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PROTECTIVE EFFECT OF EXTRACT OF *BLETILLA STRIATA* ON ISOFLURANE INDUCED NEURONAL INJURY BY ALTERING PI3K/AKT PATHWAY

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Abstract

Background: Present investigation evaluates the neuroprotective effect of *Bletilla striata* on isoflurane induced neuronal injury rat model. **Methodology:** Neuronal injury was induced by exposing the pups (P7) isoflurane (0.75%) in oxygen (30%) for the period of 6 hr and rats were treated with *Bletilla striata* at a dose of *35, 70 and 140 mg/kg, p.o. for the period of 21 days.* At the end of protocol neurological score was estimated and serum concentration of inflammatory cytokines was estimated. Isolated brains tissue was prepared to perform immunohistochemical analysis, TUNEL assay and western blot assay. **Results:** Result of the study reveals that treatment with BS significantly ($p<0.01$) reduces the neurological score compared to negative control group. Level of inflammatory cytokines in the serum and the expression of p-Akt, Bcl-xL and Bad protein were significantly attenuated in BS treated group. Moreover the cleaved caspase-3 and TUNEL positive cell was significantly ($p<0.01$) reduced in BS treated group compared to negative control group of rats. **Conclusion:** Present study concludes that ethanolic extract of *Bletilla striata* protects the neuronal injury by reducing apoptosis in isoflurane induced neuronal injury rats.

Keywords

Bletilla striata • Isoflurane • Neuroprotective • Cytokines • Apoptosis

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Introduction

Isoflurane is used clinically for the induction of anesthesia during surgical procedure [1]. Studies suggested that in children below the age of 4 year use of anesthesia causes learning, writing and reading disability [2]. Reported studies reveals that use of isoflurane on the developing brain causes neuronal damage [3]. Isoflurane reported to induce apoptosis in neuronal cell at a regular anesthetic dose by reducing the ratio of ratio of Bcl-2/Bax [4]. Bcl-2 is a protein that regulates the integrity of mitochondrial membrane and thus it maintain the death and survival of cell [5]. Moreover, Akt protein does the phosphorylation of Bcl-associated death protein (Bad) and thereby enhances the release of Bcl-xL. Bcl-2 and Bcl-xL proteins contribute in the maintainance of mitochondrial membrane potential and thereby reduce the apoptosis [6]. Thus it is important to protect the developing brain from the injury induced due to exposure of anesthesia.

In the recent years medicines from the herbal sources has shown potential effect for the management of neurodegenerative disorders including neuronal injury induced due to anesthesia on developing brain. *Bletilla striata* (*Orchidaceae*) is traditionally used as a medicine in China [7]. *Bletilla striata* reported to posses strong anti inflammatory, antioxidant and anti microbial activity [8-10]. *Bletilla striata* reported to contain several phytochemical compounds such as phenolic compound, dihydrophenanthrene, phenanthrene, bibenzyl and polysaccharides [11]. Moreover the compounds present in *Bletilla striata reported for their antiviral, antibacterial and anti inflammatory activity [12-13]. Thus present study evaluates the neuroprotective effect of Bletilla striata* on isoflurane induced neuronal injury.

Material and methods

Animals

Sprague-Dawley rat pups of (15-20 g) 7 days old age were procured from Changzhou Cavens Laboratory Animal Co. Ltd. All the rat pups were maintained as per the guidelines. All the protocols of the study were approved by The Institutional Animal Care and Use Committee of China-Japan Union Hospital of Jilin University, China (IACUC/C-JUH/JU/2017/11). The given study followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use [14].

Extraction of *Bletilla striata*

Bletilla striata whole plant was shade dried and coarsely powdered. 50 g of *Bletilla striata* was applied for the extraction in alcohol (95 %) by refluxing it for 60 min. Extract was filtered and further ethanol was evaporated by using vacuum evaporator. The practical yield of ethanolic extract of *Bletilla striata* was found to be 10.7 % w / w.

Experimentation

Neuronal injury was induced by exposing the pups (P7) isoflurane (0.75 %) in oxygen (30 %) for

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the period of 6 hr. All the animals were separated in to five groups such as control group, Negative control group which receives only isoflurane and saline solution, *BS 35, 70 and 140 mg / kg treated group receives Bletilla striata* at a dose of *35, 70 and 140 mg / kg, p.o. for the period of 21 days. At the end of protocol pups were sacrificed and* ice-cold saline and 4% paraformaldehyde was used in the perfusion of animal transcardially.

Neurological examination

Behavior and motor changes were assessed using the 20-point neuro score. The behavior assessments included the response to and circling of nociceptive stimuli, postural and walking reflexes, extremity tonus, performance in a smooth climbing platform, and consciousness.

Estimation of cytokine levels

The plasma interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) concentrations were measured using enzyme-linked immunosorbent assays kits (CUSABIO, Wuhan, China), as per the manufacturer's instructions.

Tissue Preparation

Brain was isolated from each animal and hippocampus was harvested for western blot assay. However for TUNEL and immunohistochemistry study *pups were sacrificed and* ice-cold saline and 4 % paraformaldehyde was used in the perfusion of animal transcardially. Brains were embedded in paraffin wax and later section it in to 5 mm thickness.

Immunohistochemical staining

Tissue sections were incubated with anti-cleaved caspase-3 primary antibody for the period of overnight at 4 °C and thereafter further incubate it for 40 min with a secondary antibody. Tissue sections were colorized with diaminobenzidine. NIS-Elements BR imaging processing and analysis software was used for the analysis of Cleaved caspase-3 positive cells in the isolated brain tissues.

Western blot assay

Tissue homogenate brain tissue was done and BCA protein assay kit was used for the estimation of protein in the tissue sample. Protein sample (60 µg) was incubated with the primary antibodies such as anti- Bcl-xl, anti-p-Bad, anti-Bad, anti-p-Akt, anti-Akt and anti-β-actin (Proteintech Group Inc, Wuhan, China) for the western blot assay. Images were scanned by an Image Master II scanner and were analyzed using Image Quant TL software.

TUNEL Assay

Dead End TM fluorometric TUNEL system kit was used to perform the TUNEL fluorescent assay as per the instructions of manufacturer. Slides were avoided to get expose under the direct sun light and cellular nuclei was stained using Hoechst staining. NIS-Elements BR image processing and analysis software was used for the estimating of TUNEL positive cell in the hippocampal region.

Statistical Analysis

All data are expressed as mean ± SEM (n= 6). Statistical analysis was performed using one way ANOVA. Post-hoc comparison of means was carried out by Dunnett's post hoc test (Gradpad Prism 6.1, CA, USA). The level of statistical significance was set at $p<0.05$.

Result

Effect of ethanolic extract of *Bletilla striata* on the neurological function

Effect of ethanolic extract of *Bletilla striata* on the neurological function of isoflurane induced neuronal injured rat model was shown in Fig. 1. It was observed that neurological function score was significantly ($p < 0.01$) enhanced in negative control group (10.5 ± 0.72) compared control group (1.7 ± 0.19). However neurological function score was significantly reduced up to 8.1 ± 0.41, 6.3 ± 0.33 and 3.1 ± 0.23 in BS 35, 70 and 140 mg / kg treated group respectively compared to negative control group.

Effect of ethanolic extract of *Bletilla striata* on the level of inflammatory cytokines

Fig.2. Shows the effect of *Bletilla striata* on the concentration of inflammatory cytokines (TNF-αand IL-1β) in the serum of isoflurane induced neuronal injured rat model. There was significant ($p < 0.01$) increase in the serum concentration of TNF-α (46.8 pg / ml) and IL-1β (36.9 pg / ml) in negative control group control group of rats. Treatment with BS significantly attenuates the altered level of TNF-α and IL-1β in a dose dependent manner.

Effect of ethanolic extract of *Bletilla striata* on the cleaved caspase-3

Effect of ethanolic extract of *Bletilla striata* on the cleaved caspase-3 of neuronal cells was estimated by immunohistochemical staining as shown in Fig. 3. There was significant ($p < 0.01$) increase in the cleaved caspase-3 positive cells in the hippocampus CA1 region of the negative control group (159 mm^2) compared to control (12 mm^2). However caspase 3 positive cell quantities was significantly reduced in BS treated group compared to negative control group of rats in a dose dependent manner.

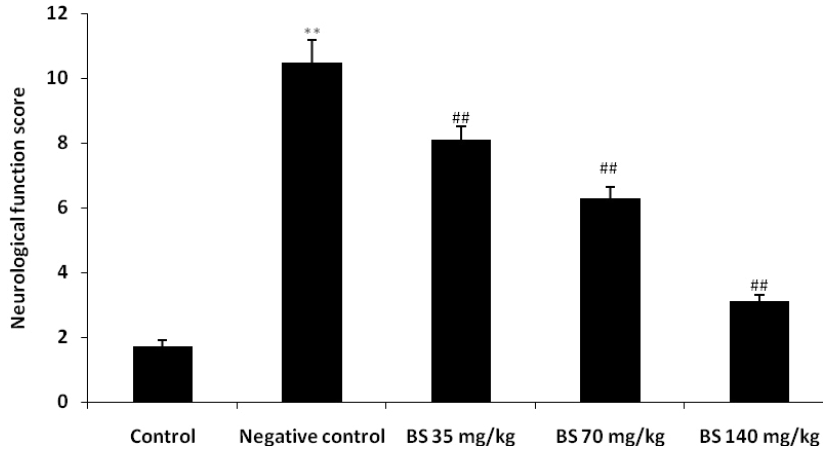

Fig. 1. Effect of ethanolic extract of *Bletilla striata* on the neurological function of isoflurane induced neuronal injured rat modelMean ± SEM (n = 10), ** p < 0.01 than control group; ## p < 0.01 than negative control group

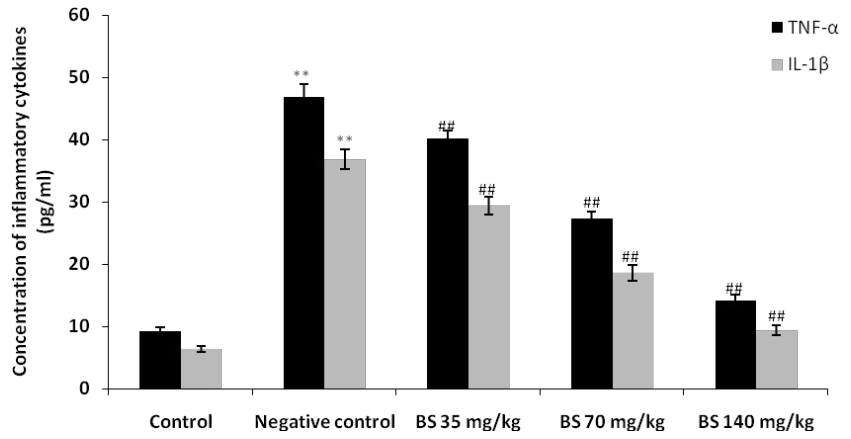

Fig. 2. Effect of ethanolic extract of *Bletilla striata* on the level of inflammatory cytokines in the serum of isoflurane induced neuronal injured rat model. Mean ± SEM (n = 10), .** p < 0.01 than control group; ## p < 0.01 than negative control group

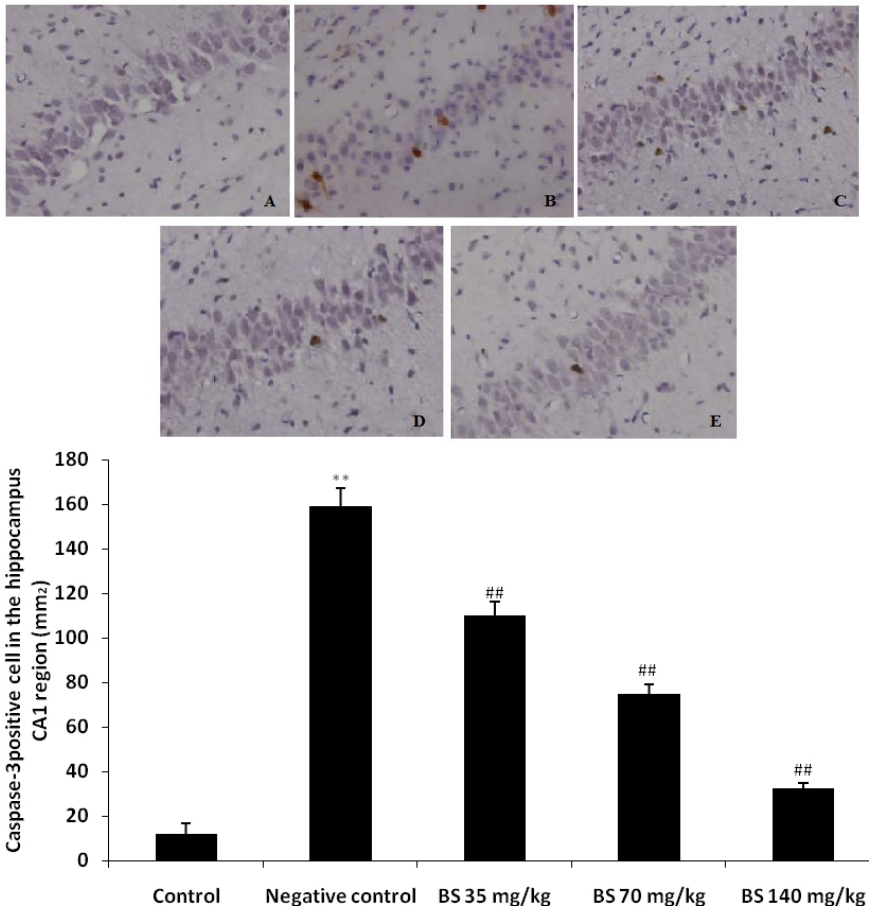

Fig. 3. Effect of ethanolic extract of *Bletilla striata* on the cleaved caspase-3 of neuronal cells. A: Control; B: Negative control; C: BS 35 mg / kg; D: BS 70 mg / kg; E: BS 140 mg / kg. Mean ± SEM (n = 10), ** p < 0.01 than control group; ## p < 0.01 than negative control group

Effect of ethanolic extract of *Bletilla striata* on the expression of Akt, Bcl-xL and Bad

Effect of ethanolic extract of *Bletilla striata* on the expression of Akt, Bcl-xL and Bad in the brain tissue of isoflurane induced neuronal injured rat model was estimated by western blot assay (Fig. 4.). Data of the western blot study reveals that the expression of ratio of p-Akt / Akt and Bcl-xL / Bad was found to be significantly reduced ($p < 0.01$) and the expression of p-Bad protein was significantly enhanced in the brain tissue homogenate of negative control group compared to control group of rats. Whereas treatment with BS treated group attenuates the altered expression of Akt, Bcl-xL and Bad in the brain tissue of isoflurane induced neuronal injured rat in a dose dependent manner.

Effect of ethanolic extract of *Bletilla striata* on the neuronal apoptosis

Effect of ethanolic extract of *Bletilla striata* on the neuronal apoptosis of isoflurane induced neuronal injured rat was estimated by determining the TUNEL positive cells (Fig. 5.I&II). It was observed that number of TUNEL positive cell was significantly enhanced ($p<0.01$) in the hippocampus CA1 region of negative control group compared to control group of rats. Data suggest that TUNEL positive cells were significantly reduced in the hippocampus CA1 region of BS treated group compared to negative control group of rats.

Discussion

Present study evaluates the neuroprotective effect of ethanolic extract of *Bletilla striata* in isoflurane induced neuronal injury in developing brain in rat. Rat pups of 7 days age were exposed isoflurane (0.75 %) in oxygen (30 %) for the period of 6 hr. and thereafter rats were treated with BS for the period of 21 days. At the end of protocol neurological score was estimated and serum concentration of inflammatory cytokines was estimated. Isolated brains tissue was prepared to perform immunohistochemical analysis, TUNEL assay and western blot assay.

Literature reveals that developing brain exposed to inhale anesthetic causes neuronal

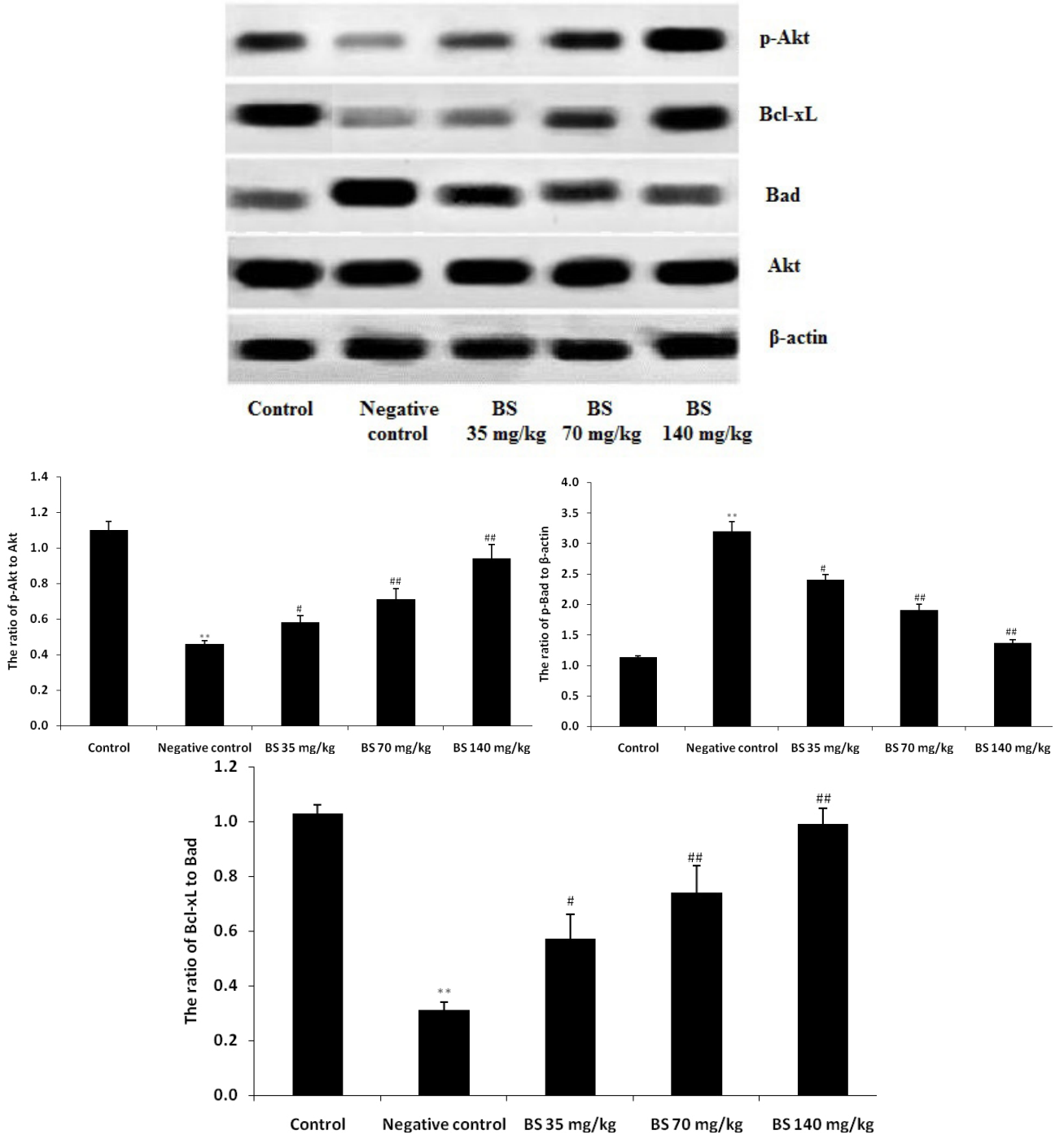

Fig. 4. **Effect of ethanolic extract of *Bletilla striata* on the expression of Akt, Bcl-xL and Bad in the brain tissue of isoflurane induced neuronal injured rat model.** Mean ± SEM (n = 10), $^{**}p < 0.01$ than control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ than negative control group

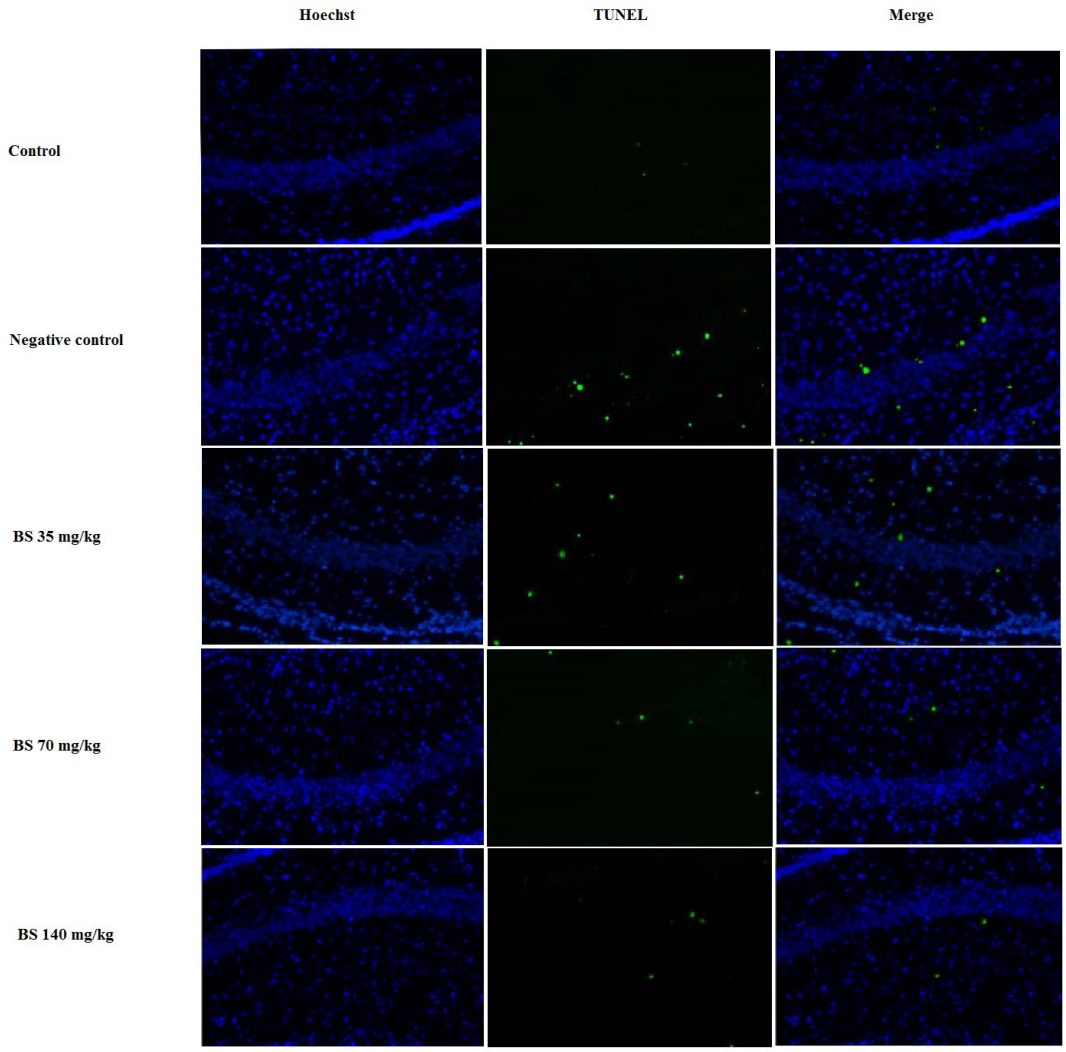

Fig. 5. **Effect of ethanolic extract of** *Bletilla striata* **on the neuronal apoptosis of isoflurane induced neuronal injured rat model. I: TS of hippocampus by TUNEL staining, II: TUNEL positive cells.** Mean ± SEM (n = 10), $^{**}p < 0.01$ than control group; $^{\#\#}p < 0.01$ than negative control group

injury [15] and data of this study suggest that isoflurane induces neuronal injury in the negative control group compared to control group of rats. Several reports reveal exposure of isoflurane causes impairment of cognitive function depending up on hippocampus region of the brain [16]. Our study evaluates the neuronal function and biochemical parameters were estimated in the hippocampus region of the brain. Injuries occur due to anesthetics expression of cleaved caspase-3 was used as a marker of neuronal apoptosis [17] and in our study expression of cleaved caspase-3 was significantly reduced in BS treated group compared to negative control group in a dose dependent manner.

Anesthetics are enhancing the intracellular Ca^{2+} ion due to over activation of IP3 receptor [18]. Cellular Ca^{2+} ion overload activates the apoptosis of neuronal cells and thereby causes neuronal injury [19]. Moreover inflammatory cytokine enhances the functional and morphological impairment of mitochondria [20]. Data of this study reveals that the level of inflammatory cytokines significantly reduced in BS treated group compared to negative control group. Bcl-2 family protein was responsible for the apoptosis and maintenance of integrity of mitochondrial membrane [21]. Result of the study reveals that expressions of pAkt, Bcl-xL and Bad protein were attenuated in BS treated group in isoflurane induced neuronal injury rats. Thus treatment with BS reduces the apoptosis of neuronal cell in isoflurane induced neuronal injury rats.

Conclusion

Present study concludes that ethanolic extract of *Bletilla striata* protects the neuronal injury by reducing apoptosis in isoflurane induced neuronal injury rats. Neuronal apoptosis was reduced by attenuating the altered PI3K/Akt pathway.

Conflict of interest

No

Acknowledgement

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