Boeravinone B Protects Brain against Cerebral Ischemia Reperfusion Injury in Rats: Possible Role of Anti-inflammatory and Antioxidant
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Abstract: It is well known that inflammatory reactions and oxidative stress play a key role in the pathogenesis of cerebral ischemia and secondary injury. Boeravinone B (BB) proofed their anti-inflammatory and antioxidant effect, but their neuroprotective effects still unknown. In this experimental study, we explore the neuro-protective effect of Boeravinone B on the ischemia/reperfusion and explore the possible mechanism. Male Wistar rats were used for the current experimental study. First induces natural I/R injury in rats and treated with BB and nifedipine, respectively. Rats were subjected to ischemia after 6 consecutive days by occlusion of the bilateral common carotid arteries (BCCAO). Neurological score, biochemical, antioxidant, pro-inflammatory cytokines and inflammatory parameters were estimated in the serum and brain tissue. BB treatment significantly (p < 0.001) suppressed neuronal injury, dose-dependently decreased the cerebral water content. BB treatment altered the pro-inflammatory cytokines, antioxidant and inflammatory mediators in the serum and brain tissue. BB regulated the expression of glycine (Gly), glutamic acid (Glu), taurine (Tau), aspartic acid (Asp) and γ-aminobutyric acid (GABA) and enhanced the activity of Na$^+$, K$^+$ ATPase and Ca$^{2+}$ ATPase. BB significantly (p < 0.001) reduced antioxidant enzymes such as glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), malondialdehyde (MDA), glutathione reductase (GR); inflammatory cytokines include interleukin-4 (IL-4), interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), interleukin-6 (IL-6) and interleukin-1β (IL-1β); inflammatory mediators include prostaglandin (PGE$_2$), nuclear kappa factor B (NF-κB) and cyclooxygenase-2 (COX-2), respectively. In this study, we have found that Boeravinone B exhibited protection against cerebral I/R by reducing oxidative stress and inflammatory reaction.

Key words: cerebral Ischemia reperfusion, Boeravinone B, neuroprotection, inflammation, oxidative stress, BCCAO

1 Introduction

Cerebral ischemic stroke (CIS) has been confirmed to be the third leading cause of death worldwide, the leading cause of disability. Cerebral ischemic stroke is linked with the high incidence of mortality and morbidity$^{1,2}$. CIS boosted evidence leads to neurological deficits such as impairment of memory and learning. In the same region of the brain, CIS is often caused by blockage or suppression of blood flow, which is induced by vascular reflow after organ transplantation, contraction, precutaneous transluminal coronary angioplasty, etc$^{3,4}$. Vascular recanalization is often used to restore blood flow, according to clinical reports; however, this reperfusion can cause more brain damage and this procedure is called the injury of ischemia/reperfusion (I/R)$^{4,5}$. I/R injury caused much damage in the cerebral microvasculature and boost the production of oxygen free radicals, endothelial cell damage and mast cell degranulation$^{6,7}$. Thrombolytic treatment is considered

Abbreviations: BB: Boeravinone B, BCCAO: Bilateral common carotid arteries, Gly; Glycine, Glu; Glutamic acid, Tau; Taurine, Asp; Aspartic acid, GABA; γ-aminobutyric acid, GSH; Glutathione, GPx; Glutathione peroxidase, CAT; Catalase, MDA; Malondialdehyde, GR; Glutathione reductase, IL-4: Interleukin-4, IL-1: Interleukin-1, TNF-α: Tumor necrosis factor-α, IL-10: Interleukin-10, IL-6: Interleukin-6, IL-1β: Interleukin-1β, PGE$_2$: Prostaglandin E$_2$, NF-κB: Nuclear kappa factor B, COX-2: Cyclooxygenase-2, CIS: Cerebral ischemic stroke, I/R: Ischemia/reperfusion, BBB: Blood brain barrier, ROS: Reactive oxygen species, CMC: Carboxy methylcellulose, AMPA: α-amino-3-hydroxy-methyl-4-isoxazole propionic, NMDA: N-methyl-D-aspartic acid
the most effective treatment for stroke, but this treatment having limitation due to small percentage of patients because strict exclusion criteria and narrow range of therapeutic time. Complex sequences are involved in the pathophysiology of stroke\(^\text{5,7}\). Due to the limitation of treatment, and urgent need of the more effective treatment.

Clinically, ischemic brain edema is commonly estimated for the determination of ischemic stroke features. Na\(^+\), K\(^+\), ATPase dysfunction in the cell membrane induced by excessive influx of water and Na\(^+\), finally cause the edema in the nerve cell\(^\text{8}\). Clinically, the induction of ischemic brain edema is significant membrane depolarization attributed to metabolic failure induced via ischemia and then there was the induction of Ca\(^{2+}\) influx by voltage-sensitive Ca\(^{2+}\) channels and increases the amino acid neurotransmitters, particularly aspartic acid and glutamate, secreted from the synaptic cleft, which induces the reaction that causes the cell death\(^\text{9,10}\).

During the CIS, blood brain barrier (BBB) breakdown and start the induction of brain edema. An inflammatory reaction exhibited an essential role in the induction of cerebral ischemia brain injury\(^\text{11,12}\). This reaction further leads to release of several pro-inflammatory cytokines and inflammatory mediators in the ischemic region via immune cell activation, that further boosted the additional inflammatory reactions, including activation of pro-inflammatory genes involves IL-1, IL-4, TNF-\(\alpha\), IL-6, IL-10 and IL-1\(\beta\); inflammatory mediators include PGE\(_2\), NF-\(\kappa\)B and COX-2, respectively\(^\text{13-15}\). Due to the activation of the above reaction and molecules, they boost the accumulation of neutrophils, macrophages and microglia activation; they boost infiltration in the ischemia area and lead to injury in the brain tissue and neuronal cells. Moreover, pharmacological improving the inflammatory injury was considered the best approach to treat stroke\(^\text{13,14}\).

Oxidative stress is considered a significant factor that is closely related to ischemic stroke pathogenesis. Post reperfusion induces prominent oxidation of lesions occurs due to generated reactive oxygen species (ROS), which further lead to dysfunction of neural, boost inflammatory reactions, induces oxidative stress and finally cause cell death\(^\text{16,17}\). Previous research has shown that excitotoxicity and oxidative damage play a major role in cerebral I/R pathogenesis\(^\text{18,19}\). Oxidative stress is an imbalance condition between the endogenous antioxidant and pro-oxidant. During the CIS, excess generation of ROS start from the mitochondria and boosted the chains of lipid peroxidation, which further take part in the damaging neuronal\(^\text{20}\). Cerebral ischemia reperfusion cause the oxidative stress, which is categorized via reduced GPx, CAT, GSH, SOD and boosted the level of MDA in the tissue and serum\(^\text{2,21}\). On the basis of above facts, we can say that potential antioxidant and anti-inflammatory drugs play a significant and preventive role in treating ischemic stroke.

2 Methods
2.1 Chemical
Boeravinone B (98%) and Nimodipine were procured from Sigma Aldrich (St. Louis, USA).

2.2 Experimental animals
Swiss Albino Wistar rats (sex-male; weight 200–240 g, clean grade) were used for the current protocol. The rats were kept from the Institutional animal house in the standard laboratory experimental condition. The rats were kept in the 12/12 h light/dark cycle with temperature (22 ± 2°C) and relative humidity (65 ± 5%). The rats were access to water and food. The whole protocol was conducted in accordance with the Guide for the Care and Use of Laboratory rodent and related to ethics regulations of our institution.

2.3 Experimental protocol
Before the experimental protocol, the rats were acclimated for 7 days to adopt the laboratory conditions. After the intragastric administration, the rats were anesthetized with 350 mg/kg, b.w. intraperitoneal injection of choral hydrate. After successfully anesthetized rats, bilateral common carotid arteries (BCCAO) were separated from the sympathetic nerves and vagos via ventral cervical incision (on the 7th day)\(^\text{4}\). For inducing cerebral ischemia, 2 bilateral common carotid arteries were ligated with the thread (30 min) and left for 15 min (performed 2 times). Normal control rats, isolated the bilateral common carotid arteries without induction the occlusion. After the surgery, the rats were kept on the heating plate (37 ± 0.5°C) to maintain the body temperature.

2.4 Drug preparation
Boeravinone B (BB) (tested drug) and nimodipine (standard drug) were dissolved in the carboxy methylcellulose (CMC; 0.25%) and every time freshly prepared for administration to the animals.

2.5 Drug treatment
The rats were divided into the following groups after successful surgery and each group includes the 14 rats in each group presented in Table 1. In the group rats were anesthetized at complete the experimental study (after 21 h reperfusion).

2.6 Cerebral water content and cerebral index
All group rats were anesthetized using choral hydrate (10%) and sacrificed by cervical decapitation for the behavioral tests performed. The cerebrum was separated from the skull and the frontal pole (2 mm thick) was removed and the wet weight of the cerebral hemispheres was measured\(^\text{4}\).
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Table 1 The group treatment.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>Group name</th>
<th>Dose</th>
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<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>Normal group</td>
<td>CMC</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>BCCAO Control</td>
<td>CMC</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>BCCAO + BB</td>
<td>1.25 mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>BCCAO + BB</td>
<td>2.5 mg/kg</td>
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<td>5</td>
<td>V</td>
<td>BCCAO + BB</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>6</td>
<td>VI</td>
<td>BCCAO + Nimodipine</td>
<td>.3 mg/kg</td>
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Cerebral index = \( \frac{\text{Wet weight}}{\text{Body weight}} \times 100 \)

After that, using ice cold water to acquire homogenates (10 percent w/v), the remaining left hemisphere portion was homogenized and held at \(-80^\circ\)C. After that, tinfoil and final weight were covered with the right hemisphere segment using the balance to achieve wet weight and dry weight estimation(tissue was dried at 100°C for 24 h).

The cerebral water content was presented using the below formula\(^{20}\)

\[
\text{Cerebral water content (\%) = } \frac{\text{Wet weight} - \text{Dry weight}}{\text{Body weight}} \times 100
\]

2.7 Morris water maze test

Morris water maze analysis has been widely used to assess memory and learning efficiency\(^{20}\). Briefly, during the secret mission, the hidden platform(1 cm) was submerged under the water surface in the maze apparatus and preserved the water temperature(25 ± 2°C) and rendered opaque using non-toxic white paint. For the current experiment, the position of the secret platform was set for all test series. Randomly chosen were the acquisition process of all groups carried out for four trials per day and the starting position of each path. An average of 4 trials is calculated and assessed for the estimation of escape latency \(\text{(time taken to hit the hidden platform)}\) \(\text{estimation of learning performance}\). Each group of rats was allowed to swim freely(60 s) in the maze to find the hidden platform on day 1 for rat training. The rats were trained to search the platform from day 2 onwards\(\text{(hidden)}\). If the rats on the platform\(\text{(within 60 s)}\) were reached and allowed to remain on the platform\(\text{(10 s)}\). If the rats were unable to locate the hidden platform, they were taught to find the platform manually and allowed to remain on the platform for 10 seconds. We maintain the minimum 2 h recovery times between the 2 trials when the trial is performed. The secret platform was immediately removed from the spatial probe experiment, enabling the rats to swim\(\text{(60 s)}\) to find the target spot\(^{20}\).

2.8 Sample preparation

For the determination of biochemical parameters in the serum and brain tissue of the rats\(^{20}\). In the pre-incubated test tube, the rats were anesthetized and blood was extracted from the retro-orbital plexus and centrifuged for 10 min at 10 g rpm to isolate the supernatant. For, the brain tissue, brain tissue homogenate was kept in cold physiological saline (900 µL).

2.9 Na\(^{+}\), Ca\(^{2+}\), K\(^{+}\)-ATPase

Na\(^{+}\), Ca\(^{2+}\), K\(^{+}\)-ATPase was determined using the appropriate detection kits following the manufacture protocol (Abcam).

2.10 Antioxidant enzymes

GPx, GSH, GR, SOD, GSH and MDA were estimated using the appropriate detection kits following the manufacture protocol (Abcam).

2.11 Pro-inflammatory cytokines

ELISA kits (eBiosciences) were used for estimating pro-inflammatory cytokines such as IL-1, IL-4, TNF-α, IL-6, IL-10 and IL-1β following the manufacture instruction (eBiosciences).

2.12 Inflammatory mediators

ELISA kits (eBiosciences) were used for estimating inflammatory parameters, including PGE\(_2\), NF-κB and COX-2 following the manufacture instruction.

2.13 Statistical analysis

Graph Pad prism 7 (USA) was used for the static analysis. All the result presented as mean ± SEM. Dennett comparison was used for estimating statistical analysis using one-way ANOVA. *p<0.05 was considered as the significant.

3 Results

3.1 Boeravinone B enhance memory deficits and spatial learning induced via BCCAO

Figure 1a showed the reduced mean latency in finding the hidden platform during the day 4 on the water maze trials. On the day 5, the model group showed the increased latency time as compared to other. Figure 1b showed the reduced latency time and BB treatment significantly \(p<0.001\) increased the latency time.

Figure 1c exhibited the probe trails. During the probe trials, memory was scrutinized via evaluating the hidden platform (previously available). Model control rats showed the reduction in the crossing and BB treatment significantly \(p<0.001\) enhanced the crossing.

3.2 Boeravinone B effect on brain parameters

Figure 2 demonstrated the effect of Boeravinone B on the brain parameters. Model group rats exhibited the enhanced brain edema, brain water content and evans blue.
leakage as compared to control rats. BB treatment significantly $p<0.001$ suppressed the level of brain edema (Fig. 2a), brain water content (Fig. 2b) and evans blue leakage (Fig. 2c) as compared to model group rats. Nimodipine significantly $p<0.001$ suppressed the brain edema, brain water content and evans blue leakage.

3.3 Boeravinone B effect on cerebral water and cerebral index content

Figure 3 illustrated the cerebral water and cerebral index content in different group of rats. Model group rats showed the almost similar level of cerebral water level as compared to control rats (Fig. 3a). BB and nimodipine treatment exhibited the almost similar level of cerebral water (data not significant). Model group rats showed the increased cerebral index as compared to control group rats. BB and Nimodipine treatment significantly $p<0.001$ reduced the cerebral index as compared to model control (Fig. 3b).
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3.4 Determination of sodium (Na\(^+\)), potassium (K\(^+\)) and Na\(^+\), K\(^+\)-ATPase activity

During the cerebral IR injury, altered the activity of Na\(^+\), K\(^+\) and Na\(^+\), K\(^+\)-ATPase. In this current experimental study, model group rats exhibited the increased level of Na\(^+\) (Fig. 4a), K\(^+\) (Fig. 4b) and reduced the Na\(^+\), K\(^+\)-ATPase (Fig. 4c). BB treatment considerably reduced the level of Na\(^+\), K\(^+\) and increased the activity of Na\(^+\), K\(^+\)-ATPase. A similar result was observed in the Nimodipine treatment group rats.

3.5 Effect of boeravinone B on Ca and Ca\(^{2+}\) ATPase activity

Figures 5a and 5b demonstrated the level of Ca (Fig. 5a) and Ca\(^{2+}\) ATPase (Fig. 5b). Model group rats showed increased level of Ca and reduced the activity of Ca\(^{2+}\) ATPase. BB and nimodipine significantly (p < 0.001) boosted the Ca level and reduced the activity of Ca\(^{2+}\).
ATPase.

3.6 Effect of boeravinone B on the level of amino acids

Figures 6a and 6e exhibited the level of amino acids on the different group of rats. Model group rats showed the boosted level of glu (Fig. 6a), tau (Fig. 6b), Asp (Fig. 6c), GABA (Fig. 6d) and gly (Fig. 6e) as compared to other treated or non treated group. Model treated group rats treated with the BB exhibited the down-regulated level of amino acid includes glu (Fig. 6a), tau (Fig. 6b), Asp (Fig. 6c), GABA (Fig. 6d) and gly (Fig. 6e). Nimodipine treatment significantly ($p < 0.001$) reduced the level of amino acid.

3.7 Effect of boeravinone B on the level of antioxidant parameters

Figure 7 showed the level of antioxidant parameters in term of MDA (Fig. 7a), SOD (Fig. 7b), CAT (Fig. 7c), GSH-Px (Fig. 7d), GSH (Fig. 7e) and 8-OhdG (Fig. 7f). Model control group rats demonstrated the increased level of MDA, 8-OhdG and decreased the SOD, CAT, GSH-Px, GSH as compared to control rats. BB treatment significantly ($p < 0.001$) declined the level of MDA, 8-OhdG and augmented the SOD, CAT, GSH-Px, GSH level. Nimodipine treated group rats exhibited the similar types of result, like BB treatment.

3.8 Effect of boeravinone B on pro-inflammatory cytokines

Model group rats demonstrated the increased boosted level of TNF-α (Fig. 8a), IL-1β (Fig. 8b), IL-6 (Fig. 8c) and IL-10 (Fig. 8d). BB and Nimodipine treatment significantly ($p < 0.001$) reduced the level of TNF-α (Fig. 8a), IL-1β (Fig. 8b), IL-6 (Fig. 8c) and boosted the level of IL-10 (Fig. 8d).
**3.9 Effect of boeravinone B on inflammatory mediators**

The level of inflammatory parameters such as COX-2 (Fig. 9a), PGE₂ (Fig. 9b) and NF-κB (Fig. 9c) boosted in the model group and BB and nimodipine treatment significantly ($p<0.001$) down-regulated the level of inflammatory parameters.

**4 Discussion**

Cerebral ischemia reperfusion is complex conditions occur due to excess production of free radicals and inflammatory reactions that plays a significant role in initiating the disease by inducing injury to biological macromolecules leading to tissue and cell injury. Moreover, researcher used the antioxidant and anti-inflammatory drug treat cerebral I/R injury. In the current experimental study, we investigated the neuro-protective effect of Boeravinone B on...
cerebral ischemia reperfusion in rats and explore the prevention of cognitive function.

Cerebral edema is the clinical feature of cerebral I/R injury. The neuronal cell membrane usually contains Na⁺, K⁺-ATPase and plays an important role in balancing the intracellular levels of K⁺ and Na⁺. The concentration of Na⁺, K⁺-ATPase in the brain decreased during the induction of ischemia due to a decrease in ATP content and increased the production of enzymatic inhibitors that take part in the ionic disorder. During ischemia, Na⁺, K⁺-ATPase dysfunction occurred in the cell membrane, which further induced membrane depolarization and deposition of water and Na⁺ in the cell, ultimately causing cytotoxic edema in the ischemic cell. In this study, we have observed that boeravinone B considerably prevented the boosting level of Na⁺ and reduced K⁺ by protecting cells against decrease the Na⁺, K⁺-ATPase activity and by inducing the decline net ion shift, which resultant occur the less cerebral edema formation.

The imbalance between the production of reactive oxygen species and the scavenging of endogenous antioxidant enzymes has induced oxidative stress. Previous studies suggest that antioxidants commonly used for scrutinize the neuroprotective effect and they are also capable of declining the incidence of cerebral injury in I/R area. The basal level of ROS played an important role in various significant physiological conditions during normal physiological conditions and could be easily scavenged through endogenous antioxidant enzymes, including GSH (low molecular weight antioxidants), GPx and SOD. Moreover, during cerebral ischemia, after reperfusion, excess amount of ROS generated from the mitochondrial respiratory chain, sequence catalyzed via lipoxygenase and cyclooxygenase, which boosted the lipid peroxidation and start the production of numerous inflammatory proteins, which take part in damaging neuronal cells and finally cause the death. The current investigation showed a significant reduction in MDA (a lipid peroxidation marker) and boosted the level of SOD, GPx and GSH after treatment with boeravinone B to cerebral I/R injury. The current investigation indicated that boeravinone B exhibited neuroprotection against the cerebral I/R injury at least due to its antioxidant nature.

Various studies have shown that excitotoxicity is a well-established feature of brain ischemia damage, leading to pathophysiology through excessive neuronal excitation by pathological release of excitatory neurotransmitters from dying cells. Excitatory amino acid includes aspartic acid and glutamic acid, which is secreted excessive quantities from astrocytes and neurons, during cerebral I/R, both receptors such as α-amino-3-hydroxy-methyl-4-isoxazole propionic (AMPA) and N-methyl-D-aspartic acid (NMDA) boost. The increased level of both receptors causes the elevation the level of intracellular Ca²⁺ that further activated the Ca²⁺ dependent enzymes and starts the production of free radicals and toxic nitric oxide. Moreover, the deposition of Ca²⁺ in the cells and increase the calcium concentration in the mitochondrial and terminate the production of ATP and finally boosting receptors in a vicious cycle. Glycine is significant amino acid that boosts the Gln's action at the NMDA receptor on the allosteric site. The concentration of glycine considerably boosted during the cerebral ischemia. Previous studies suggest that the phyto-constituent such as breviscapine could boost the ATP level and ATPase activity during I/R in rats and suppress the excitatory amino excitotoxicity expression through reduction the intracellular Ca²⁺ deposition and increases the expression of XIAP in hippocampal neurons. Various mechanisms could attenuate ischemic neuronal cells through GABA transporter inhibitors, GABA agonists, GABA transaminase inhibitors and GABA modulators. Release of inhibitory amino acids such as taurine and gamma-aminobutyric during cerebral I/R. Inhibitory amino acid reduces cerebral I/R injury and neutralize the excitatory amino acid toxicity. From the above mentioned, we may recognize that the severity of ischemic brain damage could be decreased by actions that resulted in inhibiting the release of EAAs. The current study showed that boeravinone B treatment attenuated the enhance glutamate, glycine and intracellular aspartic, meantime, it reduces the overload of Ca²⁺ and boosts the Ca²⁺ ATPase activity in the brain during the cerebral I/R.

An inflammatory reaction boosted during cerebral I/R injury due to boost the level of vascular macrophages and endogenous microglia. Cerebral I/R injury exhibited the increased deposition of pleiotropic mediators such as cytokines, chemokines and prostaglandins. Due to boost the increase in production of pro-inflammatory cytokines, enhanced the burden of anti-inflammatory cytokines in the brain area and most of the researcher targeting the inflammatory reaction to treat cerebral I/R injury. During cerebral I/R injury, increase the production of pro-inflammatory cytokines and start the deposition into the serum. During cerebral I/R injury, pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β and anti-inflammatory cytokines such as IL-10 were altered. Boeravinone B considerably altered the pro-inflammatory and anti-inflammatory cytokines.

5 Conclusion

Our experimental results showed that the neuroprotective effect of boeravinone B on cerebral ischemia reperfusion. Boeravinone B reduces the brain, and Evans blue leakage edema and brain water content.

Boeravinone B showed the neuroprotective effect via

- Antioxidant potential and reduce the production of
free radicals and increase the level of endogenous antioxidant enzymes.

- Altering the imbalance of inhibitory amino acids and excitatory amino acids.
- Reducing the pro-inflammatory cytokines and inflammatory mediators.

The result clearly indicated that Boeravinone B exhibited the neuroprotective effect against the I/R induced brain injury. Boeravinone B targeted the amino acid and excitatory amino acids, inflammatory cytokines, antioxidant enzymes and inflammatory mediators. According to the obtained result, more molecular investigation might be carried out to identify the possible gene responsible for neuroprotective effect.

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**References**


