

## Original Research Article

# Bisleuconothine A potentiates the effect of hyperbaric oxygen therapy against traumatic brain injury by enhancing P2X4 protein expressions

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## Abstract

**Purpose:** To investigate the effect of bisleuconothine A (BA), alone and in combination with hyperbaric oxygen (HO), on traumatic brain injury (TBI) in rats.

**Methods:** Traumatic brain injury (TBI) was induced by dropping a 200-g weight of steel on the left anterior frontal areas of Sprague-Dawley rats. The synergistic effect of BA and HO was determined by assessing neurological score, as well as parameters of oxidative stress and inflammation, expressions of P2X4 protein and other proteins, and levels of reactive oxygen species (ROS) in the brain tissues of TBI rats.

**Results:** Neurological function score, levels of inflammatory mediators and oxidative stress parameters were significantly reduced in rats treated with BA alone, and in those treated with a combination of BA and HO, when compared with untreated TBI rats ( $p < 0.01$ ). Moreover, treatment with BA alone, and BA-HO combination attenuated the altered protein expressions of P2X4, Akt, PI3K and TLR-4 in the TBI rats, and also upregulated the mRNA expression of P2X4 in the brain tissue, when compared with untreated TBI rats ( $p < 0.01$ ).

**Conclusion:** These results suggest that BA, when used alone or in combination with HO, reduces neuronal injury through upregulation of the protein expression of P2X4 in rats with traumatic brain injury. Thus, BA may be used clinically with HO therapy for the management of traumatic injury.

**Keywords:** Bisleuconothine A, Hyperbaric oxygen, Neuronal injury, Oxidative stress, Inflammatory mediators

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## INTRODUCTION

Traumatic brain injury (TBI) is one of the major causes of death worldwide. Incidents of TBI are on the increase in China every year, probably due to development of the Chinese society and

economy [1]. Neuronal injury occurs in TBI through two pathways: direct pathway through trauma (mechanical injury), and secondary pathway involving injury due to many pathological changes such as oxidative stress, inflammation and enhanced calcium overload in the brain cells [2]. The upregulation of the P2X4

receptor, a member of the purine receptor family, protects the brain against neuronal injury in ischemia and TBI [3]. Studies have shown that stimulation of P2X4 receptor enhances the release of brain-derived neurotrophic factor and reduces the levels of inflammatory cytokines [4]. In contrast, blockage of P2X4 receptor leads to mitochondrial damage by reducing the production of ROS species and oxidative stress [5].

It is well documented that although hyperbaric oxygen ameliorates neuronal damage, it has many limitations. In the last few decades, alternative medicine has shown promising role against neuronal injury. Chemically, bisleuconothine A is an alkaloid isolated from *Leuconotis griffithii* [6]. Bisindole alkaloids possess antibacterial, anti-inflammatory, antimalarial and anticancer properties [7]. Bisleuconothine A modulates the AKT-mTOR signaling pathway and induces autophagosomes [8]. The present investigation was carried out to study the effects of bisleuconothine A and hyperbaric oxygen on traumatic neuronal injury in rats.

## EXPERIMENTAL

### Animals

Male Sprague-Dawley rats weighing 200–230 g were kept at 12-h light/12-h dark cycle under standard conditions as per the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) [9]. All protocols used in the study were approved by the institutional animal ethical committee of the Sixth Medical Center of PLA General Hospital, China (no. IAEC/SMC/PLA-GH/2017/18).

### Treatments

Traumatic brain injury was induced in the rats after removing their fur. A midline incision was made on the scalp of each rat, and a metallic disc was fixed in a central position with bone wax. Thereafter, a 200-g weight of steel was dropped from a height of 15 cm on the left anterior frontal area of each rat placed on a foam rubber platform.

The rats were randomly assigned to 5 groups: sham group, TBI group, rat group treated with BA (2 mg/kg, i.p.) for 30 min prior to induction of TBI, HO group treated for 30 min with HO before the induction of TBI, and BA+HO group treated for 30 min with BA + HO prior to induction of TBI.

### Administration of HO

Hyperbaric oxygen (HO) was administered as per previously published reports [10]. The rats were treated with pure oxygen for 10 min by placing them in a chamber containing 95% oxygen. A pressure of 0.12 MPa was maintained in the chamber for 60 min, and thereafter, the pressure was slowly normalized within 20 min. Hyperbaric oxygen therapy was given two times at an interval of 10 h.

### Assessment of neurological function

The modified neurological severity score (mNSS) was used to determine balance, touch, vision, abnormal behaviour, muscle mass, sensation and motion, as previously reported [11]. The mNSS was calculated on a 0–18 scale, where 0 indicated normal brain function, and 18 indicated a severe deficit.

### Evaluation of biochemical parameters

Brain tissue levels of the inflammation mediators (NF- $\kappa$ B, IL-6, IL-16 and TNF- $\alpha$ ), levels of the oxidative stress markers: malondialdehyde (MDA), and activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD) were determined using ELISA assay kits as per the manufacturer's instructions.

### Western blot assay

Proteins were isolated from homogenised tissues and quantified using bicinchoninic acid (BCA) assay kit. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% was used to separate the proteins. The proteins were then transferred to a nitrocellulose membrane using an electroblotting technique. The membrane was blocked with 5% solution of non-fat milk and then incubated in a blocking buffer with the following primary antibodies overnight at 4°C: anti-TLR-4, Akt, PI3K, anti-P2X4, and anti- $\beta$  actin.

Thereafter, goat secondary antibody-conjugated with horseradish peroxidase (HRP) was added to the blocking buffer (1:1,000 dilution) and incubated for 24 h at room temperature. Chemiluminescence kit (Thermo Fisher Scientific, Shanghai, China) was used to detect the protein blots.

### Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from separated spinal cord tissue using TRIzol Reagent (Thermo

Fisher). Then, Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher) was used to reverse-transcribe the RNA to cDNA. Specific primers (Table 1) were mixed with RT-2 SYBR green master mix to evaluate gene expression using qRT-PCR. The conditions used for all samples were as follows: 98 °C for 2 min, followed by 25 – 40 cycles of 98 °C for 10 s, then 55 °C for 5 s, and 72 °C for 20 s. The mRNA expression levels of P2X4 and 18S rRNA were calculated according to relative standard curves, which were generated by plotting the quantification cycle (Cq) against the log amount of total cDNA used in the reaction. The relative expressions of the target genes were calculated using the  $2^{-\Delta\Delta C_q}$  method.

### Determination of ROS

Brain tissue homogenate (50 µL) was mixed with potassium phosphate buffer (4.85 mL) and incubated with 2',7'-dichlorofluorescindiacetate dye at 37 °C for 15 min. The mixture was centrifuged at 12000 g at 4°C for 10 min, and 5 mL of 100 mM PBS was added to the mixture, with vortexing at 0 °C, followed by further incubation at 37 °C for 60 min. Thereafter, the absorbance was read at 488 nm (excitation) and 525 nm (emission).

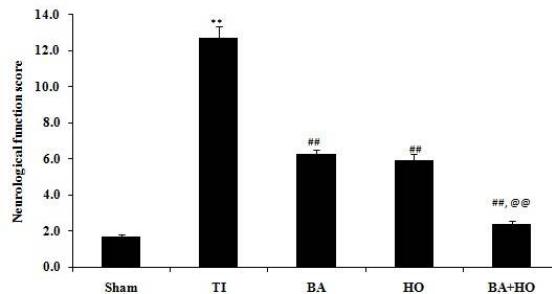
### Statistical analysis

The results are expressed as mean ± SEM (n=10). Statistical analysis was performed using one-way ANOVA. Post-hoc comparison of means was carried out with Dunnett's post hoc test (GradPad Prism 6.1, CA, USA). The level of statistical significance was set at  $p < 0.05$ .

## RESULTS

### Bisleuconothine A (BA) alleviated cognitive function deficit in TBI rats

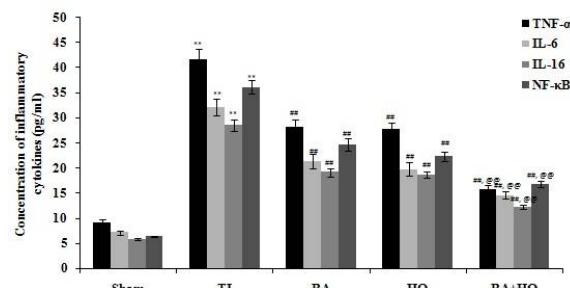
The rats were treated with bisleuconothine BA alone, or in combination with HO, and the effects of the treatments on neurological function score in TBI rats were determined. The results are shown in Figure 1. The mNSS was enhanced up to 12.7 in untreated TBI group of rats. However, there were significant reductions in mNSS of rats in BA, HO and BA+HO groups, when compared with the untreated TBI group of rats. Moreover, rats in BA+HO group showed higher reduction in mNSS than those in TBI and HO groups. These results suggest that the treatment with combination of BA and HO produced synergistic effect on mNSS in TBI rats.



**Figure 1:** Effect of bisleuconothine A on neurological function of TBI rats given hyperbaric oxygen therapy. Results are presented as mean ± SEM (n = 10); \*\* $p < 0.01$ , compared to sham group; ## $p < 0.01$ , compare to TI group; @@ $p < 0.01$ , compared to BA+HO group

### Bisleuconothine A reduced levels of mediators of inflammation

The effects of treatment with BA alone and BA + HO on levels of inflammation mediators in TBI rats are shown in Figure 2. It was observed that the levels of IL-6, IL-16 and NF-κB were enhanced more than 400 % in TBI group, when compared with the sham group of rats. However, the levels of these mediators of inflammation were significantly reduced ( $p < 0.01$ ) in BA, HO and BA+HO groups, relative to TBI group. Furthermore, it was observed that treatment with BA + HO led to higher degree of amelioration of the altered levels of mediators of inflammation, relative to when BA and HO were used separately.

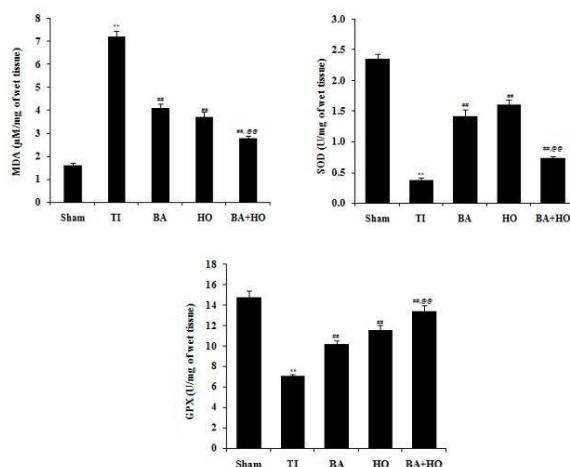


**Figure 2:** Effects of BA alone, and BA+HO on the levels of mediators of inflammation in TBI rats. Results are expressed as mean ± SEM (n = 10); \*\* $p < 0.01$ , compared to sham group; ## $p < 0.01$  compared to TBI group; @@ $p < 0.01$ , compared to BA+HO group

### Bisleuconothine A alleviated oxidative stress

The effects of BA and HO on the activities of SOD and GPx and MDA levels in the brain tissue of TBI rats are shown in Figure 3. In TBI group, brain tissue MDA level was increased to 7.2 µmoles/mg, while the activities of SOD and GPx were reduced to 0.37 and 7.1 U/mg, respectively, relative to the sham group. However, treatment

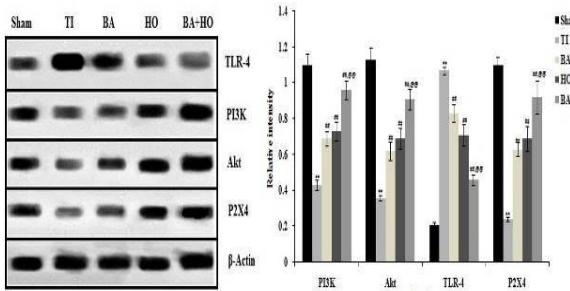
with BA and HO alone, and BA+HO combination reversed the altered level of MDA, as well as the activities of SOD and GPx.



**Figure 3:** Effects of BA and BA+HO on parameters of oxidative stress in TBI rats. Results are shown as mean  $\pm$  SEM ( $n = 8$ ); \*\* $p < 0.01$ , compared to sham group; ## $p < 0.01$ , compared to TBI group; @@ $p < 0.01$ , compared to BA+HO group

#### Effect of BA on the protein expressions of TLR-4, Akt, PI3K and P2X4

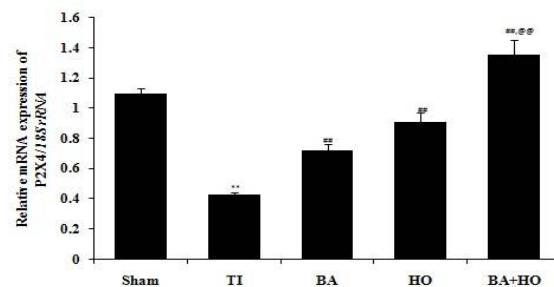
As shown in Figure 4, the protein expressions of P2X4, Akt and PI3K were reduced in the TBI groups, while protein expression of TLR-4 was enhanced, when compared to the sham group of rats. However, treatment with BA and HO alone and in combination (BA + HO) led to upregulations in protein expressions of P2X4, Akt and PI3K, and reduction in the expression of TLR-4 protein in TBI rats. It was also observed that the increases in expressions of P2X4, Akt and PI3K, and decrease in the expression of TLR-4 protein in BA+HO treated group were higher than those in the groups treated alone either with BA or HO.



**Figure 4:** Effects of BA alone, and BA+HO on the protein expressions of TLR-4, Akt, PI3K and P2X4 in the brain of TBI rats. Data are presented as mean  $\pm$  SEM ( $n = 10$ ); \*\* $p < 0.01$ , compared with sham-operated group; ## $p < 0.01$ , compared with TBI group; @@ $p < 0.01$ , compared with BA + HO group

#### Bisleuconothine A upregulated mRNA expression of P2X4 and 18S rRNA

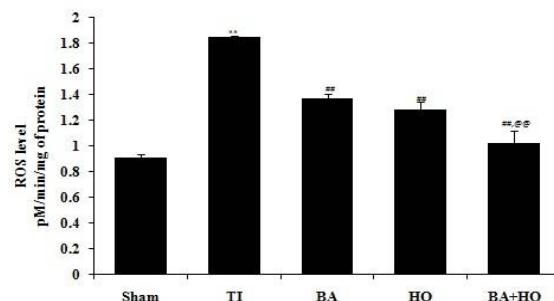
There was a significant ( $p < 0.01$ ) decrease in the mRNA expression of P2X4 in the TBI group, when compared to sham group of rats. Rats treated with BA and HO alone, and in combination had enhanced expressions of P2X4, when compared to TBI rats. Moreover, rats in the BA+HO-treated group had higher expression of P2X4 than rats in BA or HO alone group.



**Figure 5:** Effect of BA alone, and BA+HO on the mRNA expression of P2X4 in TBI rats. Data are shown as mean  $\pm$  SEM ( $n = 10$ ); \*\* $p < 0.01$ , compared to sham-operated group; ## $p < 0.01$ , compared to TBI group; @@ $p < 0.01$ , compared to BA + HO group

#### Bisleuconothine A reduced ROS levels in TBI rats

As shown in Figure 6, there was an increase in the level of ROS in the brain tissue homogenate of TBI group, when compared to sham group of rats. However, treatment with BA and HO, alone and in combination, significantly reduced the level of ROS in the tissue homogenate, when compared to TBI group of rats. The level of ROS was reduced to a greater extent in the BA+HO-treated group than in the groups treated separately with BA and HO.



**Figure 6:** Effect of BA alone, and BA+HO on the level of ROS in TBI rats. Data are shown as mean  $\pm$  SEM ( $n = 10$ ); \*\* $p < 0.01$ , compared with sham-operated group; ## $p < 0.01$ , compared with TI group; @@ $p < 0.01$ , compared with BA+ HO

## DISCUSSION

The present study determined the protective effect of bisleuconothine A (BA) alone, and in combination with hyperbaric oxygen (HO), on secondary injury in TBI rats. The synergistic effect of BA and HO was determined by estimating neurological score in TBI rats, as well as parameters of oxidative stress, mediators of inflammation, expression of P2X4 protein and level of ROS. Moreover, western blot assay and RT-PCR were performed by estimating protein expressions in the brain tissue of TBI rats. Secondary injury in traumatic brain injury leads to cognitive dysfunction, for example memory loss [12]. In this study, treatment with BA alone, and in combination with HO reduced neurological function score of the TBI rats. Inflammatory and oxidative stress pathways are involved in TBI-induced neuronal degeneration [13]. Studies have revealed that HO therapy is used in the management of TBI rats [14]. However, there are many limitations associated with HO therapy. It has been shown that BA possesses anti-cancer and anti-inflammatory properties [7]. The results of the present investigation showed that treatment with BA reduced the levels mediators of inflammation in the brain tissue of TBI rats. Moreover, BA produced synergistic effect with HO on the mediators of inflammation in TBI rats. In traumatic brain injury, the expression of TLR-4 is significantly increased, and it has been reported that inhibition of TLR-4 attenuates TBI. The present study has shown that BA, when used alone or in combination with HO, reduced the protein expression of TLR-4 in TBI rats. Oxidative stress plays an important role in the development of neuronal injury in traumatic brain injury [15]. Drugs used for the management of TBI reduce the level of oxidative stress [16]. In this study, BA alone, and in combination with HO, reduced levels of the markers of oxidative stress and ROS in the brain of TBI rats.

It has been reported that the expression of P2X4 is unregulated in TBI rats [17]. Inflammatory cytokine levels were reduced, while BDNF and oxidative stress were enhanced due to stimulation of P2X4 receptor. Mitochondrial damage is mitigated by blocking the P2X4 receptor [18]. The results of this study showed that BA, alone and in combination with HO, attenuated the expression of P2X4 protein in TBI rats.

## CONCLUSION

The findings of this study indicate that BA, alone and in combination with HO, reduce neuronal injury in TBI rats by upregulating the expression

of P2X4 protein. Moreover, BA showed synergistic effect with HO therapy in the management of TBI. These results indicate the beneficial potential of BA in combination with HO therapy for the clinical management of traumatic brain injury.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this study.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiangen Meng and Chunyang Zhang conducted the experimental work and literature review related to presented work. Na Li, Yu Zhang, Danfeng Fan and Chen Yang performs the molecular assay and histopathological analysis. Hang Li and Dazhi Guo performed the statistical analysis and Shuyi Pan supervised the presented work and written the manuscript. Xiangen Meng and Chunyang Zhang contributed equally to this work.

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