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Cerebrovascular Protective Effect of Boldine Against Neural Apoptosis via Inhibition of Mitochondrial Bax Translocation and Cytochrome C Release

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Background: In the present study, we explored the protective effect and mechanism of action of boldine (BOL) against neural apoptosis, which is a mediator of TBI.

Material/Methods: The effect of BOL on mitochondrial and cytosol proteins of extracted from cerebral cortical tissue of mice was evaluated. The grip test was used to assess the neurological deficit and brain water content of the subjects after administration of BOL to assess its effect on SOD, GSH, and MDA activity in brain ischemic tissues. To further confirm the effect of the BOL, the histopathological analysis and morphology of neurons were studied by Nissl staining. The effect of BOL against TBI-induced neural apoptosis by immuno-histochemistry and Western blotting assay were also studied.

Result: BOL showed significant improvement against TBI in a dose-dependent manner. In the BOL-treated group, the apoptotic index was significantly reduced, but the level of caspase-3 was greatly diminished. Additionally, the level of the Bax in mitochondria (mit) and cytosol was elevated in the TBI-treated group as compared to the sham group. Further BOL at the test dose causes significant reduction in the level of mitochondrial MDA together with increase in SOD activity as compared to the TBI alone group.

Conclusions: BOL showed a cerebroprotective effect against TBI by attenuating the oxidative stress and the mitochondrial apoptotic pathway. It also inhibited mitochondrial Bax translocation and cytochrome c release.

MeSH Keywords: **Apoptosis Inducing Factor • Cytochromes c • Malondialdehyde**

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Background

As a vital organ of the body, improper functioning of the brain because of any traumatic injury has very deleterious effects on the affected individual [1]. Survivors of traumatic brain injury (TBI) often have reduced physical, behavioral, and cognitive efficiency and must heavily rely on long-term health-care support [2,3]. According to recent estimates, TBI is a major cause of morbidity and mortality worldwide, affecting approximately 10 million people [4,5]. In the USA, an estimated 5 million people live with disability associated with TBI [6]. TBI causes neurodegeneration as a result of brain damage because of cellular impairment, necrosis, and apoptotic cellular death [7]. Despite recent advances and the well-established etiology of TBI, there is currently no effective treatment for TBI. The current therapeutic strategies target various pathways to regulate the deleterious effects of TBI [8]. Optimal control of reactive oxygen species (ROS) under stress condition is very beneficial because its impairment damages mitochondrial proteins, DNA, and lipids, disrupting cellular Ca^{2+} homeostasis, inducing apoptosis, and causing metabolic failure [9,10].

The use of the potent antioxidants to prevent the production of ROS and its effects be an effective approach [11]. In numerous studies, plants and plant-based products have been identified as excellent source of antioxidants and are helpful in counteracting the free-radical-based damage [12,13]. Among such plant-based compounds, boldine, which is chemically known as (S)-2,9-Dihydroxy-1,10-dimethoxy-aporphine, is a compound isolated from the boldo tree (*Peumus boldus* Molina, Monimiaceae) [14]. This plant is native to the Mediterranean climatic region of central Chile and extends into the northern half of the much rainier Chilean lake district. Earlier studies have reported the excellent antioxidant potential of boldine [15]; for instance, inhibition of nonenzymatic peroxidation of microsomal lipids and attenuation of CYP1A-dependent 7-ethoxyresorufin *O*-de-ethylase in a mouse hepatoma Hepa-1 cell line [16].

Concerning the utility of antioxidants in TBI and excellent free-radical scavenging activity of boldine, in the present study we investigated the protective effect and mechanism of action of boldine (BOL) against the neural apoptosis, a mediator of TBI.

Material and Methods

Animals

Male ICR mice weighing 25–30 g were selected and obtained from the animal house. They were housed in polypropylene cages and food and water were provided *ad libitum*. They were acclimatized in the laboratory condition for at least 6–7 days prior to the start any experiment. The study was approved by the Institutional Animal Ethics Committee.

Induction of traumatic brain injury

TBI was induced using the Marmarou weight-drop model, where the mice under anaesthesia were administered with choral hydrate intra-peritoneally. A longitudinal incision was made in the scalp to expose the skull and locate the anterior frontal area for impact. The pre-determined weight was dropped on the skull, and after the impact the wound was re-packed using sutures.

Experimental design

Mice were divided into 3 groups (12 in each group): a sham group, a TBI (no drug) group, and a TBI + boldine (BOL) group. The mice in the TBI + BOL group (10, 20, and 30 mg/kg) received BOL (Sigma-Aldrich, USA) i.p. 30 min after TBI. The mice were euthanized 24 h after TBI.

Isolation of mitochondria

Briefly, the proteins of mitochondria and cytosol were extracted from left cerebral cortical tissue using a mitochondria isolation kit. Freshly obtained tissue samples were homogenized in ice-cold homogenization buffer and centrifuged to isolate the nuclear fraction in the supernatant. The resulting supernatants were further centrifuged to isolate the mitochondria and cytoplasmic proteins. The brain tissue was weighed, homogenized, and centrifuged at 12 000 *g* for 15 min at 4°C. Finally, the protein content of each sample was determined using a protein assay kit.

Neurological deficit

The grip test was used to assess the neurological deficit. Mice were placed on a special device consisting of thin metal wire tied between 2 vertical poles. The scoring was given based on the following behavior.

| Score | Behavior |
|-------|---|
| 0 | Unable to hold on to the wire for more than 30 s |
| 1 | Unable to hold the wire with both forepaws and hindpaws together |
| 2 | Held the wire with cooperation of forepaws and hindpaws but not the tail |
| 3 | Held the wire with the tail as well as with both forepaws and both hindpaws |
| 4 | Moved along the wire on all 4 paws plus tail |
| 5 | Scored 4 points but also ambulated down one of the posts used to support the wire |

The grip test was performed 3 times, and a total value was calculated for each mouse.

Assessment of brain water content

The brainstem and cerebellum were removed from the brain of the anesthetized subject. The wet weight (ww) of left and right hemispheres were noted and then dried at 80°C for 72 h to determine the dry weight (dw). The water content was calculated as a percentage using the formula: $(ww-dw)/ww \times 100\%$.

Nissl staining

Coronal sections of brain tissue (5- μ m-thick) were stained with cresyl violet. The normal neurons had large cell bodies and cytoplasmic volume, with large, round nuclei. Damaged cells had emaciated cell bodies, compressed nuclei, and dark cytoplasm, including many empty vesicles. The histological examination was performed by an observer blinded to the group assignment.

Western blot analysis

Mitochondrial, nuclear, and cytosolic proteins were extracted from the cerebral cortex and quantified. Cerebral cortex nuclear protein samples were subjected to electrophoresis on 10–15% SDS-PAGE for 45 min at 80 V, followed by 100 min at 100 V, and then transferred onto nitrocellulose for 1 h at 100 V. The membrane was blocked with 5% skimmed milk for 2 h at room temperature and then incubated with primary antibodies overnight at 4°C. The antibodies used were H3 at 1: 1000 dilution (Cell Signaling Technology, Danvers, MA, USA), cytochrome c at 1: 5000 dilution (Abcam, Cambridge, MA, USA), Bax at 1: 400 (Abcam, Cambridge, MA, USA), COX IV at 1: 1000 (Cell Signaling Technology) and cleaved caspase-3 at 1: 1000 (Cell Signaling Technology). Subsequently, the membranes were incubated for 2 h with corresponding secondary antibodies.

Immunohistochemistry

Briefly, the brain tissue samples were fixed in formalin for 24 h and then entrenched in paraffin. Sections were cut at 5- μ m thickness, deparaffinized in xylene, dehydrated in a graded series of ethanol, and subjected to antigen retrieval in citrate buffer (pH 6.0) for 30 min in a 37°C chamber. The sections were quenched in 3% H₂O₂ and blocked with PBS containing 10% goat serum (Sigma-Aldrich) for 1 h at 37°C and then incubated with rabbit monoclonal anti-caspase-3 antibody at 1: 300 (Cell Signaling Technology) overnight at 4°C, followed by 3 washes (for 15 min each) in PBS and incubation with horseradish peroxidase-conjugated IgG at 1: 500 dilution (Bioworld Technology, Inc., St. Louis Park, MN, USA) for 60 min at room temperature. Sections were then stained with hematoxylin, dehydrated, and cleared with xylene before mounting. The control tissue received similar treatment but without primary antibody.

Estimation of superoxide dismutase, malondialdehyde, and glutathione concentration

The activity of superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) activity in the brain ischemic tissues were estimated following instructions provided with assay kits obtained from Nanjing Jiancheng Bioengineering Institute, China.

Histopathological analysis

Brains of rats were detached and immobilized in 4% PBS-buffered formaldehyde for 48 h. Thereafter, a 4-mm coronal section of the brain was cut 2.0 mm anterior and posterior to the bregma and the block was implanted in paraffin. The block was then cut into 5- μ m coronal sections that were stained with hematoxylin-eosin (HE) using standard methods.

Statistical analysis

All results are reported as mean \pm SD of 3 independent experiments. One-way analysis of variance (ANOVA) followed by Bonferroni test for multiple groups or Student's *t* test between 2 groups were used for statistical analysis, with $P < 0.05$ considered to be significant.

Results

BOL affects neurological function and alleviates cerebral edema

The initial part of the study was intended to elucidate the cerebroprotective effect of BOL after TBI. Thus, to understand its pharmacological effect, we formed a group of animals based on the treatment they received: Group I (control), Group II (TBI), and Group III (TBI + BOL). The latter group was further classified into 3 sub-groups receiving 10 mg/kg, 20 mg/kg, and 30 mg/kg of BOL.

The mice in the experimental groups were trained on the test task just 1 day before the TBI insult. The control (sham) group showed no significant changes during the study. As shown in Figure 1A, mice in all experimental groups showed improvement in motor coordination over time. Moreover, the group treated with BOL showed significantly more improvement than the TBI alone group, and the effect was dose-dependent. The next phase of the study was concentrated for the determination of the effect of BOL on a broader scale. For this, the water content of the brain of animals was measured at 24 h after the insult. As presented in Figure 1B, the result showed that the TBI alone group had a considerable increase in the water content compared to the control group, but the groups receiving BOL

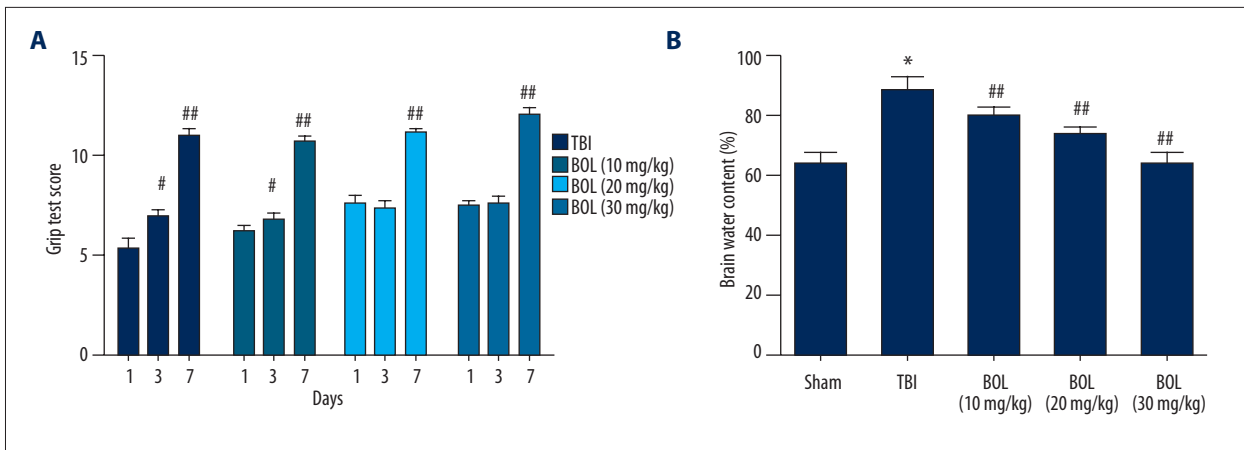


Figure 1. Effect of BOL on the neurological function of treated and un-treated group. Data are presented as mean \pm SEM. * $p < 0.05$ vs. sham group; # $p < 0.05$; ## $p < 0.01$ vs. TBI alone group.

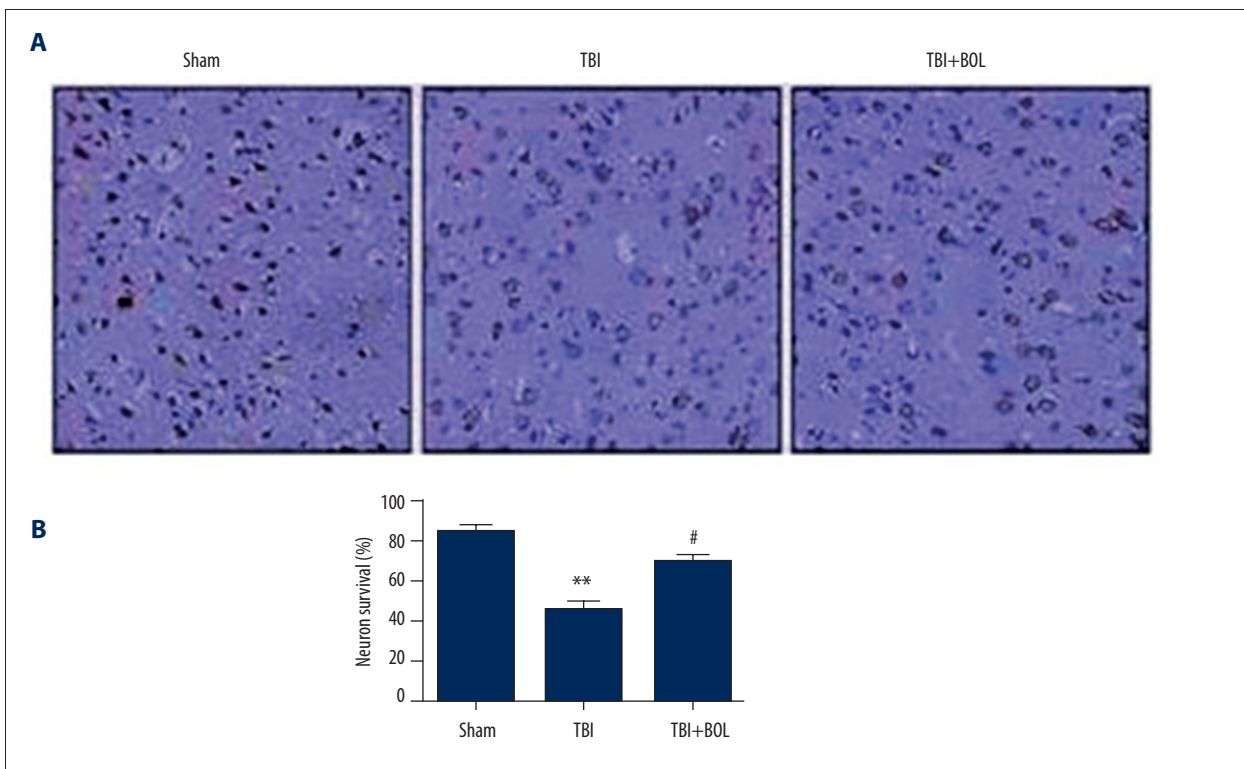


Figure 2. Effect of BOL (30 mg/kg) on the neuronal apoptosis followed by TBI insult. (A) Nissl staining of the cortical tissue under different treatments; (B) Quantitative scoring of neuron survival. Data are presented as mean \pm SEM. ** $p < 0.01$ vs. sham group; # $p < 0.05$ vs. TBI alone group.

showed significant decreases in water content as compared to the TBI group, with a maximum at 30 mg/kg, and the effect was dose-dependent. These results suggest that BOL at all the tested doses showed considerable neuroprotection, with maximum effect at 30 mg/kg. Therefore, the 30 mg/kg dose was selected to perform subsequent experiments.

Effect of BOL on neuronal apoptosis

To further confirm the effect of the BOL in a more precise way, the next part of the study was aimed at determination of the effect of BOL on the histopathological level and morphology of neurons by Nissl staining. As shown in Figure 2, the neurons of the sham group were clear and not distorted, but, in the TBI group the morphology of neurons was damaged,

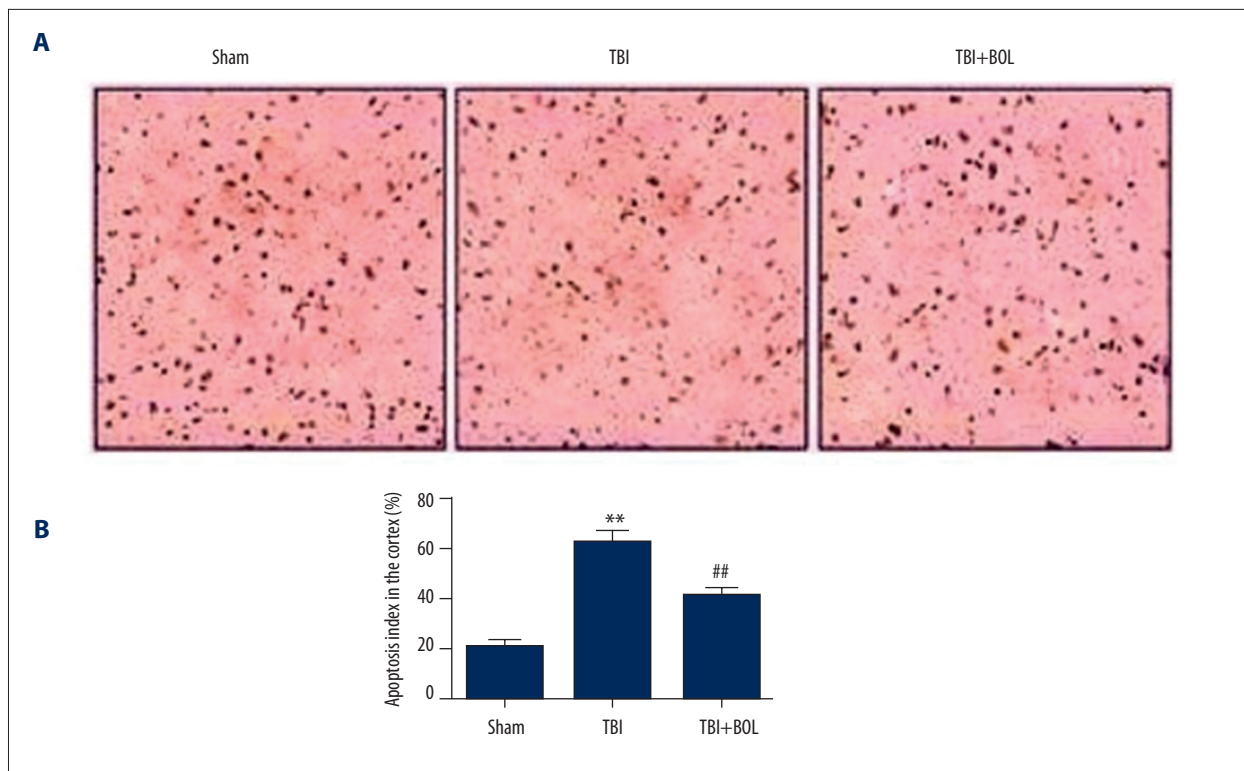


Figure 3. Effect of BOL (30 mg/kg) on apoptotic index as determined by TUNEL assay. (A) Immunohistochemistry of the apoptotic cells; (B) Apoptosis index in the cortex (%). Data are presented as mean \pm SEM; ** $p < 0.01$, vs. sham; ## $p < 0.01$ vs. TBI alone group.

showing broad changes in the cellular arrangements with dis-morphed nuclei, emaciated cytoplasm, and inflamed cellular bodies. These changes were reversed to near normal in a significant manner in the BOL-treated group.

TUNEL staining was further used to elucidate the effect of BOL on neural cells in the brain after the insult. As shown in Figure 3, there were fewer TUNEL-positive cells in brain slices of the sham group. As expected, the TBI alone group showed significantly higher apoptotic index in comparison to the sham group. In the BOL-treated group, the apoptotic index was significantly reduced, suggesting it reduces cellular apoptosis and protects against TBI.

Effect of BOL on proapoptotic factors

The next part of the study was intended to determine the effect of BOL against TBI-induced neural apoptosis by immunohistochemistry and Western blotting assay. As shown in Figure 4, in the TBI alone group the expression of caspase-3 was elevated, as confirmed by Western blotting, but in the BOL-treated mice, the level of caspase-3 was greatly diminished. Additionally, the level of the Bax mitochondria (mit) and cytosol was elevated in the TBI-treated group as compared to the sham group, together with reduction in mitochondrial and cytosolic cytochrome c levels as compared to sham subjects. The BOL-treated mice showed reversal of these changes, in which the release of cytochrome c

from cytosol was inhibited, together with inhibition of mitochondrial translocation. The last phase of the study was aimed at elucidating the effect of BOL on antioxidant activity. As shown in Figure 6, BOL at the test dose caused significant reduction in the level of mitochondrial MDA, together with increase in SOD activity as compared to the TBI alone group.

Discussion

The present study was conducted to explore the beneficial effect of BOL against neuronal apoptosis to provide cerebroprotective effects. The results of the current investigation show that BOL improves cerebral edema and neurological function, and inhibited apoptosis [17]. BOL at the test dose attenuated the oxidative stress induced by TBI and attenuated the mitochondrial apoptotic pathway after TBI insult.

Previous results showed that boldine prevents both enzymatic- and nonenzymatic-mediated destruction of biological systems [18]. Particularly, in *in vitro* assay, it causes inhibition of the free-radical induced beginning and proliferation of peroxidative harm in a variety of membranes, such as liver homogenates, hepatic microsomes, and ghost erythrocytes [19]. Moreover, it also blocked the free-radical induced lysis of red blood cells and intact hepatocytes [20]. Boldine has also showed

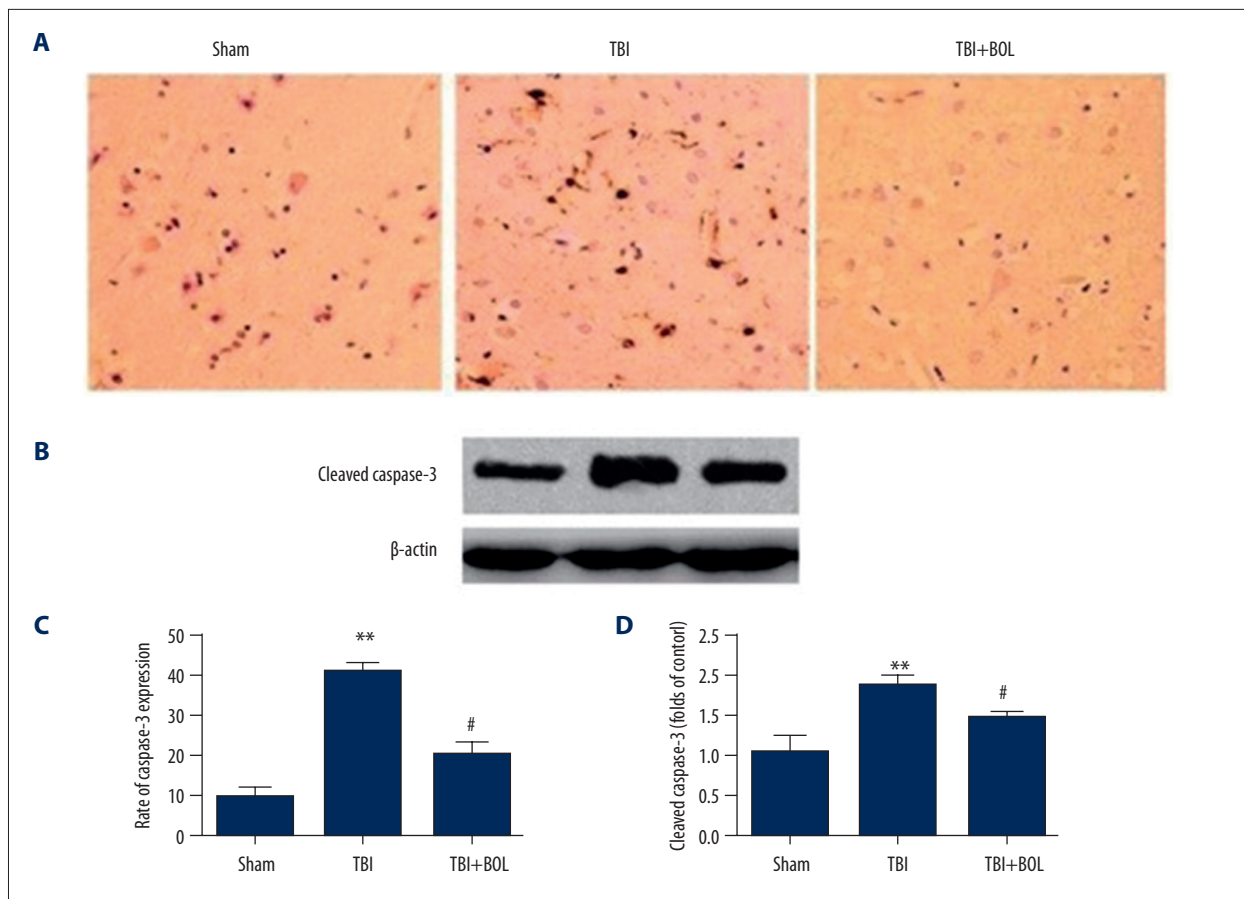


Figure 4. Effect of BOL (30 mg/kg) as determined by Western blot analysis of the (A) expression of caspase-3 following TBI. (B) and (C) cleaved caspase-3 expression in cortical neural cells. Data represent the mean \pm SEM. ** $p < 0.01$ vs. sham; # $p < 0.05$ vs. TBI alone group.

excellent anti-inflammatory [21], hepatoprotective [22], anti-trypansomal [23], and cytotoxic activities [24]. However, its effect on traumatic brain injury (TBI) has not previously been elucidated.

Despite significant advances in medicine, there is no effect drug or strategy that can circumvent the damage caused by TBI [4]. Various studies have emphasized that no single strategy will be able to counteract this damage because of the multifactorial etiology of TBI [25]. It has been found that the brain undergoes secondary damage originating from the oxidative stress, metabolic dysfunction, and inflammatory response following traumatic injury [26]. TBI leads to the production of free-radicals, which induces lipid peroxidation and DNA damage [27]. The extent of the lipid peroxidation is typically correlated with the level of MDA activity in the brain. Immediately after brain injury, the MDA level was found to be elevated and remain in that condition for 48 h after the insult. The level of oxidative stress is also determined with the help of SOD activity, an antioxidant enzyme [28]. In the present study, BOL causes significant decrease in MDA activity with elevation of

SOD activity, suggesting its antioxidant behavior helps overcome the damage caused by TBI. Growing evidence suggests the role of mitochondrial disturbances in neuronal apoptosis in TBI. In neuronal apoptosis, the mitochondrial cascade plays a significant role in cytochrome c release [29]. Moreover, cellular death (e.g., exo-cytotoxicity) and oxidative stress have significant effects on mitochondrial function. Thus, the current therapeutic strategies are aimed at modulating and limiting mitochondrial dysfunction.

Particularly, the mitochondria-mediated apoptosis involves Bax (a Bcl-2 family member), which forms a channel that facilitates permeability of cytochrome c from the mitochondrial intermembrane [30]. Thus, its release from the inner space leads to the cascade of events, including the activation of caspases (e.g., caspase-3) which terminates in apoptosis [31]. In the present study, we have shown that Bax is easily translocated to the mitochondrial membrane and the release of cytochrome c in cytosol is increased after TBI, suggesting activation of the mitochondrial apoptotic pathway. Our results show that BOL protects neurons by inhibition of the mitochondrial apoptotic pathway.

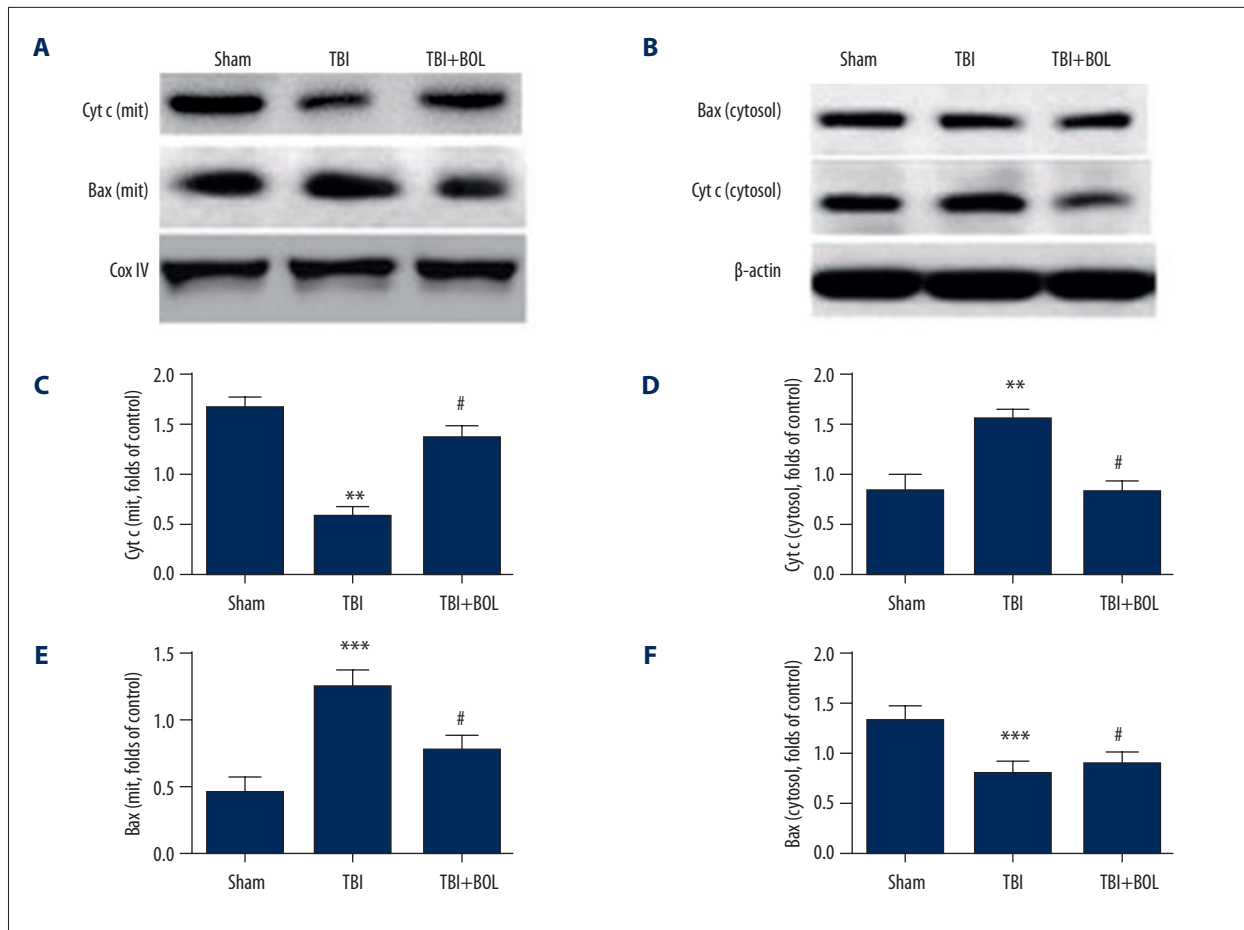


Figure 5. Effect of BOL (30 mg/kg) on proapoptotic protein expression as determined by Western blot analysis. (A, B) the expression of Bax and cytochrome c (Cyt c) in the ipsilateral cortex, respectively; (C–F) relative expression of Bax and cytochrome in mitochondria (mit) and cytosol. Data represent the mean \pm SEM. ** $p < 0.01$; *** $p < 0.001$ vs. sham; # $p < 0.05$ vs. TBI alone group.

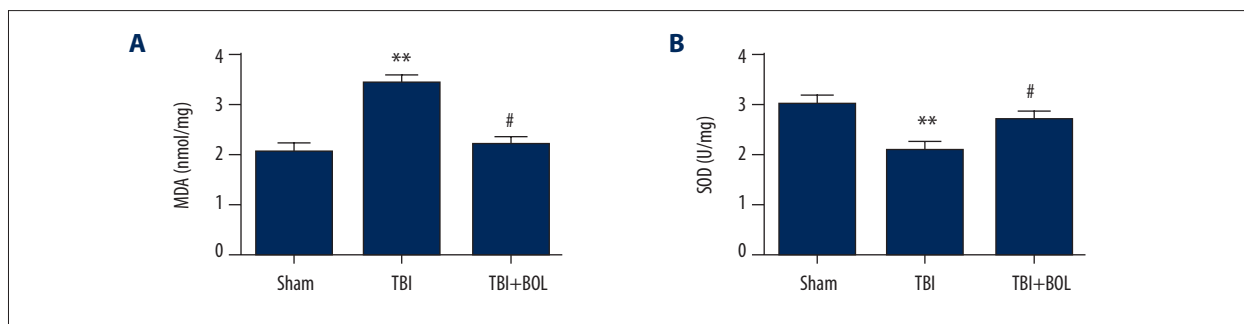


Figure 6. Effect of BOL (30 mg/kg) on oxidative stress. (A) MDA activity; (B) SOD level. Data represent the mean \pm SEM. ** $p < 0.01$; *** $p < 0.001$ vs. sham; # $p < 0.05$ vs. TBI alone group.

Conclusions

Boldine has cerebroprotective effect against TBI via attenuating oxidative stress and mitochondrial apoptotic pathway by inhibiting mitochondrial Bax translocation and cytochrome c release. Boldine has potential in mitigating brain damage after traumatic brain injury.

Conflict of interest

The authors have declared no conflict of interest.

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