NMDA receptor antagonist MK-801 reduces neuronal damage and preserves learning and memory in a rat model of traumatic brain injury

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Abstract: Objective NMDA receptor channel plays an important role in the pathophysiological process of traumatic brain injury (TBI). The present study aims to study the pathological mechanism of TBI and the impairment of learning and memory after TBI, and to investigate the mechanism of the protective effect of NMDA receptor antagonist MK-801 on learning and memory disorder after TBI. **Methods** Forty Sprague-Dawley rats (weighing approximately 200 g) were randomized into 5 groups ($n = 8$ in each group): control group, model group, low-dose group (MK-801 0.5 mg/kg), middle-dose group (MK-801 2 mg/kg), and high-dose group (MK-801 10 mg/kg). TBI model was established using a weight-drop head injury mode. After 2-month drug treatment, learning and memory ability was evaluated by using Morris water maze test. Then the animals were sacrificed, and brain tissues were taken out for morphological and immunohistochemical assays. **Results** The ability of learning and memory was significantly impaired in the TBI model animals. Besides, the neuronal caspase-3 expression, neuronal nitric oxide synthase (nNOS)-positive neurons and OX-42-positive microglia were all increased in TBI animals. Meanwhile, the number of neuron synapses was decreased, and vacuoles degeneration could be observed in mitochondria. After MK-801 treatment at 3 different dosages, the ability of learning and memory was markedly improved, as compared to that of the TBI model animals. Moreover, neuronal caspase-3 expression, OX-42-positive microglia and nNOS-positive neurons were all significantly decreased. Meanwhile, the mitochondria degeneration was greatly inhibited. **Conclusion** MK-801 could significantly inhibit the degeneration and apoptosis of neurons in damaged brain areas. It could also inhibit TBI-induced increase in nNOS-positive neurons and OX-42-positive microglia. Impairment in learning and memory in TBI animals could be repaired by treatment with MK-801.

Keywords: traumatic brain injury; MK-801; learning and memory; caspase-3; microglia; neuronal nitric oxide synthase

1 Introduction

Nowadays, traumatic brain injury (TBI) is one of the main diseases that threaten the human health^[1]. The brain injury from TBI can be divided into the primary and the secondary. The secondary brain injury occurs within several

hours or days after injury, and consists of a series of neuron damage and nervous dysfunction caused by nervous biochemical process. The clinical symptoms of TBI include ischemia and hypoxia following damage, brain edema, brain death, *etc.* The symptoms are determined by the final outcome of TBI to a large degree^[2]. Learning and memory disorder is the severe sequela of TBI secondary damage, and could seriously lower the life qualities of the patients^[3]. The pathological mechanism of the secondary damage remains unclear. However, more attention has been paid to other fields, including: (1) excitotoxic effect of excitatory amino acid, (2)

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intracellular Ca^{2+} overloading, (3) overoxidation of oxygen free radical and lipid peroxide, (4) inflammatory mediators, (5) apoptosis, *etc.*^[4-6]. These mechanisms not only occur simultaneously, but also are mutually promoted, finally inducing neuronal death. To study the mechanisms above, the noncompetitive glutamate receptor antagonist MK-801 (dizocilpine) was adopted in the present study. As has been previously reported^[7], MK-801 can block N-methyl-D-aspartate (NMDA) receptor, and alleviate the calcium overload in cerebral cortex nerve cells with hypoxic ischemic brain damage, inhibiting cell apoptosis in brain. Moreover, although it has been reported that MK-801 could exert cerebral protective effects in TBI model rats, reports on its influence on the sequela of learning and memory are very rare^[7,8]. Based on the changes of learning and memory ability in animals after the weight-drop head injury in this experiment, the effects of MK-801 on attenuating brain injury, and the morphological and immunohistochemical changes were observed. The pathological mechanism of learning and memory disorder after head injury is further analyzed.

2 Material and methods

2.1 Materials Antibodies used in the present study included rabbit anti-rat caspase-3 monoclonal antibody (Calbiochem), anti-nitric oxide synthase (nNOS) antibody (Santa Cruz), mouse anti-rat CD11b (OX-42) monoclonal antibody (Sigma) and peroxidase-labeled rabbit anti-mouse IgG antibody (Bio-Technology). DAB Chromogen was purchased from Boster. Other equipments included Morris water maze video analysis system (DigBehv-MM, Shanghai JiLiang Software Technology CO., LTD.), Freezing microtome (AS620 type, U.K.), and RS IMAGE Image Analysis System (Roper Scientific, USA). Observation and analysis of electrical microscope slice and electron microscopy were conducted by Electron Microscopy Laboratory in Medical College of Shanghai Jiaotong University.

2.2 TBI model establishment and animal grouping Forty healthy male Sprague-Dawley rats, weighing (200 ± 30) g, were purchased from Animal Centre of Medical College of Shanghai Jiaotong University. Rats were raised under relatively stable conditions at the temperature of (25 ± 1) °C, with a hu-

midity of $(50 \pm 2)\%$ under a 12:12 h day/night cycle.

The rats were randomly divided into 5 groups ($n = 8$ in each group), including control group (cont), model group, low-dose group, middle-dose group, and high-dose group. Local contusion and laceration of the upper area of the brain was induced by the impact of weight-drop equipment, with a 3-mm depth of impact, causing the impact of $600 \text{ g} \times \text{cm}$ ($20 \text{ g} \times 30 \text{ cm}$), as described by Feeney method^[9]. The rats in control group received no impact from a weight-drop equipment. After TBI modeling, intraperitoneal injection was immediately taken. Proper physiological saline was injected into the rats in control and model groups. MK-801 was injected into the rats in low-dose, middle-dose, and high-dose groups, at the doses of 0.5 mg/kg, 2 mg/kg and 10 mg/kg, respectively. The duration of the therapy was 2 months.

2.3 Behavior test After the 2-month treatment, learning and memory ability was evaluated by using Morris water maze test.

2.3.1 Navigational test The navigational test was conducted in a dark and airtight room. The pool was divided into 4 imaginary quadrants, and a platform was placed in one of the quadrants. The rats entered the water from 4 directions. Then they were allowed 90 s to find the platform, and stayed on the platform for 30 s. If the rats failed to find the platform within 90 s, they were placed on the platform and stayed for 30 s. The test was conducted once per day for 4 d. The platform was removed after the test.

2.3.2 Probe test After the platform was removed, the rats were placed in the center of the pool to find the platform from their memories. The number of times that the rat passed the platform and the percentage of swimming distance in the platform quadrant to the total distance were recorded by the computer.

2.4 Morphological and immunohistochemical assays The rats were anaesthetized by pentobarbital sodium injection into abdominal cavity. Springe apical was intubated to aorta for hyperthermic saline perfusion to wash the blood *in vivo*. Then 250 mL formaldehydum polymerisatum (4%, 4 °C) was perfused. The brain was taken out and fixed in 4% formaldehydum polymerisatum (4 °C) for 2 h, and then it was placed in 20% sucrose at 4 °C overnight. The serial trans-

verse sections were cut by a freezing microtome, with a thickness of 15 μm for immunohistochemical staining of caspase-3, OX-42 and nNOS. The slices were routinely stained, cleared, and finally mounted. Staining results were observed and photographs were taken by Leica DM2500 microscope. Image analysis was performed by RS Image Software.

In each group, 2 rats were used for morphological observation by transmission electron microscopy. Brain tissues were fixed in 2% glutaraldehyde instead of 4% formaldehydum polymerisatum. The sections of hippocampus were cut. The morphological variations of mitochondria, cell nuclear and synapse were observed by transmission electron microscopy.

2.5 Statistical analysis Data were presented as mean $\pm$ SEM and analyzed by SPSS10.5 statistical software and one-way ANOVA-LSD.

3 Results

3.1 Results of behavior test

3.1.1 Navigational test Training results of 4 tests in place navigation test (evasive latency) were shown in Fig. 1.

3.1.2 Probe test The percentage of swimming distance in the platform quadrant to the total distance, and the number of times passing the platform were shown in Fig. 2. The control group and the 3 treatment groups all showed significantly higher levels in both the number of times passing the platform and the percentage of the swimming distance than

the model group did.

3.2 Expression of caspase-3 in neurons in cerebral cortex

After brain injury, neuronal caspase-3 expression was greatly increased, and neuronal apoptosis was also induced. After the treatment with MK-801, the increase of caspase-3 expression in the damaged brain region was significantly inhibited, as shown in Fig. 3A. Two photographs were taken from one

Fig. 1 The average escape latency of rats in 4 tests in each group. With the increase of training time, evasive latency was shortened in all the 5 groups, and the slope of evasive was the least in model group. The difference in the escape latency among the 5 groups was the most significant on day 4 of the training. Moreover, none of the 3 MK-801 treatment groups showed any significant difference with the control group. Compared to the other 2 treatment groups, HD group showed the best protective effect, as revealed by the greatest slope of evasive. * $P < 0.01$ vs model group; LD: low-dose group; MD: middle-dose group; HD: high-dose group.

Fig. 2 Results of the spatial search test. A: the number of times that rats passed the platform. B: the percentage of the swimming distance in platform quadrant to the total distance. The control group and the 3 treatment groups were all significantly different from model group in both the number of times passing the platform and the percentage of the swimming distance. None of the treatment groups showed any significant difference with the control group. * $P < 0.05$, ** $P < 0.01$ vs model group. LD: low-dose group; MD: middle-dose group; HD: high-dose group.

Fig. 3 A: the caspase-3-positive neurons in cerebral cortex in (A1) control group, (A2) model group, and (A3) high-dose group. The increase of caspase-3-positive neurons in model group could be significantly inhibited by MK-801 treatment. B: the expression of nNOS in hippocampus in (B1) control group, (B2) model group, and (B3) high-dose group. The nNOS-positive neurons in HD group were significantly less than those in model group. C: the OX-42-positive microglia in cerebral cortex in (C1) control group, (C2) model group, and (C3) high-dose group. The increase of OX-42-positive microglia in model group was significantly inhibited by MK-801 treatment. Scale bar, 50 μ m. D: the ultrastructures of hippocampal neurons (24 500 $\times$). D1: mitochondria in the cytoplasm were eumorphism in control group. D2: in model group, cristae were shortened, and even disappeared in mitochondria, and vacuolar degeneration was also detected. D3: vacuolar degeneration did not happen in mitochondria in high-dose group. E: the configuration and number of synapses in the nerve fiber in (E1) control group, (E2) model group, and (E3) middle-dose group, as observed under the electron microscopy at a magnification of 25 000 $\times$.

Table 1. Grey levels of caspase-3 and OX-42, and the number of nNOS-positive cells and OX-42-positive cells in different groups

Group	Control	Model	LD	MD	HD
Grey level of caspase-3	0.12±0.02**	0.16±0.03	0.13±0.03*	0.12±0.02**	0.13±0.02**
Number of nNOS cells	9.50±1.35**	11.60±1.26	10.09±1.04*	9.78±1.20*	9.90±1.10*
Grey level of OX-42	0.22±0.04**	0.27±0.04	0.23±0.03**	0.22±0.03**	0.23±0.03**
Number of OX-42 cells	17.25±1.95**	21.19±2.32	19.25±1.44*	18.63±1.82**	18.25±1.95**

* $P < 0.05$, ** $P < 0.01$ vs model group. $n = 16$ in each group. LD: low-dose group; MD: middle-dose group; HD: high-dose group.

rat, and each group included 16 sections. Image analysis of the grey levels was conducted by RS Image Software. The quantification results were shown in Table 1.

3.3 Number of nNOS-positive neurons in hippocampus The nNOS-positive neurons could be seen in various regions of the brain, among which hypothalamus, hippocampus and cortex were the most common. Hippocampus was selected for the comparison of nNOS-positive neurons between different groups (Fig. 3B). Two photographs were taken in one rat, and each group had 16 sections. Neurons were observed at a magnification of 400× and counted. The quantification results were shown in Table 1.

3.4 OX-42-positive microglia in brain OX-42-positive microglia represented the degree of inflammation in brain. The grey levels of positive staining of OX-42 in cerebral cortex were compared between different groups (Fig. 3C). Two photographs were taken from one rat, and each group had 16 sections. The grey levels of staining and the number of cells were analyzed by RS Image Software for image analysis. The quantification results were shown in Table 1.

3.5 Ultrastructural observation In control group, mitochondria were rod-like in shape in the cytoplasm in hippocampus, and mitochondrial cristae was uniform and dense, with no vacuole. Besides, the number of synapses was greater. In model group, vacuoles appeared in mitochondria in the cytoplasm, and the number of synapses was reduced. In MK-801 treatment group, no vacuolar degeneration occurred in mitochondria, and the number of synapses was increased. The ultrastructures of hippocampal cells were shown in Fig. 3D. The configuration and the number of synapses in the nerve fiber were shown in Fig. 3E. The quantification results of the number of synapses in each group at a magnification of 25 000× in 10 visual fields were shown in Fig. 4.

Fig. 4 Quantification results of the number of synapses in each group, as observed under the electron microscopy. Synapses were counted in 10 visual fields in each group. * $P < 0.05$, ** $P < 0.01$ vs model group. LD: low-dose group; MD: middle-dose group; HD: high-dose group.

4 Discussion

TBI is one of the hotspots in medical research nowadays. However, in brain injury research, much emphasis has been paid on injury mechanism and treatment, while little has been reported on the influence of TBI sequelae: learning and memory impairment. Clinical data and animal studies indicate that the impairment of learning and memory ability exists in different degrees after brain injury^[3,9]. Improving the learning and memory ability after brain injury is of great significance in improving the living qualities of the patients.

Kline^[10] and Phillips^[11] have found that the 15-d administration of NMDA receptor antagonist MK-801 could improve the learning and memory ability after experimental cerebral trauma. In the present study, MK-801 was injected i.p. to weight-dropped TBI rat model. The improvement of learning and memory ability after MK-801 treatment was studied. The results showed that after the treatment with MK-801 at different doses for 2 months, the evasive latencies in the 3

MK-801 groups were all significantly shortened, as compared to that in model group. Moreover, the number of times that the rats passed the platform and the percentage of swimming distance in the platform quadrant to the total distance were both significantly higher than those in model group. Besides, there was no significant difference among the 3 MK-801 groups, which might be due to the block of the NMDA receptor in the brain by MK-801, although it was not in a dose-dependent manner. In addition, Kline and Phillips have reported that there is no difference between MK-801 treatment group and TBI model group when MK-801 was administered for 1 or 2 months, which has been confirmed by our former study. This could be explained that the TBI model animals could recover from the injury even without any treatment. However, the treatment would have obvious effects if the injury is heavy.

During TBI, approximately 10%-15% of the neurons undergo apoptosis. The caspase family members participate in apoptosis with strict specificity in cell type and in death signal. During apoptosis of different tissue cells or the same cells induced by different stimulations, the active caspase is not the same. Caspase-3 is in an essential position, and different proteases cut caspase-3 discretely, inducing the activation of caspase-3. Moreover, the active caspase-3 cuts different substances, which could enlarge the reaction and induce cell death. Activation of caspase-3 has important biological significance in the process of apoptosis. Active caspase-3 can specifically cut DNA, activating PARP and DNA-PK (DNA-dependent protein kinase) that participate in the injury-repair process of DNA inactivation, leading to chromatin condensation and ribozyme activation, and eventually the cell apoptosis. Here we demonstrate that the application of NMDA receptor antagonist—MK-801 could efficiently inhibit caspase-3 expression and neuronal apoptosis in injured brain, thereby improving the function of learning and memory in the TBI model animals.

There are important relationships between neuronal injury and nNOS after brain injury. However, the influence of nNOS on neuron apoptosis is generally not predictable after brain injury. nNOS was firstly found by Bredt in 1990^[12] in nerves around the blood vessels. The research by Kim *et al.*^[13] has

shown that the application of nNOS inhibitor 30 min before occlusion of the middle cerebral artery could obviously decrease the infarct volume in mouse. In the study of Hag *et al.*^[14], the inhibitor of nNOS was applied 30 min after rat brain injury, and results have shown that at 4 h and 24 h after brain injury, the brain injury is obviously relieved. On the other hand, in the early stage of brain injury, the degree of brain injury is obviously relieved in nNOS gene knock-out rats^[15]. The neuronal injuries following brain injury include apoptosis and necrosis. It has been reported that in the early stage of brain injury, the increase of NO can lead to neuronal apoptosis^[16] through ONOO^[17]. Meanwhile, the expression of nNOS in neurons also increases within this period^[18]. In this experiment, MK-801 can inhibit the expression of nNOS in brain after brain injury, which indicates that MK-801 can inhibit neuron necrosis after brain injury by inhibiting the expression of nNOS, thus improving the function of learning and memory. This may be achieved through blocking the NMDA receptor signaling pathway and the following ONOO⁻ damage pathway, and finally neuron apoptosis is inhibited.

Microglia can secrete IL-1 β , TNF- α , TGF- β , *etc.*, and play an important role in the pathogenesis of brain injury. After brain injury, both protein and mRNA levels of IL-1 β increase. IL-1 β can drive the local deposition of polymorphonuclear cells during inflammation, and activate them in the injured location, aggravating brain injury. IL-1 β can also induce the activation of microglia, which can aggravate brain injury by releasing cytokines. IL-1 β not only stimulates the production of NO and the release of TNF- α , but also induces abundant production of NO and the activation of NMDA receptor combined with TNF- α ^[19]. TNF- α not only participates in the damage of injured brain tissue, but also accelerates the synthesis of platelet activating factors, inhibiting the anticoagulant mechanism such as thrombomodulin C protein, increasing profibrinolysin and the formation of thrombosis^[20]. Moreover, TNF- α stimulates the expression of nerve growth factor in fibroblast cells, glial cells and astrocytes, facilitating neuronal survival in ischemic area and peripheral zone. It has been reported that microglia can release a great amount of H₂O₂, leading to the death of cultured neurons. Meanwhile, the superoxide produced by activated microglia and NO can

form the high toxicity anion and nitrogen peroxide^[21]. *In vitro* experiment has also shown that NO produced by activated microglia can directly kill cultured human embryonic cortical neurons, and the oligodendrocytes can be dissolved by activated microglia through NO^[22]. Glutamate is another neurotoxin produced by microglia. It can pass the active glutamate-gated channel to induce Na⁺ excessive influx and cellular edema, leading to neuronal acute injury. Through the interaction with NMDA receptor, glutamate can activate the Ca²⁺ channels regulated by NMDA receptor gate channel, inducing Ca²⁺ influx and delayed death of neurons^[23]. In the present study, rats with traumatic brain injury were treated with MK-801. We find that MK-801 can inhibit the expression of OX-42 in microglia and the activation of microglia. The mechanism may be that MK-801 inhibits Ca²⁺ influx and activation of microglia by inhibiting glutamate-gated channel, thus decreasing brain damage and improving the functions of brain.

In the pathological changes of neurons, the injury of mitochondria which is closely correlated with oxygen metabolism plays a determinant role. The pathological manifestations of mitochondria in the early stage are reversible, such as the swelling of mitochondria and broken of the mitochondria crista. Subsequently, the manifestations are irreversible, such as degeneration of mitochondria. The damage of mitochondria then causes a series of ultrastructural changes of neurons^[24]. Many studies have suggested that mitochondria is the key point in neuron degeneration after brain injury, and the injury and dysfunction of mitochondria will decide the survival state of neurons^[25]. It has been reported that Cytochrome C released from mitochondria can directly affect the apoptotic factors Bcl-2, Bax and caspase, and activate death protease caspase-3, promoting apoptosis^[26]. Some researchers propose that in the process of brain injury, NO can activate PARP which exhausts the energy substrate of mitochondria-NA⁺, thereby breaking the electronic respiratory chain of mitochondria, aggravating energy metabolic disturbance, and promoting cell death^[27]. Clinical research has shown that the delayed cerebropathy can be attributed to the delayed energy failure by mitochondria injury and excessive accumulation of lactic acid after brain injury as revealed by spectral analysis^[28]. In the model of cerebral anoxia in neonatal rats,

Gilland *et al.* have found that the activation of NMDA receptor can induce Ca²⁺ overloading, which is the main cause of mitochondria damage. Moreover, application of NMDA receptor antagonist MK-801 could alleviate this injury of mitochondria. Since TBI is accompanied by cerebral ischemia and energy metabolism abnormality, it needs to be clarified whether MK-801 has some protective effects on mitochondria after TBI. Here we found similar results to those of others, that is different degrees of mitochondria damage were observed in hippocampal neurons, including swelling of mitochondria, broken of the mitochondria crista and mitochondria degeneration, and degeneration of synapse. Moreover, after the treatment with MK-801, mitochondria degeneration did not happen, and the number of synapses significantly increased. These findings indicate that MK-801 can inhibit neuronal injury caused by the damage of mitochondria after TBI, and prevent synapse degeneration, accelerating the recovery of learning and memory after TBI.

In conclusion, NMDA receptor antagonist MK-801 can inhibit the expression of nNOS in brain, the activation of microglia and the activity of caspase-3 in neurons after TBI, and protect the injured neurons. MK-801 may be the first-line drug on clinical treatment of TBI and cerebral ischemia.

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NMDA 受体拮抗剂 MK-801 对创伤性脑损伤学习记忆障碍的保护机制研究

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摘要: **目的** NMDA型谷氨酸受体通道在创伤性脑损伤(trumatic brain injury, TBI) 的病理生理过程中具有重要的作用。本研究探讨 TBI 后的学习记忆损伤及其病理特点, 并观察 NMDA 型谷氨酸受体拮抗剂 MK-801 对 TBI 引起的学习记忆障碍的保护机制。**方法** 体重 200 g 左右的 SD 大鼠 40 只, 随机分为 5 组: 正常组(未创伤)、模型组、低剂量组(MK-801 0.5 mg/kg)、中剂量组(MK-801 2 mg/kg)、高剂量组(MK-801 10 mg/kg), 每组 8 只。后 4 组经自由落体形成脑损伤模型。经 MK-801 治疗 2 个月后, 进行 Morris 水迷宫测试学习记忆能力。测试结束后处死, 取脑组织进行形态学及免疫组织化学检测。**结果** 在 TBI 后, 大鼠学习记忆力显著下降。形态学和免疫组织化学研究表明, TBI 大鼠神经细胞内 caspase-3 表达增加, 神经源性一氧化氮合酶(nNOS)阳性神经元增加, 并且 OX-42 阳性小胶质细胞增加, 同时神经元突触减少, 线粒体空泡样变性。经 3 种不同剂量的 MK-801 治疗后, TBI 模型动物学习记忆能力得到显著改善, 神经元 caspase-3 的表达量降低。同时, nNOS 阳性神经元和 OX-42 阳性小胶质细胞均显著减少, 线粒体空泡样变性减少。**结论** MK-801 明显减少损伤脑区神经元变性死亡, 抑制 nNOS 阳性神经元和小胶质细胞增生活化等炎症反应, 改善 TBI 模型动物学习记忆力。

关键词: 创伤性脑损伤; MK-801; 学习记忆; caspase-3; 小胶质细胞; 神经源性一氧化氮合酶