

Original Article

Safe doses of MK-801 enhanced the antioxidant ability in the central nervous system of obstructive jaundice rats through the Keap1-NF-erythroid 2-related factor 2-antioxidant response element (Keap1-Nrf2-ARE) pathway

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Abstract: This study aims to explore the role of the Keap1-NF-erythroid 2-related factor 2-antioxidant response element (Keap1-Nrf2-ARE) pathway in the central nervous system of obstructive jaundice rats and evaluates the antioxidative effect of MK-801. Twenty rats were divided into four equal groups: sham group (Group I), bile duct ligated group (Group II), low and high dose of MK-801 group (Group III and IV). Groups II, III, and IV were the obstructive jaundice groups (OJ Groups). Rats were treated with different doses of MK-801 injected intraperitoneally daily for 10 days after bile duct ligation in Group III and Group IV. Then, Nrf2 mRNA and levels of Nrf2, HO-1, and NQO1 proteins in the rat cerebral cortex were measured. The expression of Nrf2 mRNA and Nrf2 and NQO1 proteins increased in MK-801 groups compared to that in the control group. The expression of HO-1 increased in the low dose group, but decreased in the high dose group. The expression of Nrf2 mRNA decreased and that of NQO1 protein increased in the high dose group compared to that in the low dose group; however, this was not statistically significant. In contrast, the expression of Nrf2 and HO-1 protein decreased in the high dose group with a statistical significance. After bile ligation, the Keap1-Nrf2-ARE pathway is activated and the transcriptional activity of Nrf2 increases. Safe doses of MK-801 reduced the oxidative stress in obstructive jaundice through the Keap1-Nrf2-ARE pathway. High dose of MK-801 did not have better antioxidative effect than the safe dose.

Keywords: Dizocilpine maleate, oxidative stress, obstructive jaundice, hyperbilirubinemia, nuclear factor E2 related factor 2, entral nervous system

Introduction

Obstructive jaundice occurs after the occlusion of the common bile duct. Aggregates of bilirubin can accumulate in the neuronal membrane and damage the nearby membrane-bound enzymes; this could be one of the mechanisms of bilirubin toxicity [1, 2]. Bilirubin-induced oxidative stress has already been verified in many studies.

Researchers have shown that bilirubin-induced neurotoxicity could be prevented by the admin-

istration of MK-801 (dizocilpine maleate) a potent open-channel antagonist of the N-methyl-D-aspartate (NMDA) receptor. However, some other studies demonstrated that MK-801 does not protect against bilirubin neurotoxicity [3]. However, the effect of MK-801 on obstructive jaundice-induced oxidative stress in the central nervous system has not been investigated thus far.

NF-erythroid 2-related factor 2 (Nrf2) is a recently discovered nuclear factor, which forms the Keap1-Nrf2-ARE pathway after decoupling

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with Keap1 and conjugation with the antioxidant response element (ARE). The Keap1-Nrf2-ARE pathway plays an important role in oxidative damage by regulating the intracellular redox balance and protecting against oxidative glutamate toxicity and apoptosis induced by hydrogen peroxide. However, its role in obstructive jaundice, particularly in response to NMDA receptor antagonists has not been studied extensively.

This study was designed to explore the role of the NMDA receptor antagonist MK-801 and the Keap1-Nrf2-ARE pathway in obstructive jaundiced rats to determine the pathophysiological mechanism of obstructive jaundice and the appropriate therapeutic targets.

In this study, we determined whether NMDA receptor antagonists had protective effects on oxidative stress-induced brain injury in obstructive jaundice after successful establishment of rat model of obstructive jaundice. In addition, the effects of MK-801 on Keap1-Nrf2-ARE pathway were evaluated.

Materials and methods

Animals

Twenty adult male Wistar rats, weighing between 250 and 300 g were obtained from the Experimental Animal Centre of Hebei Medical University (Hebei Province, China). The animals were housed in stainless-steel cages with two or three rats per cage, under controlled temperature and humidity conditions ($23\pm 2^{\circ}\text{C}$ and $55\pm 15\%$ relative humidity), with 12 h dark/light cycles. The rats were maintained on a standard laboratory diet with tap water throughout the experiment except for an overnight fast the night before surgery. The animals were then randomly divided into four groups based on their weight: Group I (sham operation group), Group II (control group), Group III (low dose group), and Group IV (high dose group). Group II, III, and Group IV are the obstructive jaundice groups (OJ groups). From the second day, animals in group III were treated with MK-801 (0.025 mg/kg/d, intraperitoneal) while those in group IV with a dose of 0.25 mg/kg/d, for 10 days. Meanwhile, animals in group I and group II were injected with the same volume of saline daily for 10 days. MK-801 (5R, 10S-(4)-5-methyl-10, 11-dihydro-5H-dibenzo [a, d] cyclo-

hepten-5, 10-imine hydrogen maleate) was supplied by Sigma (St. Louis, USA).

The experiments were carried out according to the international standards on animal welfare (86/609/EEC) and conformed to the guidelines of the Ethics Committee of the Second Hospital of Hebei Medical University, Shijiazhuang, China. The study protocol was approved by the local ethics committee.

Surgical procedures

All surgical procedures were performed under strict sterile conditions at the same time of the day, between 8:00 and 10:00. Under ketamine (40 mg/kg, intraperitoneal) anesthesia, all rats underwent laparotomy on day 0. Through a midline abdominal incision about 1 cm in length, the gastroduodenal ligament was isolated, after which the common bile duct was mobilized and double-ligated in its middle third with 4-0 silk suture. Thereafter, the common bile duct was transected between the two ligatures. In the sham operation group, the common bile duct was isolated without ligation or transection after a midline abdominal incision, similar to the other three groups. The abdomen was then closed in two layers with 4-0 and 2-0 nylon sutures, respectively.

Tissue treatment

On day 10, all the animals were anesthetized with intraperitoneal injection of ketamine (60 mg/kg). They were then decapitated and the brains were excised quickly on ice. The cerebral cortex was dissected and placed into screw-capped vials, which were then stored at -70°C until preparation.

The expression of Nrf2 mRNA in brain tissues by RT-PCR

The total RNA was extracted by Trizol method and was then used to synthesize cDNA through reverse transcription. The cDNA was used for PCR amplification of Nrf2. The upstream primer was 5'-TTCCTCTGCTGCCATTAGTCAGTC-3' and the downstream primer was 5'-GCTCTCCAT-TCCGAGTCACTG-3'. The total volume of the PCR reaction mixture was 25 μL , with 0.5 μL Taq DNA polymerase, 0.5 μL upstream primer, 0.5 μL downstream primer, 0.5 μL dNTP, 1.0 μL cDNA, and 2.5 μL 10 \times PCR buffer (containing 20 mM Mg^{2+}). The PCR reaction conditions

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Table 1. Expression of HO1, NQO1, Nrf2 protein and Nrf2 mRNA in the rat brain of four groups ($\bar{x} \pm s$)

Groups	Nrf2 mRNA	Nrf2	HO1	NQO1
Group I	0.007339±0.001611	4.70±0.47	7.38±0.48	5.16±0.23
Group II	0.009944±0.001703	6.50±0.37	14.542±0.51	7.13±0.46
Group III	0.01514±0.001985	12.84±1.37	16.50±1.96	12.01±0.66
Group IV	0.01358±0.001589	11.52±0.59	10.52±1.16	12.96±1.12

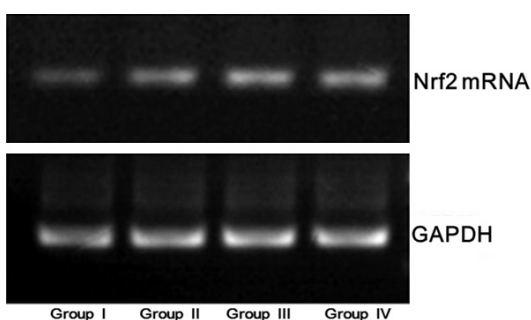


Figure 1. Expression of Nrf2 mRNA in brain tissues (RT-PCR picture).

were predegeneration for 3 min at 94°C; denaturation for 30 s at 94°C, annealing for 30 s at 57°C, and elongation for 30 s at 72°C, with 35 cycles in total; elongation again for 10 min at 72°C and end at 4°C. A pair of GAPDH internal reference primers (the upstream primer was 5'-ATTGCTCTCAATGACAACCTT-3' and the downstream primer was 5'-GGCGGTCTTAGCCTCTTCTGT-3') were added when target gene augmentation and the internal reference DNA was augmented as control. Ten microliters of the amplified product was transferred to 2% sepharose gel. After 20 min of electrophoresis, the Gel Doc EQ imaging analysis system was adopted for scanning and analyzing the strips, and the absorbance ratio of the target strip and internal reference GAPDH was calculated.

The expression of Nrf2, HO-1, and NQO1 proteins in the brain by western blotting

The total protein in the brain tissues was extracted and protein lysate was added. After schizolysis for 1 h at 4°C and centrifugation for 15 min (8000 r/min at 4°C), the supernatant was collected, and Coomassie Brilliant Blue protein assay was used to detect the concentration of Nrf2, HO-1, and NQO1 proteins. After denaturation of a 50 µg sample of total protein for 5 min, the SDS-PAGE gel electrophoresis was performed. After protein transfer and seal-

ing, diluted primary monoclonal antibodies to Nrf2, HO-1, and NQO1 (Bioworld Technology, USA) were added. After incubation for 16 h at 4°C and washing the membrane thrice, horseradish peroxidase-conjugated secondary antibodies to Nrf2, HO1, and NQO1 were added, with incubation for 2 h at 37°C followed by membrane washing thrice. Chemiluminescence detection was used for analysis of the results.

Statistical analysis

The experimental data was analyzed by SPSS19.0 statistical software and expressed as mean \pm s. Differences between the four groups were assessed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. The acceptance level of significance was $P < 0.05$ using a two-tailed distribution. Each value was obtained from five individual rats.

Results

The results are summarized in **Table 1**. None of the rats died and no obvious behavioral changes were observed during the experimental procedure. All samples were successfully analyzed.

Expression of Nrf2 mRNA in brain tissues

Compared with the sham operation group, the OJ groups showed significantly increased Nrf2 mRNA expression. The expression of Nrf2 mRNA significantly increased in the high dose and low dose group relative to the control ($P < 0.05$). However, there was no significant difference ($P > 0.05$) between the high dose and low dose group (**Figures 1, 2**).

Expression of Nrf2 in the brain tissues

The expression of Nrf2 increased significantly in the OJ groups in comparison to that in the sham operation group. Compared with the control group, the high dose and low dose group showed increased Nrf2 expression ($P < 0.05$). The expression of Nrf2 decreased markedly in the high dose group compared to that in the low dose group, with a significant difference (**Figures 3, 4**).

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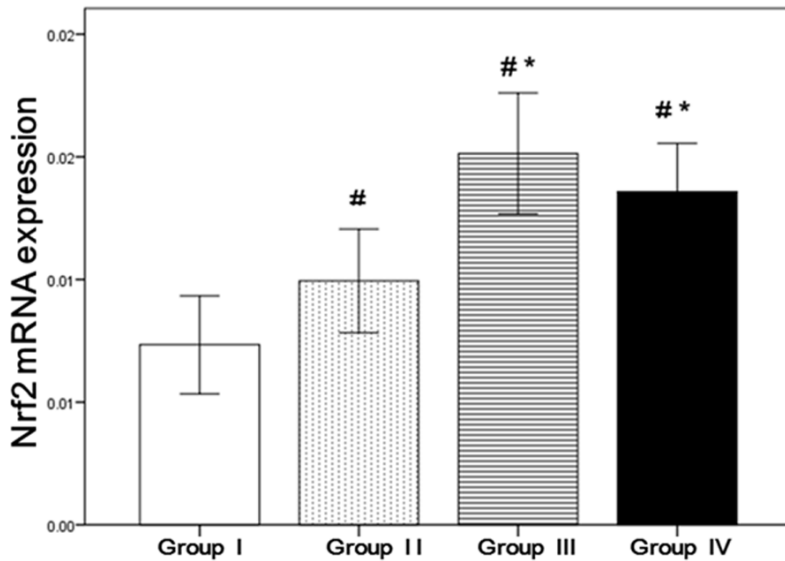


Figure 2. Expression of Nrf2 mRNA in the rat brain of four groups. Note: #P<0.05, compared with Group I; *P<0.05, compared with Group II.

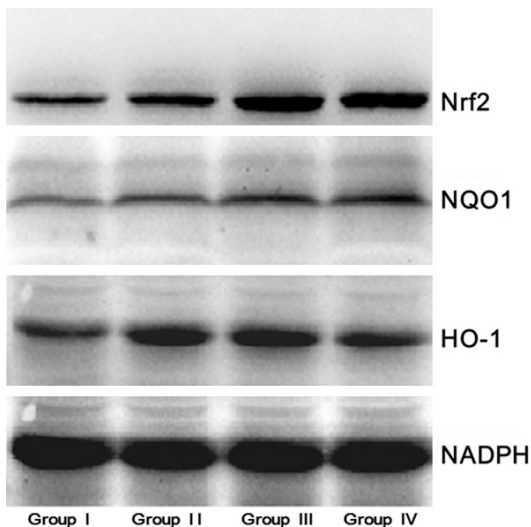


Figure 3. Expression of HO1, NQO1, Nrf2 protein in the rat brain of four groups (Western blot).

Expression of HO-1 in the brain tissues

Compared with the sham operation group, the OJ groups demonstrated significantly increased expression of HO-1. The expression of HO-1 increased in the low dose group, but decreased in the high dose group with a statistical significance when compared with either the low dose group or the control group (**Figures 3, 5**).

Expression of NQO1 in the brain tissues

Compared with the sham operation group, the control groups exhibited increased expression

of NQO1 (P<0.05). The expression of NQO1 increased in the high dose and low dose group relative to the control group (P<0.05). However, there was no statistical difference (P>0.05) between the high dose and low dose group (**Figures 3, 6**).

Conclusion

The results demonstrated the presence of oxidative stress in obstructive jaundice rats.

After bile ligation, the Keap1-Nrf2-ARE pathway is activated and the transcriptional activity of Nrf2 increases. Nrf2 protein expression and the II-phase detoxification enzymes HO-1 and NQO1 are upregulated, thereby enhancing the antioxidative ability of the body.

Safe doses of the NMDA receptor antagonist MK-801 can promote the expression of HO-1 and NQO1 through the Keap1-Nrf2-ARE signaling pathway and thus exert its neuroprotective effect. High doses of MK-801 decreased the expression of HO-1 and the Nrf2 protein, possibly because of the side effects, which warrant further research.

Discussion

In obstructive jaundice, hyperbilirubinemia causes various life-threatening pathologies in different organs [4]. Owing to its lipophilic properties, bilirubin markedly affects the central nervous system, which has a high lipid content [5], especially in preterm newborns [6]. Patients with obstructive jaundice show signs of central nervous system dysfunction, such as tremor, alteration in mental status and behavior, together known as hepatic encephalopathy [7]. Bilirubin concentration can interfere with the normal metabolism of brain cells, which downregulates the concentration of closed protein in the brain [8], causing damage to the blood brain barrier (BBB) [9].

Oxidative stress and its damage to the brain tissue in various conditions of the central nervous

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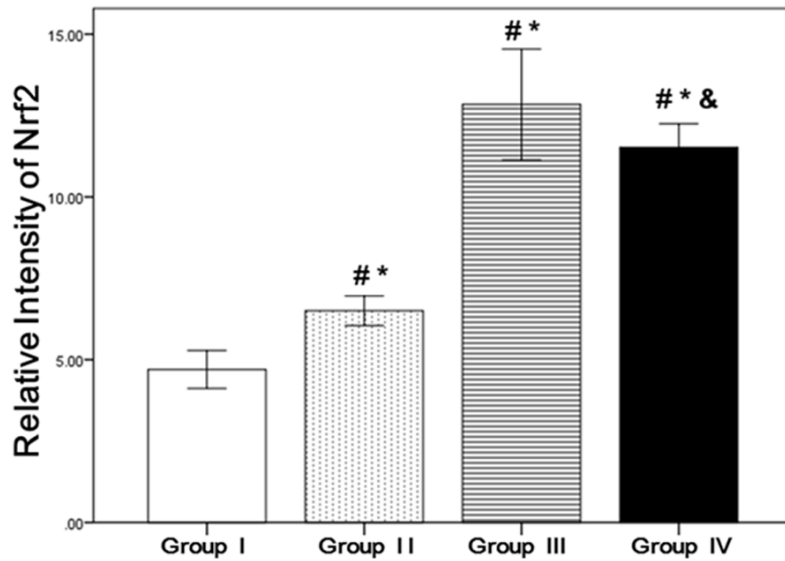


Figure 4. Expression of Nrf2 protein in the rat brain of four groups: Note: #P<0.05, compared with Group I; *P<0.05, compared with Group II; &P<0.05, compared with Group III.

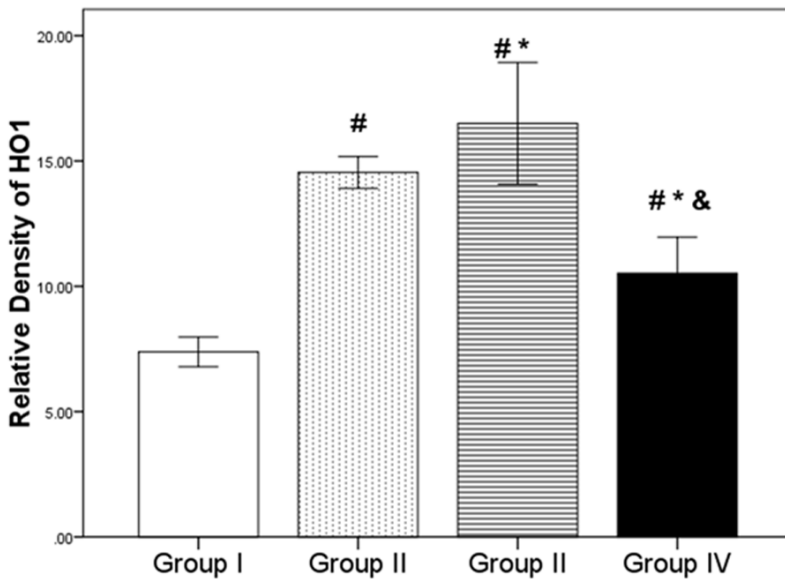


Figure 5. Expression of HO1 protein in the rat brain of four groups. Note: #P<0.05, compared with Group I; *P<0.05, compared with group II; &P<0.05, compared with group III.

system, such as trauma [10], ethanol [11] and drug abuse [12] have been increasingly discussed in recent years. Oxidative stress occurs when the production of reactive oxygen and/or nitrogen species overwhelms the antioxidant defense capacity [13]. Researchers have shown that oxidative stress has played an important role in the pathogenesis of cholesta-

sis [14-17]. The strong affinity of neuronal membranes for bilirubin leads to bilirubin-mediated neurotoxicity and results in short- or long-term disruption of the neuronal function [18] and induces oxidative stress causing the change of direct or indirect oxidative stress markers in the rat's brain [15]. Reactive oxygen species (ROS) have a biological importance in that they can lead to cell injury through several mechanisms, including direct damage to the DNA, lipid peroxidation generating vasoactive and pro-inflammatory molecules, protein oxidation, and which can finally lead to an altered protein activity [19, 20]. The removal of pathological ROS during the course of brain disorder seems to be a viable approach to neuroprotection [21].

We chose cerebral cortex tissue as our testing area in this experiment, because there were researches showing that after bile duct ligation, O_2^- increased significantly in the cerebral cortex, but less so in the midbrain and not at all in the cerebellum. However, the reason for this region-dependent specific pattern of O_2^- formation in the rat's brain, in the context of obstructive jaundice, was not clear [15, 22].

Antioxidants such as vitamin C, vitamin E and N-acetyl-L-cysteine (NAC) have been suggested as to exerting neuro-protective effects, but the effects have been attributed to a reduced lipid peroxidation and/or oxidative damage [7]. Resveratrol could also decrease intracellular ROS production, most likely by the mechanisms involving NMDA, AMPA/KA, intracellular Ca^{2+}

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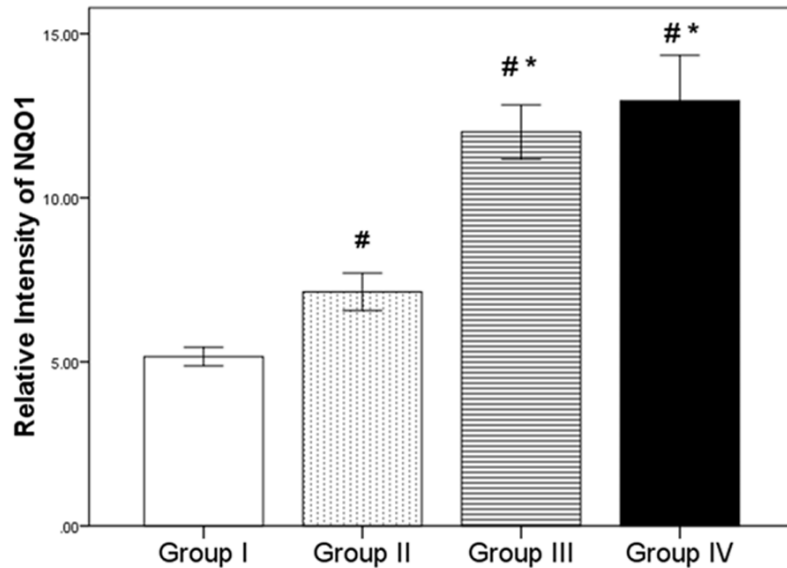


Figure 6. Expression of NQO1 protein in the rat brain of four groups. Note: #P<0.05, compared with Group I; *P<0.05, compared with group II.

and the HO-1 pathway, and therefore, preventing mitochondrial dysfunction, oxidative stress, and neuroinflammation [23].

We choose MK-801 to carry out our experiment because there were conflicts over this material on the protective effects of the central nervous system. In addition, we thought MK-801 would have an anti-oxidative effect based on the theories and research experiments.

Glutamate is the major excitatory neurotransmitter in the brain [24] and over-stimulation of the glutamate receptors may cause excitotoxic neuronal degeneration [25] and death [26]. Studies have already shown that damages from many neural insults, such as stroke, the status epilepticus hyperammonemia and traumatic brain injury were mediated through NMDA channel activation [3]. The NMDA receptor is an ion/channel complex located on the synaptic surfaces of neuronal membranes [27], which has an important role during brain development [28]. Overactivation of the NMDA-type glutamate receptors may increase Ca^{2+} influx into neurons. Additionally, Ca^{2+} can also be taken up by mitochondria, where it may stimulate the generation of oxidative/nitrosative species, and damage the mitochondria and the cell [29, 30].

Hoffman found that the NMDA channel was more open in the presence of bilirubin [31]. In

neonatal jaundice, increased bilirubin was supposed to cause damage to the neurons by increasing the activation of NMDA [2, 32] and bilirubin enhanced the neuronal excitability by increasing the glutamatergic transmission [33]. In other words, the NMDA receptor mediated bilirubin-induced neurotoxicity [34].

An NMDA-R-mediated oxidative stress has also been implicated in blood-brain barrier (BBB) disruption in a variety of neuropathological diseases. Disruption of the BBB may be mediated by reactive

oxygen species, nitric oxide and excitatory amino acids, such as glutamate and aspartate [35, 36]. Oxidative stress upregulates the NMDA-R on the cerebrovascular endothelium and thus heightens susceptibility to glutamate-induced BBB disruption [37], which generates a vicious spiral, and in turn, contributes to the neurotoxicity [38]. What is more, the activation of neuronal NMDA receptors induces superoxide-mediated oxidative stress in neighboring neurons and astrocytes, which means that the activation of NMDA receptors on one neuron can lead to the oxidative stress and cell death in neighboring neurons and astrocytes, by a process involving the extracellular release of superoxide [39].

Some studies have shown that Bilirubin did not modulate ionotropic glutamate receptors or glutamate transporters [40] and did not induce neuronal death by acting directly on the NMDA or AMPA receptors nor indirectly by blocking the glutamate uptake and thus raising the extracellular concentration of glutamate. In vivo studies assessing the role of the NMDA channel in obstructive jaundice have shown MK-801 pretreatment doses ranging from 0.1-4.0 mg/kg did not protect against brainstem auditory-evoked potential (BAEP) abnormalities [3].

MK-801 is a potent non-competitive antagonist of the NMDA receptor, which can easily pass

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the blood brain barrier and acts on the site of phencyclidine in the NMDA receptor channel and then induce allosteric regulation, to reduce the Ca^{2+} internal flow, which finally makes the function of the NMDA receptor weak and reduces the toxicity of the glutamic acid. Numerous studies have confirmed that MK-801 has a protective effect *in vitro* [6, 41] and *in vivo* [34] on neuronal injury and decrease oxidative stress in situation such as lipopolysaccharide-induced acute lung injury [19], ischemia [42], and methylmercury-induced [43] and ammonia-induced [11] neuronal injury. Post-ischemic treatment with a lower dose of MK-801 (3 mg/kg *i.p.*) has been shown to provide significant neuroprotection against global cerebral ischemia-induced oxidative stress in all tested brain structures of the adult gerbil in normothermic conditions [42].

Besides the protective effect, MK-801 also has a neural toxicity, which has been shown by the increased levels of several markers of oxidative damage in the rat prefrontal cortex. In Ozyurt's study [44], MK-801 was injected intraperitoneally at the dose of 0.5 mg/kg /day for 5 days. Within a few minutes after the intraperitoneal injection of MK-801, the rats showed an increased locomotor activity, a sniffing of the floor and walls, circling and head weaving which proved that MK-801 could induce severe ataxia (the inability to maintain body posture and body rolling). Another study [45] has shown that MK-801 (5 mg/kg *SConce*) rapidly induces a large and prolonged elevation in blood flow within the posterior cingulate and that occurs by the administration of a number of antioxidants with differing properties. In addition, MK-801-induced neuronal necrosis was attenuated without modulating regional blood flow.

A study has suggested that the dosage range of 0.3-10 mg/kg could improve functional recovery and reduces the histological damage following a global brain ischemia [46]. The dose of MK-801 that we used (0.025 mg/kg body wt. for 10 days) in the third group caused no overt behavioral changes according to Bondy's study [47], although the results showed no behavioral changes in the high dose group (0.25 mg/kg body wt. for 10 days) too. On the other hand, we can see that the anti-oxidant indicators decreased in the fourth group; maybe the reason for that is the too high concentration of MK-801 damaged the neurons. The results

were controversial with some studies, maybe because of differences in the indexes they observed and the time or frequency of application (before or after the insult, in minutes, hours, days, even weeks). According to our findings in the third and fourth group, let us just call the dose used in the third group "the safe dose." More work such as the histological changes of the brain need to be done in the future.

NF-erythroid 2-related factor 2 (Nrf2) is a newly discovered nuclear factor, which is found to play a critical role in some pathological reactions in recent years [10]. In a physiological state, it is mainly distributed in the cytoplasm and it forms a complex combining with cytoplasmic protein Keap1. When oxidative stress stimulus occurs, it is decoupled with the Keap1 through phosphorylation and then it will be transferred into a nucleus and then combines with the antioxidant response element (ARE) sequence to form the Keap1-Nrf2-ARE pathway and then starts the gene expression of the phase II detoxifying enzymes (HO-1, NQO1 etc.), and antioxidants regulated by the ARE [10]. At present, the roles of the Keap1-Nrf2-ARE pathway in stroke [48], in an acute lung injury [49], in a drug-induced liver [50], in an intestine injury [51], in a traumatic brain injury [52], and other aspects have been confirmed and further its role in the central nervous system has attracted increasing attention.

This work found that after common bile duct ligation the expressions of Nrf2 mRNA and Nrf2 protein, as well as the endogenous antioxidant enzymes HO-1 and NQO1 significantly increased ($P < 0.01$), which verified the existence of an anti-oxidative stress procedure in the central nervous system of obstructive jaundice in rats. After having been given MK-801 no matter whether the dose was low or high the Nrf2 mRNA, the Nrf2 protein, HO-1 and NQO1 increased which suggests that the MK-801 up-regulated anti-oxidative capacity of the brain through the Keap1-Nrf2-ARE pathway. So Nrf2 transcription may be the ideal treatment target for some diseases in theory [10] for it can induce the expression of phase II detoxifying enzymes, hemoglobin metabolism and the expression of antioxidant factors [53, 54] and at the same time, it can regulate multiple mechanisms of secondary brain injury.

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We also found that only the safe dose of MK-801 promotes the expression of Nrf2 mRNA and the Nrf2 protein as well as the anti-oxidants NQO1 and HO-1 through the Keap1-Nrf2-ARE signaling pathway in the central nervous system of obstructive jaundice in rats, to enhance the anti-oxidative ability. However, a high dose of MK-801 has no better effect on the low dose group. What is more, a high dose of MK-801 decreased some indexes in the anti-oxidant system in the central nervous system of obstructive jaundice in rats. Maybe its high concentration affected the anti-oxidant system because of its side-effects.

In conclusion, we have shown that safe doses of MK-801 protects against obstructive jaundice induced oxidative stress in the cerebral cortex through the Keap1-Nrf2-ARE pathway. Much progress has been made in understanding the pathophysiology and the pathology of obstructive jaundice. Our data provides the first experimental demonstration that safe dose of MK-801 decreases oxidative stress in the central nervous system of obstructive jaundice in rats. However, more studies need to be carried out in order to evaluate the role of the NMDA receptor in the process of obstructive jaundice induced oxidative stress.

Disclosure of conflict of interest

None.

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