None of the rats had observable physical abnormalities, and all showed similar swimming abilities in the visible-platform probe test. The MWM results revealed that all the rats found the platform increasingly quickly over the 5 days of training, and they demonstrated improved performance. There was a significant effect of training day on the measures of escape latency. Both the 2-vo group ($p = 0.001$) and the M group ($p = 0.001$) required significantly more time to learn to reach the platform than the C group, implying that both of the 2 groups had different levels of impairments in spatial learning and memory (Fig. 1A).

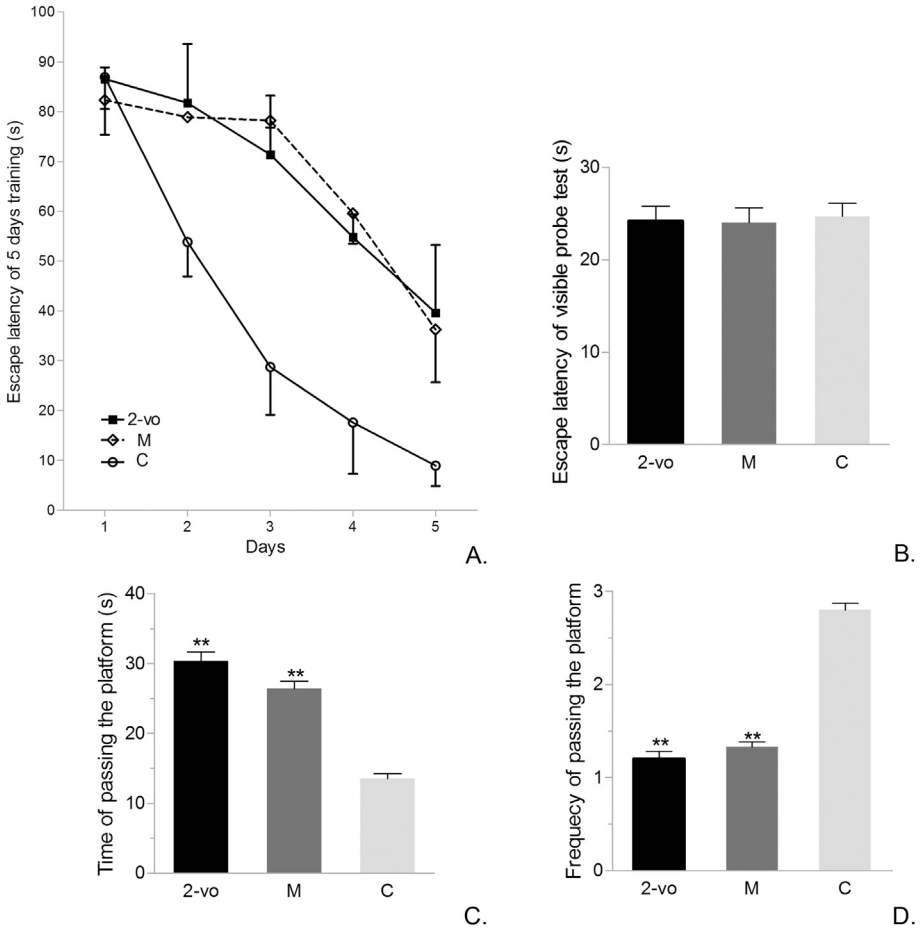

Figure 1 The 2-vo group and M group both showed impaired spatial learning and memory (n = 16). (A) There was a significant main effect of training day on the measures of escape latency in each group ($p < 0.001$). The 2-vo and M groups required significantly more time to learn to reach the platform than the C group ($p < 0.05$). (B) During the visible-platform probe test, there was no significant difference among the groups (One-way ANOVA, $p = 0.58$). (C) In the probe trial, the 2-vo and M groups took significantly more time to cross the original platform area for the first time than the C group (n = 16) (One-way ANOVA, **P < 0.01). (D) The frequencies of rats passing the original platform area in the 2-vo and M groups were significantly lower than those in the C group. The data are expressed as the mean ± SD (One-way ANOVA, **P < 0.01).

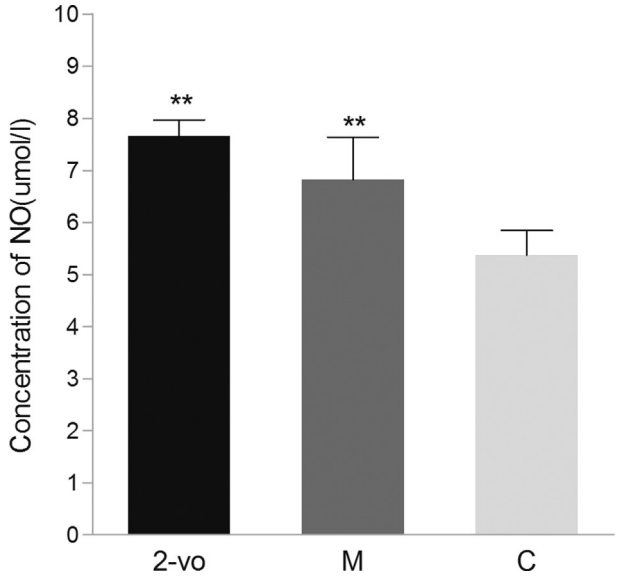

Figure 2 The molarless condition and chronic cerebral ischemia both promoted NO production in the hippocampal area of the rats (n = 6). In the hippocampus, NO production in the 2-vo and M groups was higher than in the C group. The data are expressed as the mean ± SD (One-way ANOVA, **P < 0.01).

However, there was no significant difference between the 2-vo group and the M group ($p = 0.064$). During the visible-platform test, the rats of each group had similar swimming ability (One-way ANOVA, $p = 0.58$) (Fig. 1B).

Probe trials (without a platform) were run on day 5 after training. The time it took for the rats to cross the area where the platform used to be was significantly different in the 2-vo (One-way ANOVA, $p = 0.001$) and M (One-way ANOVA, $p = 0.001$) groups compared to the C group (Fig. 1C). There was no significant difference between the 2-vo group and the M group (One-way ANOVA, $p = 0.53$) (Fig. 1C). Similar results were observed in the frequency of passing the platform (Fig. 1D).

The release of NO in the hippocampus

Using the Griess reagent assay, the amount of NO in the hippocampus after 8 weeks of surgery was estimated. The data indicated that compared to the C group, the NO concentrations in the hippocampus of the 2-vo group (One-way ANOVA, $p = 0.003$) and the M group (One-way ANOVA, $p = 0.007$) were higher (Fig. 2). There was no significant difference between the 2-vo and M groups (One-way ANOVA, $p = 0.45$).

iNOS expression in the hippocampus

The Western blot results demonstrated that the 2vo (One-way ANOVA, $p = 0.002$) and M (One-way ANOVA, $p = 0.003$) groups had significantly larger amounts of iNOS-positive cells compared to the C group (Fig. 3A,C). However, 2-vo group showed a slight but not statistically significant increase than M groups (One-way ANOVA, $p = 0.21$). The same immunohistochemistry results were found in the

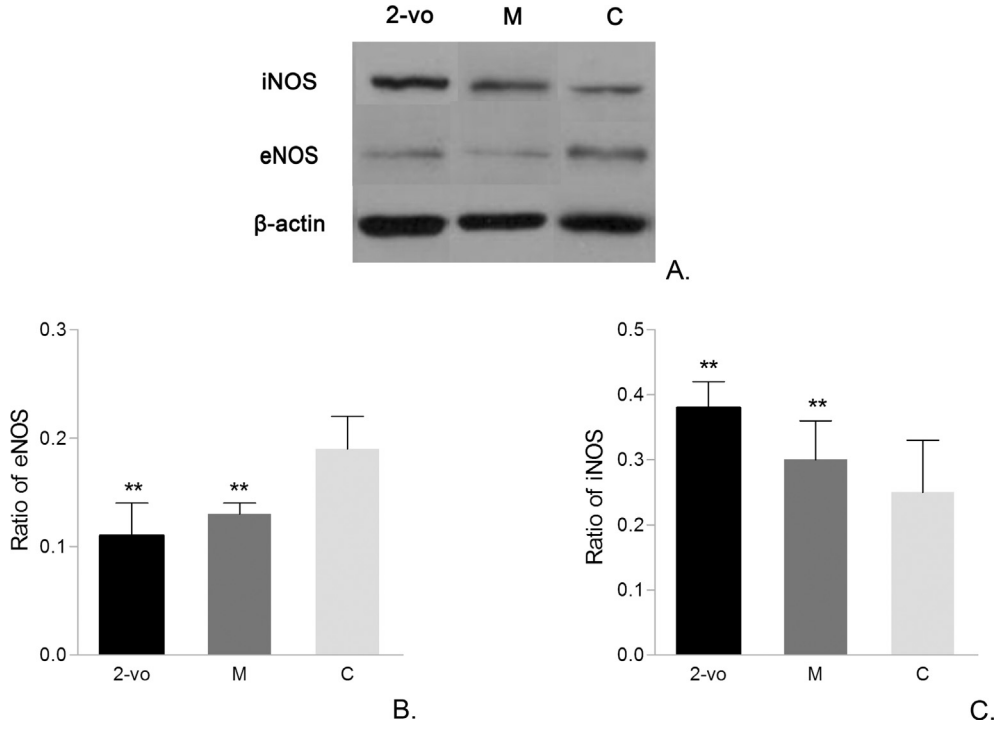

Figure 3 The expression of iNOS increased and the expression of eNOS decreased in the hippocampal areas of the rats in the 2-vo and M groups (n = 5). (A) The expression levels of iNOS and eNOS were determined by Western blot analysis. (B) The proportion of iNOS was determined through densitometric analysis. (C) The proportion of eNOS was determined through densitometric analysis. The data are expressed as the mean ± SD (One-way ANOVA, **P < 0.01).

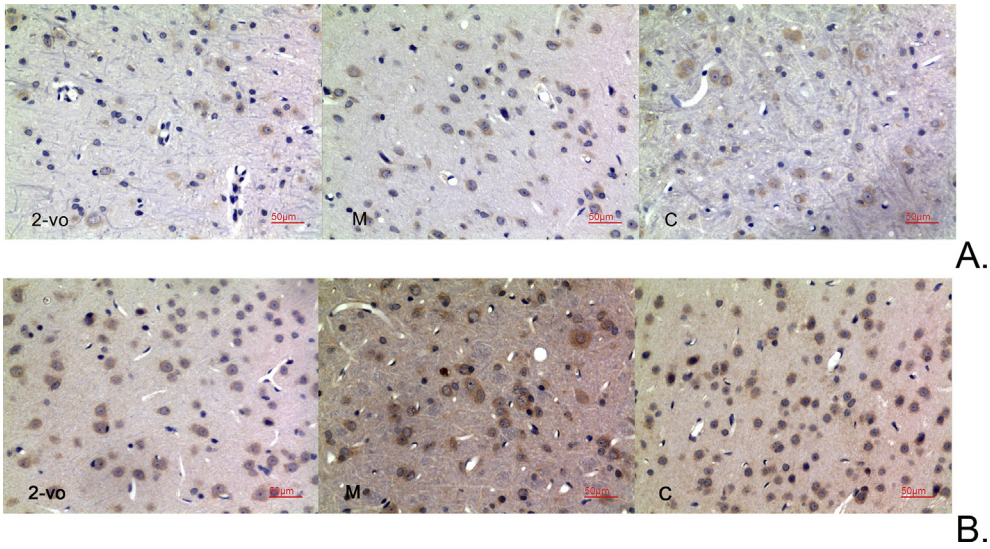

Figure 4 The expression of iNOS increased and the expression of eNOS decreased in the hippocampal areas of the rats in the 2-vo and M groups (n = 5). (A) The expression of iNOS was determined by immunohistochemistry. (B) The expression of eNOS was determined by immunohistochemistry.

expression of iNOS in the hippocampus (for 2vo group, $p = 0.003$ and M groups, $p = 0.001$) (Figs. 4A and 5A). Similarly, there was a slight but not statistically significant difference between the 2-vo and M groups (One-way ANOVA, $p = 0.35$).

eNOS expression in the hippocampus

Western blot results showed that there were significantly fewer eNOS-positive cells in both the 2vo group (One-way ANOVA, $p = 0.001$) and the M group (One-way ANOVA, $p = 0.001$) compared to the C group (Fig. 3A, B). 2-vo group expressed less eNOS than M groups, yet there was no significant difference (One-way ANOVA, $p = 0.57$). Similar results were also found in the immunohistochemistry results, which both the 2vo group (One-way ANOVA, $p = 0.003$) and the M group (One-way ANOVA, $p = 0.001$) expressed fewer eNOS than C group (Figs. 4B and 5B). And, there was also a slight but not statistically significant difference between the 2-vo and M groups (One-way ANOVA, $p = 0.35$).

Discussion

Wistar rats have been widely used in animal experiments due to their mild temperament and vitality.[19,20] This study focused on the mechanism of the spatial memory/learning abilities associated with occlusal function. The factor of age was standardized. Young Wistar rats were chosen, and none of the rats died during the study.

The two vessels that were occluded were the bilateral common carotid arteries. De la Torre[21] first established a chronic cerebral ischemia model by simultaneous permanent ligation of the bilateral carotid arteries in Wistar rats.

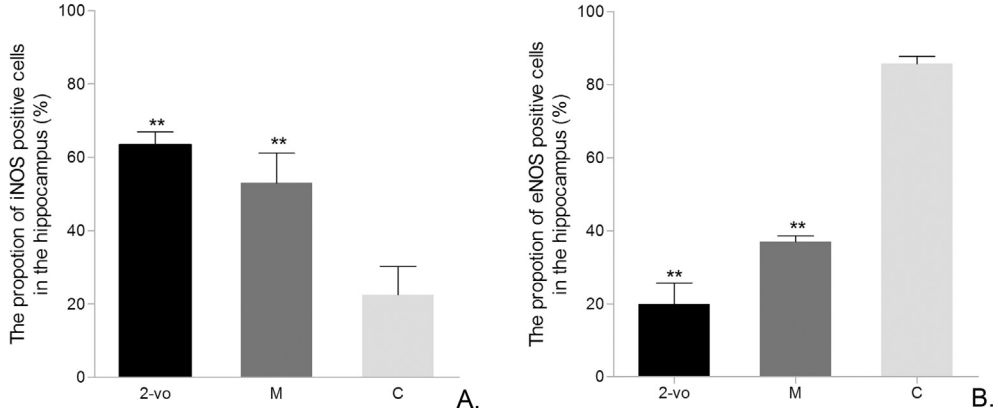

Figure 5 The expression of iNOS increased and the expression of eNOS decreased in the hippocampal areas of the rats in the 2-vo and M groups (n = 5). (A) There were more iNOS-positive cells in the hippocampal areas of the rats in the 2-vo and M groups than in those of the rats in the C group. (B) There were fewer eNOS-positive cells in the hippocampal areas of the rats in the 2-vo and M groups than in those of the rats in the C group. The data are expressed as the mean ± SD (One-way ANOVA, **P < 0.01).

The method is simple, comparable and stable and produces a marked decrease in learning and memory. It is suitable for modeling long-term chronic cerebral ischemia. It is a commonly accepted chronic cerebral ischemia model.[22,23] It has been reported that 2 weeks after 2-vessel occlusion, the results of HE staining and Nissl staining show that the neurons in the hippocampal CA1 area are damaged, with the percent damage increasing to 55% at 4 weeks and to 67% at 8—13 weeks.[24] In addition, KM mice show impaired spatial learning and memory ability at 8 weeks postsurgery.[16]

The MWM has been used in some of the most sophisticated experiments in the study of the neurobiology and neuropharmacology of spatial learning and memory. It has been used in the validation of rodent models for neurocognitive disorders.[25] Sex hormones may play a role in the difference in cognitive performance between males and females. Male animals have better spatial learning skills than female animals. The MWM task was originally designed to study the mechanisms of spatial localization in rats. Although mice have been successfully used and showed similar performance in dry-land spatial tasks where used, mice perform worse than rats in swimming tasks.[26] Therefore, male rats were used in this study.

Evidence has been presented for the specific and disproportional involvement of hippocampal formation in the spatial aspects of MWM learning.[27] Rats with hippocampal lesions are impaired in hidden- but not in visible-platform MWM learning. In fact, the pathological changes associated with Alzheimer's disease start in the hippocampus;[28] the same is true for postischemic brain injury dementia.[29] There is strong evidence that the earliest pathological process in sporadic Alzheimer's disease may be ischemic episodic damage to the hippocampus.[30] The act of biting is not only important for chewing food but also has a wider significance. Clenching the jaw muscles may promote cardiac output.[31] Brain functional activity is enhanced by the improvement of dentures in toothless patients.[32] Jaw clenching could stimulate local blood flow into the brain.[31] There are several possible underlying biological mechanisms regarding the relationship between the loss of molars and cognitive impairments, such as diminished sensory input,[33] decreased cerebral blood flow,[34] impaired cholinergic neurotransmission[35] or increased stress responses.[19] This study found that the spatial learning and memory of Wistar rats were impaired after either tooth loss or 2-vessel occlusion. It can be inferred that vascular factors play a similar role in the effects of tooth loss and chronic cerebral ischemia.

The degrees of changes in NO and iNOS after the 2-vo procedure and tooth extraction were almost the same. These two types of surgery may stimulate the overproduction of NO and iNOS, and eNOS plays a role in nitric oxide (NO) production.[36] Generally, eNOS is expressed in endothelial cells and plays a crucial role in vasodilation.[37] During this research, the level of eNOS decreased in the 2-vo and M groups, suggesting that eNOS may participate in the pathological state of cerebral ischemia and that eNOS is usually expressed in physiological conditions.

Within the limitations of this study, it can be concluded that the molarless condition aggravates cognitive impairments, the results of cognitive impairments related to tooth loss and those that occur due to chronic cerebral ischemia were statistically not significant and that NO, iNOS and eNOS in the hippocampus are involved in both cases.

Declaration of Competing Interest

There is no potential conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2019.09.001.

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