

Ketamine ameliorates oxidative stress-induced apoptosis in experimental traumatic brain injury via the Nrf2 pathway

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Background: Ketamine can act as a multifunctional neuroprotective agent by inhibiting oxidative stress, cellular dysfunction, and apoptosis. Although it has been proven to be effective in various neurologic disorders, the mechanism of the treatment of traumatic brain injury (TBI) is not fully understood. The aim of this study was to investigate the neuroprotective function of ketamine in models of TBI and the potential role of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in this putative protective effect.

Materials and methods: Wild-type male mice were randomly assigned to five groups: Sham group, Sham + ketamine group, TBI group, TBI + vehicle group, and TBI + ketamine group. Marmarou's weight drop model in mice was used to induce TBI, after which either ketamine or vehicle was administered via intraperitoneal injection. After 24 h, the brain samples were collected for analysis.

Results: Ketamine significantly ameliorated secondary brain injury induced by TBI, including neurological deficits, brain water content, and neuronal apoptosis. In addition, the levels of malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were restored by the ketamine treatment. Western blotting and immunohistochemistry showed that ketamine significantly increased the level of Nrf2. Furthermore, administration of ketamine also induced the expression of Nrf2 pathway-related downstream factors, including hemoxygenase-1 and quinone oxidoreductase-1, at the pre- and post-transcriptional levels.

Conclusion: Ketamine exhibits neuroprotective effects by attenuating oxidative stress and apoptosis after TBI. Therefore, ketamine could be an effective therapeutic agent for the treatment of TBI.

Keywords: traumatic brain injury, ketamine, oxidative stress, Nrf2, apoptosis

Introduction

Traumatic brain injury (TBI) is defined as a mechanical injury that causes numerous deaths and has an adverse impact on families and society.^{1,2} Over the past few years, great efforts have been directed toward identifying effective ways to improve the prognosis of TBI; however, many approaches have failed during clinical trials.³ It is widely believed that TBI causes both primary and secondary brain damage. Although the primary brain injury is a major factor, the secondary brain damage precipitates a complex pathological process that can lead to a series of endogenous events, including oxidative stress, glutamate excitotoxicity, and the activation of inflammatory responses.⁴ Of these processes, oxidative stress plays the most important role in secondary damage,⁵ not only because of the excessive production of reactive oxygen species (ROS) but also due to the exhaustion of the endogenous antioxidant system.

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Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor which controls the redox state as the cell's main defense mechanism against oxidative stress.^{6,7} Nrf2 acts by activating the antioxidant response, which induces the transcription of a wide range of genes that are able to counteract the harmful effects of oxidative stress, thus restoring intracellular homeostasis.⁸ Under basal conditions, Nrf2 is localized in the cytoplasm, anchored by the Kelch-like ECH-associated protein (Keap1), and degraded by the ubiquitin-26S proteasome pathway, with a half-life of 20 min.⁹ Once the cell encounters stimulations, Nrf2 dissociates from Keap1 and is translocated into the nucleus, where it induces the expression of antioxidant enzymes. The enzymes regarded as antioxidants include nicotinamide adenine dinucleotide phosphate (NADPH), quinone oxidoreductase-1 (NQO-1), heme oxidase-1 (HO-1), superoxide dismutase (SOD), and glutathione peroxidase (GPx).^{10,11} These free radical scavenging enzymes form a powerful antioxidant defense mechanism.

Ketamine, an *N*-methyl-D-aspartate receptor antagonist, has shown potent anti-inflammatory effects in various models of systemic inflammation, including endotoxemia, ischemia, and burns.^{12,13} These effects were observed in multiple organ systems and involve the modulation of certain molecular mediators of the inflammatory response.¹⁴ In addition, when administered either before or after various pro-inflammatory responses, ketamine was found to diminish systemic production of cytokines and improve survival.¹⁵ In models of brain injury and ischemia, ketamine elicits neuroprotective effects.¹⁶ Moreover, recent evidence suggests that the use of ketamine could be beneficial for patients with TBI.¹⁷ Nevertheless, the effects of ketamine on TBI-induced oxidative stress in the brain remain to be fully elucidated, and the present study was performed to better examine these effects.

Materials and methods

Animals

Male Institute of Cancer Research (ICR) mice (Experimental Animal Centre of Fujian Medical University, Fujian, China), weighing 28–32 g, were used in this study. Mice were housed under a 12-h light/dark cycle, with free access to food and water, and were acclimatized for at least 1 week before the experiment. Experimental protocols were approved by the Animal Care and Use Committee of Fujian University and complied with the Guide for the Care and Use of Laboratory Animals by the National Institute of Health (NIH, Bethesda, MD, USA).

Models of TBI

The TBI model used in this study was a modified version of Marmarou's weight drop model.¹⁸ Mice were briefly

anesthetized with an intraperitoneal injection of 10% chloral hydrate and placed in a stereotaxic frame. Following a 1.5 cm midline scalp incision, the fascia was reflected to expose the skull. After locating the left anterior frontal area (the impact area), a 200 g weight was released onto the skull. During the operation, the dura was kept intact. The scalp incision was subsequently closed and sutured. The mice were then returned to their former cages. Sham animals underwent identical procedures, except the weight drop.

Experimental design

Mice (132 mice were used, 18 mice died) were randomly divided into five groups, namely Sham (n = 24), Sham + ketamine (n = 6), TBI (n = 24), TBI + vehicle (n = 24), TBI + ketamine (three subgroups: 30 [n = 6], 60 [n = 6], and 100 [n = 6] mg/kg). The mice in the TBI + ketamine groups were injected intraperitoneally with the respective dose of ketamine (Hengrui, Jiangsu, China), 30 min after TBI. At the corresponding time points, all mice in the TBI + vehicle group were administered equivalent volumes of vehicle solution. All mice were sacrificed 24 h after TBI.

Measurement of the brain water content

The brain water content was measured according to a previous study.¹⁹ Left brain cortical tissue samples were collected on sacrifice. The collected tissue was positioned directly over the injury site, covering the contusion and the penumbra. The fresh tissue was weighed to record the wet weight, and then dried for 72 h at 80°C and weighed to record the dry weight. The brain water content was calculated by the formula: [(wet weight – dry weight)/wet weight] × 100%.

Neurological evaluation

Neurological deficit was evaluated by the grip test, which was developed based on the test of gross vestibulomotor function, as described previously.²⁰ Briefly, mice were placed on a thin, horizontal metal wire (45 cm long) that was suspended between two vertical poles, 45 cm above a foam pad.

Every mouse was required to perform 10 different tasks that represented motor function, balance, and alertness. One point was given for failing to perform each of the tasks, and thus 0 = minimum deficit and 10 = maximum deficit. A lower score demonstrated less neurological deficits. The severity of injury is defined by the initial NSS, which is evaluated 1 h after TBI. Testing was performed by the investigator who was blinded to the experimental groups. The sequence of the behavioral tasks was randomized.

Nissl staining

For Nissl staining, the sections of paraffin-embedded brain tissue (5 μm thick) were stained by cresyl violet, according to a previous study.²⁰ “Normal” neurons had one or two large, round nuclei, located in the central soma, and abundant cytoplasm. In contrast, positive cells had irregular neuronal cell bodies, shrinking and hyperchromatic nuclei, and dried-up cytoplasm with vacuoles. The histological examination was performed by two observers who were blinded to the group assignment.

Western blot analysis

Protein extraction was performed according to the instructions provided by the manufacturer of the Protein Extraction Kit (Beyotime Biotech Inc, Nantong, China). Equal amounts of protein were loaded on a 10% or 12% sodium dodecyl sulfate–polyacrylamide gel and then transferred onto polyvinylidene fluoride membranes. The membranes were blocked for 2 h in 5% skimmed milk in TBST at room temperature. After that, the membranes were separately incubated overnight, at 4°C, with antibodies against Nrf2 (1:1,000 diluted, rabbit; Abcam, Cambridge, MA, USA), HO-1 (1:200, rabbit; Santa Cruz Biotechnology Inc., Dallas, TX, USA), and NQO-1 (1:1,000 diluted, rabbit; Abcam), Bax (1:400 diluted, rabbit; Abcam) and cleaved caspase-3 (1:1,000 diluted, rabbit; Cell Signaling Technology, Danvers, MA, USA), Bcl-2 (1:1,000 diluted, rabbit; Cell Signaling Technology), and β -actin (1:5,000 diluted, rabbit; Bioworld Technology, St Louis Park, MN, USA) in blocking buffer. Then, the membranes were washed with TBST for 15 min and were incubated with the secondary antibodies (1:5,000 diluted, goat; Bioworld Technology) for 2 h. After three 15-min washes with TBST, the protein bands were visualized by enhanced chemiluminescence (ECL) (EMD Millipore, Billerica, MA, USA) and exposure to X-ray film. All the results were analyzed with the Un-Scan-It 6.1 software (Silk Scientific Inc., Orem, UT, USA). The density of each band was separately quantified.

Immunohistochemical staining

Tissue sections (4 μm thick) were incubated overnight with the primary antibody against Nrf2 (1:100; Abcam) at 4°C. Following a 15-min wash in phosphate-buffered saline (PBS), the sections were incubated with horse radish peroxidase (HRP)-conjugated IgG (diluted 1:400, rabbit; Santa Cruz Biotechnology Inc.) for 1 h at room temperature. After three washes with PBS, the immunolabeled protein was visualized as brown staining, after staining with diaminobenzidine and the hematoxylin counterstaining. In each coronary section,

six randomly selected fields were observed under a light microscope (ECLIPSE E100; Nikon Corporation, Tokyo, Japan). Then, the number of positive neurons in each of the six fields was recorded, and the average number of positive neurons was calculated for each sample.

Malondialdehyde (MDA) content and the activities of SOD and GPx

The MDA content and the activities of SOD and GPx were measured with a spectrophotometer, using the appropriate kits (Nanjing Jiancheng Biochemistry Co, Nanjing, China), according to the manufacturer’s instructions. Total protein concentration was determined by the Bradford method. The MDA level and the activities of SOD and GPx were expressed as nmol/mg protein and U/mg protein, respectively.

Statistical analyses

The SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Data were expressed as mean \pm standard error of the mean (SEM), and the differences were evaluated by one-way analysis of variance (ANOVA), followed by Tukey’s test. Statistically significant differences were indicated by $P < 0.05$.

Results

Ketamine treatment provided protection in injured brains

The brain water content was also examined to confirm the protection of ketamine 24 h after TBI, as previously described (Figure 1A). The results showed that the TBI and TBI + vehicle groups had significantly increased brain water contents compared with the Sham group. Brain edema was attenuated in the ketamine-treated groups, which was consistent with the results of the neurological score. Specifically, the treatment with 60 mg/kg of ketamine conferred a better effect than the treatment with 30 mg/kg of ketamine. Accordingly, 60 mg/kg of ketamine was used in the subsequent experiments.

The neurological score was used to evaluate the motor function of the mice at 24 and 48 h after TBI (Figure 1B). All mice were trained 3 days pre-TBI. At 48 h after TBI, the motor scores of the TBI and TBI + vehicle groups were lower than the scores of the Sham and Sham + ketamine groups. The ketamine-treated group exhibited reduced neurological deficits compared with the TBI group at 24 h, which were also lower than the deficits of the Sham and Sham + ketamine groups. The group that received a higher dose (60 mg/kg) showed a better effect than the low dose (30 mg/kg) for the TBI + ketamine group at 24 and 48 h after TBI, but

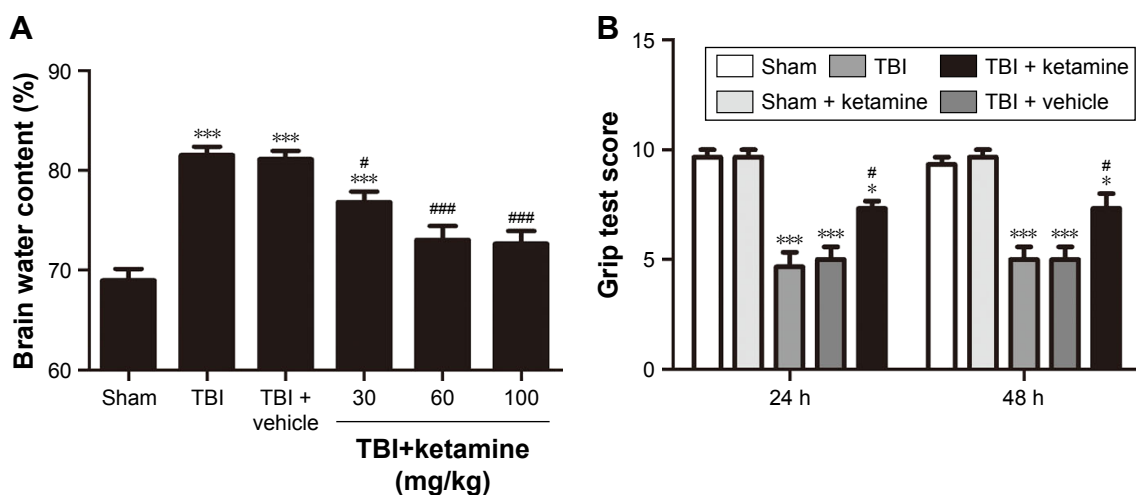


Figure 1 Ketamine protects against secondary brain injury in mice model of TBI.

Notes: Mice were subjected to TBI and then received 30, 60, and 100 mg/kg of ketamine or vehicle 30 min after TBI. The grip test was evaluated 24 and 48 h after injury, whereas the brain water content was examined at 24 hours after TBI. **(A)** The brain water content was examined at 24 h after TBI. **(B)** The grip test score was performed while brain water content was examined at 24 h and 48 h after TBI. Data represent mean \pm SEM. * $P < 0.05$, *** $P < 0.001$ vs the Sham group; # $P < 0.05$, ### $P < 0.001$ vs the TBI + vehicle group.

Abbreviations: SEM, standard error of the mean; TBI, traumatic brain injury.

the highest dose (100 mg/kg) failed to further enhance the neuroprotective effect. There was no difference between the Sham and Sham + ketamine groups. So, the Sham + ketamine group would be absent from the subsequent experiments.

Ketamine reduced neuronal apoptosis

To investigate the protective effects of ketamine, neuron morphology was examined by Nissl staining. While neurons in the Sham group were clear and intact (Figure 2A), multiple neurons in the TBI and TBI + vehicle groups were damaged, exhibiting extensive changes including oval or triangular nuclei and shrunken cytoplasm (Figure 2B and C). However, an improvement in neuronal morphology and cytoarchitecture was observed in TBI mice treated with ketamine (Figure 2D).

To investigate the mechanistic basis for the effects of ketamine, TUNEL staining was used to examine neural cells in injured brain tissue. TUNEL-positive cells were detected at a low frequency in the brains of mice in the Sham group (Figure 2E). The apoptotic index was increased in the TBI and TBI + vehicle groups compared to Sham animals (Figure 2F and G), but was reduced in the TBI + ketamine groups (Figure 2H–J). These results indicate that ketamine blocks neural apoptosis induced by TBI.

Additionally, Western blot analysis was performed to assess the expression of cleaved caspase-3, an indicator of apoptosis. Cleaved caspase-3 expression increased significantly following TBI, relative to the Sham group. However, the treatment with ketamine reduced the level of cleaved caspase-3, relative to the TBI + vehicle group (Figure 3A and B).

Expression of apoptotic factors was reduced by ketamine administration

The protective effects of ketamine against TBI-induced neuronal apoptosis were examined by Western blot analysis. These effects were reversed in TBI mice treated with ketamine, in which the translocation of Bax was inhibited. The expression of the pro-apoptotic factor Bax increased following TBI when compared with the Sham group (Figure 3A and D), whereas the expression of the anti-apoptotic factor Bcl-2 decreased when compared with the Sham group (Figure 3A and C). However, the treatment with 60 mg/kg of ketamine reversed the expression levels of Bax and Bcl-2 relative to the TBI + vehicle group at 24 h after TBI.

Ketamine reduced oxidative stress following TBI

To evaluate the effect of ketamine on oxidative stress induced by TBI, MDA level, activities of SOD and GPx, indicators of lipid peroxidation, and antioxidant levels, respectively, were assessed. The MDA level was increased in the TBI + vehicle group compared with the Sham group. This effect was mitigated by the administration of 60 mg/kg of ketamine (Figure 4A). The activities of GPx and SOD were both decreased after TBI, while ketamine treatment increased their activity (Figure 4B and C).

Ketamine promoted the expression of Nrf2 and Nrf2 downstream factors

The expression of Nrf2 was investigated by Western blot analysis and immunohistochemistry. Compared with the Sham group, both TBI and ketamine administration induced

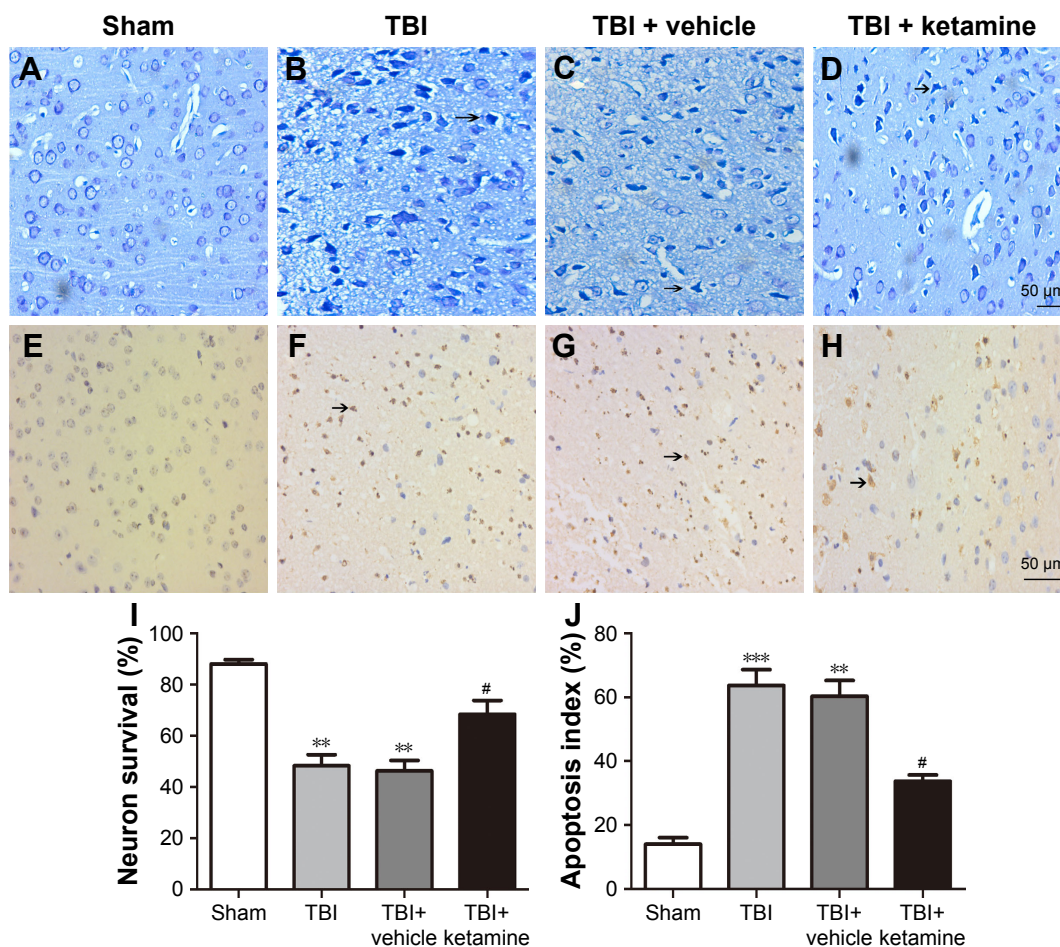


Figure 2 Ketamine suppresses neural apoptosis induced by TBI.

Notes: (A–D and I) Nissl staining of cortical tissue sections shows neuronal damage in mice subjected to TBI, which was alleviated by ketamine treatment (B, C, and D, arrows). (E–H and J) Apoptotic index was determined using TUNEL assays after TBI. Ketamine treatment significantly decreased the percentage of apoptotic cells after TBI (B, D, F–H arrows). Data are presented as mean \pm SEM. ** $P < 0.01$, *** $P < 0.001$ vs the Sham group; # $P < 0.05$ vs the TBI + vehicle group.

Abbreviations: SEM, standard error of the mean; TBI, traumatic brain injury.

Nrf2 expression (Figure 5A, B, and D–F). However, compared with the TBI + vehicle group, the TBI + ketamine groups exhibited significantly increased Nrf2 levels, which indicates that ketamine promoted the Nrf2 level following TBI (Figure 5C–F).

The expression of NQO-1 and HO-1 was also measured by Western blot analysis. At the protein level, NQO-1 and HO-1 were both upregulated after TBI (Figure 5E, G, and H). Additionally, administration of ketamine further enhanced protein expression compared with the vehicle. These results demonstrate that ketamine induced the expression of factors downstream of Nrf2 via activation of the Nrf2 and Antioxidant Responsive Element (Nrf2-ARE) signaling pathway (Figure 5E, G, and H).

Discussion

The secondary injury after TBI represents consecutive pathological processes that consist of excitotoxicity, activation

of inflammatory responses, oxidative stress, etc.²¹ Several oxidants and their derivatives are generated after TBI, which enhances the production of ROS along with the exhaustion of antioxidant defense enzymes, such as SOD, catalase, and GPx.²² Substantial evidence indicates that oxidative stress is a major contributor to the pathophysiology of TBI.²³ Thus, neuroprotective approaches must be aimed at limiting and reversing oxidative stress.

Ketamine is often used as a kind of anesthetic in clinical work and animal experiments. It appears to exert effects through not only NMDA receptors but also non-NMDA receptor mechanisms.²⁴ In a previous study, 10 or 50 mg/kg of ketamine was used as a subanaesthetic dose and 100 mg/kg of ketamine as an anesthetic dose.²⁵ The higher dose (60 mg/kg) showed a better effect than the lower dose (30 mg/kg) at 24 and 48 h after TBI, but the highest dose (100 mg/kg) failed to further enhance the neuroprotective effect. It demonstrated that the protective effect of ketamine was related to dosage

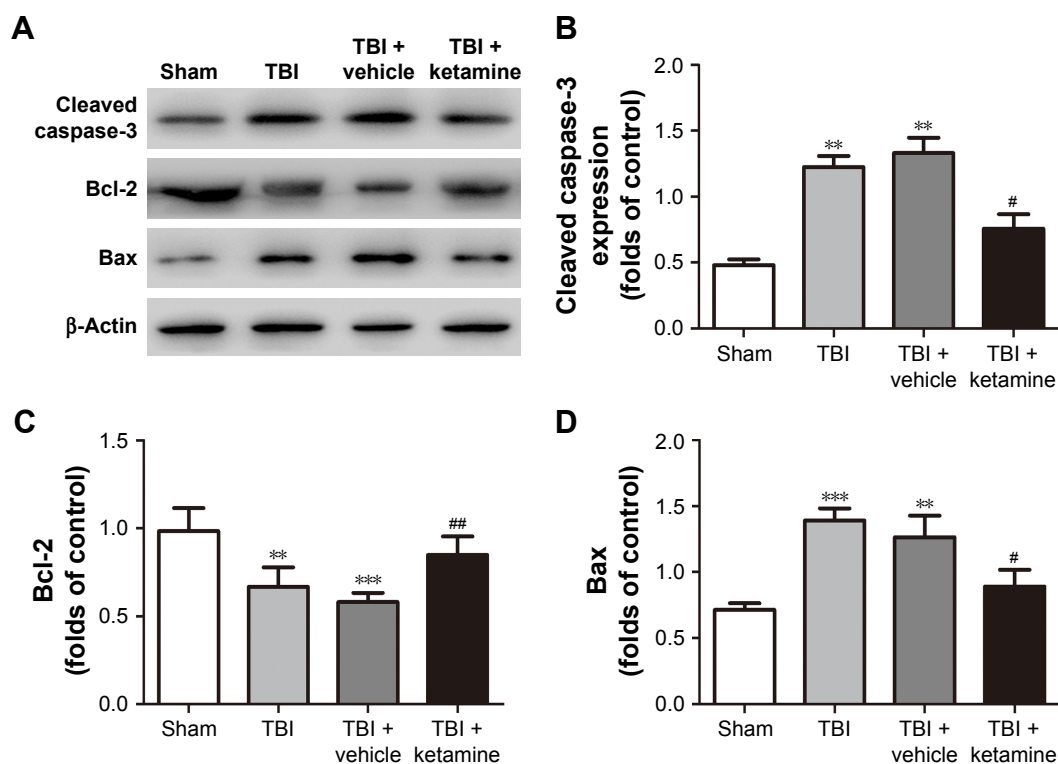


Figure 3 The effect of ketamine on neural apoptotic associated proteins after TBI in mice.

Notes: (A–D) The expression of cleaved caspase-3, Bcl-2, and Bax were evaluated by Western blot analysis. Data are presented as mean \pm SEM. ** $P < 0.01$, *** $P < 0.001$ vs the Sham group; # $P < 0.05$, ## $P < 0.01$ vs the TBI + vehicle group.

Abbreviations: SEM, standard error of the mean; TBI, traumatic brain injury.

in the case of subanaesthesia. Ketamine is a highly effective antioxidant that has been frequently used as a protectant in many kinds of traumatic injury studies although the effect is incompletely understood yet.^{26–28}

The present study showed that the treatment with ketamine after TBI attenuated brain contusion-induced oxidative insult, alleviated brain edema, improved neurological function scores, and prevented brain neuronal loss after TBI.

This beneficial role led us to consider the neuroprotective effects and the mechanisms underlying oxidative insult. The ability of ketamine to stimulate the activity of antioxidant enzymes plays a critical role in its antioxidative characteristics.²⁹ Lipid peroxidation, which refers to the oxidative degradation of lipids, increases membrane permeability, leading to cell damage. MDA has been utilized as an index of lipid peroxidation. In addition, both SOD and

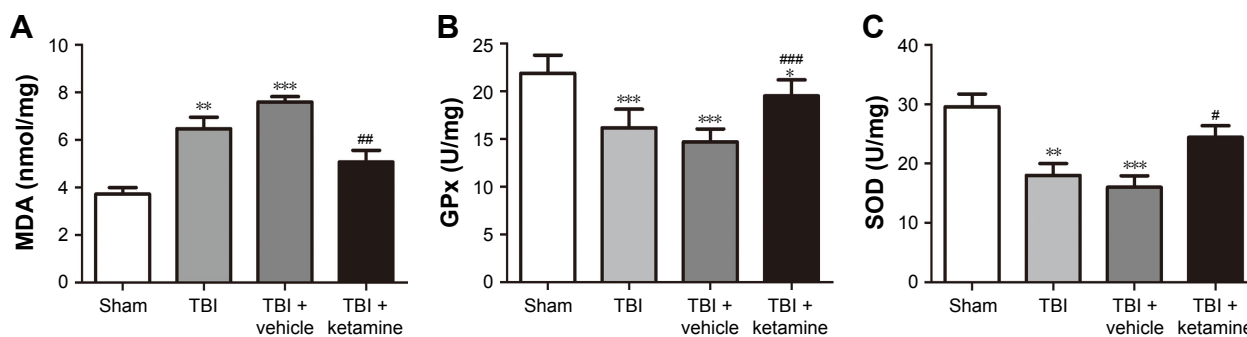


Figure 4 Ketamine attenuated oxidative stress caused by TBI.

Notes: (A) Measurements of MDA levels. (B and C) The activities of GPx and SOD. Data represent mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Sham group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs TBI + vehicle.

Abbreviations: GPx, glutathione peroxidase; MDA, malondialdehyde; SEM, standard error of the mean; SOD, superoxide dismutase; TBI, traumatic brain injury.

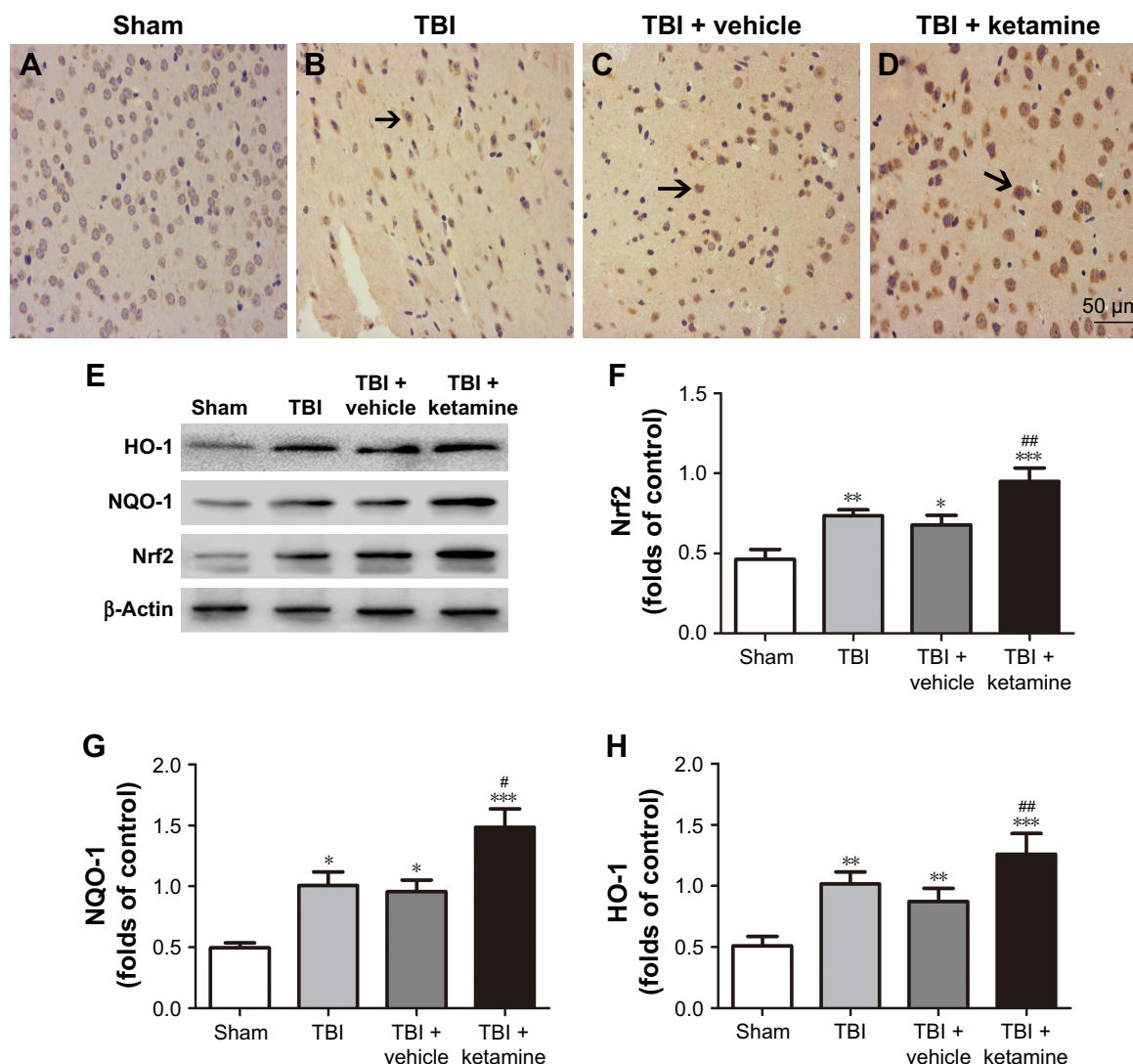


Figure 5 Ketamine promoted the expression of Nrf2 and its downstream factors in mice after TBI.

Notes: (A–D) The representative photomicrographs showing Nrf2 immunohistochemistry of tissues from different groups at 24 h after TBI. (E–H) All of the Nrf2, HO-1, and NQO-1 proteins were evaluated by Western blot analysis. Bars represent mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs Sham group; # $P < 0.05$ and ## $P < 0.01$ vs TBI + vehicle group. Expression of Nrf2 was described by the arrows in B, C and D.

Abbreviations: HO-1, heme oxidase-1; NQO-1, quinone oxidoreductase-1; Nrf2, nuclear factor erythroid 2-related factor 2; SEM, standard error of the mean; TBI, traumatic brain injury.

GPx are antioxidant enzymes that catalyze the reduction of glutathione.^{30,31} The conversion of MDA and the activities of SOD and GPx in the cortex of mice with TBI indicate that oxidative stress occurs following TBI. Apart from that, the results of this study also showed that the treatment with ketamine partly relieves this impact, suggesting that ketamine could attenuate TBI-induced oxidative stress.

The Nrf2 pathway plays an important role in cellular adaptation to oxidative stress by upregulating Phase II enzymes.^{3,32} In addition, previous studies indicated that Nrf2 protein as well as Phase II enzymes, such as NQO-1 and HO-1, are activated after TBI.^{33,34} The present study

demonstrated that exposure to ketamine (60 mg/kg) after TBI resulted in a significant increase in Nrf2 expression, as well as in the level of NQO-1 and HO-1, through the activation of the Nrf2 signaling pathway.

Conclusion

The present study demonstrated that ketamine attenuated the oxidative reaction by enhancing the expression and activity of antioxidant enzymes in a TBI mouse model. Furthermore, experimental data showed that ketamine exerted neuroprotection against TBI, by partially combating oxidative stress via the activation of the Nrf2 pathway and its downstream

proteins. However, further study is still needed to elucidate the underlying mechanism of Nrf2 pathway transformation after ketamine administration.

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Disclosure

The authors report no conflicts of interest in this work.

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