

Effectiveness of 6-shogaol in potentiating sevoflurane mediated neuroprotection against ischemia/reperfusion-induced brain injury via regulating apoptotic proteins and PI3K/Akt/mTOR/s6K signaling and HIF-1 α /HO-1 expression

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ABSTRACT

Purpose: The current research has been intended to evaluate the impacts of 6-shogaol in rodent model of ischemic-reperfusion induced- brain injury and also assessed whether 6-shogaol enhanced sevoflurane's neuroprotective effects.

Methods: Ischemic-Reperfusion (I/R) injury was induced by middle cerebral artery occlusion (MCAO) method in Sprague-Dawley rats. A separate group of animals was exposed to sevoflurane (2.5%) post-conditioning for 1 h immediately after reperfusion. 6-shogaol (25 mg or 50 mg/kg body weight) was orally administered to treatment group rats for 14 days and then subjected to I/R.

Results: 6-shogaol treatment along with/without sevoflurane post-conditioning reduced the number of apoptotic cell counts, brain edema and cerebral infarct volume. The western blotting analysis revealed a significant stimulation of the PI3K/Akt/mTOR signal pathway. RT-PCR and western blotting studies revealed improved expressions of HIF-1 α and HO-1 were also noticed at both gene level and protein levels as determined by. I/R induced neurological deficits were also alleviated on sevoflurane post-conditioning with/without 6-shogaol treatment. **Conclusion:** The study's findings reveal that pre-treatment with 6-shogaol enhanced the neuroprotective properties of sevoflurane post-conditioning, illustrating the efficacy of the compound against I/R injury. 6-Shogaol thus could be investigated further for cerebral protection following I/R.

Keywords: 6-shogaol, Hypoxia-inducible factor 1 α , Hemeoxygenase 1, Ischemia, phosphatidylinositide 3-kinase/protein kinase B (PI3K/AKT) signaling, Sevoflurane

INTRODUCTION

Ischemia/reperfusion (I/R) induced cerebral injury, is a common pathological occurrence associated with stroke and various neurological and cardiovascular procedures as intracranial aneurysm clamping, aortic arch replacement etc [1]. I/R induced brain damage is known to be associated with multiple mechanisms as oxidative stress, neuroapoptosis, inflammatory responses and excitotoxicity [2, 3]. I/R-induced brain injury critically interrupts the success rates of surgical interventions, the prognosis of the disease and survival rate of patients. This necessitates the development of efficient approaches that could effectively reduce or prevent ischemic injury [4].

Studies have shown that volatile anaesthetics like isoflurane and sevoflurane possess neuroprotective effects [5-7]. Sevoflurane is a fast-acting extensively employed for general anaesthesia. Sevoflurane has been reported to possess negligible effects on cerebral metabolism and intracranial pressure. These factors contribute to the use of sevoflurane in neurosurgery [8, 9]. Further, experimental data reveal that sevoflurane post-conditioning following I/R improved neurological function and also reduced inflammation [7, 10]. Previous works by Lai *et al.* [11] and Ye *et al.* [10] suggested neuroprotective properties of sevoflurane post-conditioning following neonatal hypoxia-ischemia (HI) were mediated through the phosphatidylinositide 3-kinase (PI3K)/AKT signaling.

PI3K/Akt signaling is one of the major pro-survival signaling pathways that also regulates various processes, including inflammatory reactions [12]. Numerous research data has revealed that post-ischemic inflammatory reactions assist in the progression of neuronal injury and cerebral infarction [13]. Initiation of PI3K/Akt signaling has been demonstrated to reduce brain injury in ischemic stroke models [14, 15]. Mammalian target of rapamycin (mTOR), a major effector protein of Akt, comprises the protein complexes - mTORC1 and mTORC2 [16]. mTORc1 phosphorylates ribosomal protein S6 kinases (s6K) activates Akt

and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). The event subsequently leads to the discharge of eukaryotic translation initiation factor 4E (eIF4E) that results in cell cycle progression. mTORc2, via a positive feedback loop, stimulates Akt activation, subsequently inducing cell proliferation and survival [17]. Activation of Akt/mTOR signal increases the survival of neurons [18] and also induces activation of hypoxia-inducible factor-1 α (HIF-1 α) [19, 20].

HIF-1 α is a significant oxygen level sensor in the cells that is involved critically in the physiological process as cell survival, cell metabolism and metabolic adaptation [21]. HIF-1 α regulates various pivotal genes, including vascular endothelial growth factor (VEGF) [22] and heme oxygenase-1 (HO-1) [23]. Increased expressions of HIF-1 α and HO-1 has been reported to offer neuroprotection [10].

Compounds that could reduce inflammation, and that could potentially activate PI3K/Akt signaling and up-regulate HIF-1 α and HO-1 could be of immense clinical value. The present work aimed in evaluating the protective effects of 6-shogaol in I/R injury and whether 6-shogaol treatment improved the neuroprotective effects of sevoflurane post-conditioning following I/R injury. 6-shogaol is a major gingerol found in the dried rhizomes of ginger [24, 25]. Studies have explored numerous pharmacological activities of 6-shogaol including antioxidant, [26] anticancer, [27, 28] neuroprotective [29] and anti-inflammatory effects [30, 31].

MATERIALS AND METHODS

Animals and cerebral I/R induction

The handling of animals, study design, grouping, methods and procedures for investigation were permitted by the University Ethical Committee and conducted in agreement with the guidelines of the National Institutes of Health on the care and use of animals [32].

From the institution's animal breeding center, adult Sprague-Dawley rats (male; n = 96; 280 - 300 g) were procured. The rats (n = 3/cage) were maintained under 12h day/12h night cycle and controlled laboratory conditions (22-23°C, relative humidity 55- 60 %). The animals were allowed freely to access standard rat diet and water. Prior initiation of the study, the animals were adapted for about 5 days to the laboratory environments.

The animals were assigned randomly to experimental groups (8 groups; n = 12/group). 6-shogaol at 25 mg or 50 mg/kg body weight was given orally to rats for 14 days prior to ischemia/reperfusion initiation. 6-shogaol was administered, 60 minutes before I/R insult on day 15 (the day of induction).

On the day before insult, the rats were subjected to 8 to 10h fasting; however, were permitted access to water. For I/R induction the animals were anaesthetized (25 mg/kg, zoletil) and subjected to cerebral infarction and I/R by middle cerebral artery occlusion (MCAO) method [33].

The left common carotid artery (CCA) was exposed via a ventral midline neck incision and the internal carotid artery (ICA) was isolated, and its extra cranial branch was tied near to its origin. A 3-0 nylon monofilament suture was introduced through the external carotid artery (ECA) into the ICA to prevent blood flow into the middle cerebral artery (MCA). The nylon filament was retained in position for 120 minutes for induction of

ischemia. For restoration of ICA-MCA blood flow, sutures were removed and for 24 h reperfusion was allowed.

Following I/R, induction, study animal body temperature was retained at 37°C by heating blanket that thermostatically regulated until the animals retrieved from the surgery

Study groups

Rats that were not subjected to I/R and administered normal saline orally via gavage served as normal control (Group 1). Rats that were administered with saline and subjected to I/R were marked as I/R control (Group 2). A separate group of rats (Group 3-4) orally administered with 6-shogaol (25 mg or 50 mg/kg body weight) were subjected to I/R. 6-shogaol (50 mg/ kg) alone treated rats not induced with I/R served as group 5. Sevoflurane post-conditioning groups (Groups 6-8) were induced with I/R and were then immediately exposed to sevoflurane (2.5% in oxygen) through a vaporizer (Sevorane Vapor 15.3, Abbott) for 1 hour following reperfusion. The supply of sevoflurane was maintained at the desired concentration and monitored (Gas analyzer; Datex-Oheda, Helsinki, Finland). Group 6- Rats were induced I/R and subjected to sevoflurane post-conditioning alone.

24 h following reperfusion, rats from each treatment group (n = 6/ group) were sacrificed by transcardial perfusion of saline and paraformaldehyde (4%) in 0.1 M phosphate buffer (ice-cold). Instantly, brain tissues were excised and stored at -80°C used for analysis.

Neurobehavioral deficit evaluation

Neurobehavioral deficits were assessed in rats at 24 h post reperfusion and prior sacrifice. The extent of the neurological deficit was graded based on parameters as - dynamic behavior, the symmetry of the forelimbs, balance of movements, climbing, touch response, and response to vibrissae touch. The behaviour of animals was graded on a scale between 0-4

as follows: 0- normal (no deficit); 1- mild deficits observed; 2- moderate deficit; 3-severe deficit; and 4, very severe deficit [34-36].

Brain water content

The sectioning of excised brain tissue at 2 mm intervals in the coronal plane was carefully executed, and the tissue sections were measured for wet weight. The dry weight of the brain slices were determined by weighing the tissue slices after drying in a hot air oven at 70 °C for 72 h. The formula that was used to determine the brain water content is $(\text{wet weight} - \text{dry weight}) / (\text{wet weight}) \times 100 (\%)$.

TTC staining

Tissue viability and infarct size were measured by 2,3,5-triphenyltetrazoliumchloride (TTC) staining of the tissues, following 24 h post reperfusion. Brain tissue sections (2 mm) were treated with TTC (30 min; 37 °C) and then were plunged in para formaldehyde (4%) overnight in darkness. Tissue sections were visualised, and the infarcted region was determined using NIH ImageJ software (Version 1.42; NIH, Bethesda, MD). The infarcted region remained unstained, while normal areas appeared stained with TTC. The extent of infarction was determined as follows $\text{infarct area } (\%) = \text{infarct area} / \text{total area of slice} \times 100$.

Assessment of neuronal apoptosis

Terminal transferase-mediated dUTP nick end-labelling (TUNEL) staining was done for evaluation of neuronal apoptosis following I/R. Tissue sections of a 5- μm thickness (n = 6/ group) were treated as per the instructions specified (DeadEnd TM fluorometric TUNEL system kit, Promega, Madison, WI, USA) and were analysed for apoptotic cells by NIS-Elements BR imaging processing and analysis software (Nikon Corporation, Japan).

Semi-quantitative RT-PCR

Brain samples excised 24 h after perfusion was used for analysing the expression by RT-PCR and western blotting. For RT-PCR process, Total tissue RNA isolated from ischemic cortical samples from the different treatment groups (n=6/ group) and were used for the assay. NucleoSpin RNA II kit and Titanium One-step RT-PCR kit from BD Biosciences were employed to RNA isolation and for RT-PCR respectively. Following primers were used: HIF-1 α – Forward: 5'-AAG TCT AGG GAT GCA GCA C-3', Reverse: 5'-CAA GATCAC CAG CAT CTA G-3' HO-1- Forward: 5'- GAG ATT GAG CGC AAC AAG GA-3', Reverse: 5'-AGC GGT AGA GCT GCT TGA ACT-3'. The amplified PCR products of the test genes were normalised with the expressions of β -actin as an internal control. Forward: 5'-CCC TCA AGA TTG TCA GCA ATG C-3', Reverse: 5'-GTC CTC AGT GTA GCC CAG GAT-3'. PCR products were visualised by Molecular Imager FX (Bio-Rad, USA) and analysed using Quantity One software (Bio-Rad).

Western blotting

The excised ischemic cortical samples (n = 6/ group) were homogenized in ice-cold cell lysis buffer containing cocktail of protease inhibitors (Cell Signaling Technology). The total protein content of the tissues were quantified using protein assay kit from Thermo Fischer Scientific. Protein samples (n = 6/group) of equal concentrations (60 μ g) from the different treatment groups were loaded on SDS-PAGE (to 10-12 %) gels and were electrophoresed. The size-fractioned proteins were electrotransferred onto a nitrocellulose membrane (ThermoFischer, USA). The blotted membranes were treated with non-fat dry milk (5%) followed by overnight incubation at 4 °C with appropriate concentration of specific primary antibodies and further incubated at room temperature (1 h) with secondary antibodies (peroxidase-conjugated; Santa Cruz Biotechnology). Primary antibodies against

the following were used: Bcl-2, Bax, Bad, β -actin, cleaved-caspase-3, HIF-1 α , HO-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt, PI3K, p-Akt, p-PI3K (Cell Signaling Technology, Beverly, MA, USA), mTOR, p-mTOR, s6K and p-s6K (Abcam, Cambridge, MA, USA). The immunoreactive bands were detected and analysed in an electrochemiluminescence system (ECL, Millipore, USA). The intensity of the bands were standardised to expressions of β -actin that was run in parallel as the internal control.

Statistical analysis

The data obtained are presented as mean \pm SD (n=6) and analysed by one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) using SPSS version 21.0 (IBM Corporation, USA); p-values < 0.05 were marked as statistically significant values.

RESULTS

6-shogaol and sevoflurane attenuated I/R-induced neurobehavioral deficits

The animals of all treatment groups exhibited normal behavioural responses and neuromuscular coordination an hour before I/R induction. However, 24 h following I/R substantial ($p < 0.05$) neurological deficits were observed (Fig. 1). The I/R induced animals were noticed to be hesitant to move about and walk. The flexibility of the left limbs was found to be reduced. The animals were noticed to struggle to respond to the test stimulus. The neurological deficits were observed to substantially ($p < 0.05$) reduce treatment with 6-shogaol given at 25 and 50 mg doses.

Further, development in the neuro-behavioural responses and muscular coordination was noted in rats that were administered with 6-shogaol and as well exposed to 2.5% sevoflurane for 60 minutes immediately after reperfusion. Animals administered 6-shogaol and exposed to sevoflurane exhibited better neuroprotective effects vs. sevoflurane or 6-shogaol when given alone. Further, the behaviour of the group 5 animals that were administered with 6-shogaol at 50 mg/kg dosage and not subjected I/R injury did not exhibit any neurological deficits. The behaviour of the rats was similar to that of normal control.

6-shogaol and sevoflurane reduced brain edema and infarct area

Infarct area following I/R was determined by TTC staining. Infarcted regions appeared as unstained regions vs. normal regions stained with TTC. The ischemic areas were observed predominantly in the striatum and the frontoparietal cortex regions (Fig. 2). Pre-treatment with 6-shogaol at 25 and 50 mg doses resulted in a substantially ($p < 0.05$) reduced brain edema and infarct volume following I/R (Fig. 2 a and b).

Nevertheless, supplementation of 6-shogaol followed by sevoflurane post-conditioning more significantly ($p < 0.05$) diminished the I/R-induced cerebral infarct size,

and brain water content in comparison to 6-shogaol alone treated I/R induced animals. Interestingly, post-conditioning with sevoflurane though exerted protective effects pre-treatment with 6-shogaol irrespective of the dosage given, was noticed to exert greater protective effects compared to sevoflurane post-conditioning alone. The infarct volume was observed to decrease from 65.15% to 5.80% on 50 mg 6-shogaol treatment and post-conditioning with sevoflurane. Further, group 5 rats that were treated with 6-shogaol at 50 mg dose alone did not exhibit any neuronal injury.

6-shogaol and sevoflurane inhibited I/R-induced neuronal cell death

To assess the neuroprotective effects of 6-shogaol pre-treatment was further evaluated by TUNEL assay. The data obtained illustrated that post-conditioning with sevoflurane following 6-shogaol pre-treatment produced a substantial ($p < 0.05$) reduction in I/R-induced nerve cell death (Fig. 3). 6-shogaol administered alone at both the doses (25 mg and 50 mg) before I/R caused a substantial ($p < 0.05$) decline in TUNEL positive cell counts. Further, significant neuroprotection following I/R brain injury was observed in rats that were treatment with 6-shogaol followed by sevoflurane post-conditioning vs. rats exposed to sevoflurane or 6-shogaol. The apoptotic cell counts decreased from 141 cells/mm² in I/R control to 58 cells/mm² on 6-shogaol treatment at 50 mg/kg dose. While 6-shogaol and sevoflurane post conditioning decreased apoptotic cell counts to 19 cells/ mm².

To further assess the neuroprotective effects of 6-shogaol treatment and sevoflurane post-conditioning, the expressions of the proteins associated with cell apoptosis were evaluated by Western blotting (Fig. 4 a-d). Markedly ($p < 0.05$) higher expressions of Bad, Bax and Cleaved caspase-3, were noticed in the I/R control group vs. normal control. The expressions of Bcl-2, the anti-apoptotic protein was reduced in I/R control in comparison with the control. These enhanced expressions of pro-apoptotic proteins possibly could have

contributed to the increased neuroapoptosis as noticed in TUNEL assay. 6-shogaol treatment prior to I/R induction resulted in decreased expressions of Bax, Bad and cleaved caspase-3 vs. I/R control (Fig. 4 a-d). However, the data revealed that 6-shogaol pre-treatment, along with sevoflurane post-conditioning more effectively down-regulated the expressions of pro-apoptotic proteins. Bcl-2 expression increased to 85% on exposure to 2.5% sevoflurane following reperfusion vs. 48% in I/R control. Interestingly, shogaol pre-treatment at doses 25 mg/kg and 50 mg/kg along with sevoflurane post-conditioning, enhanced the expressions of Bcl-2 to 98% and 100% respectively, demonstrating the anti-apoptotic efficacy of 6-shogaol. The results indicate that 6-shogaol significantly potentiated the neuroprotective effects of sevoflurane.

6-shogaol and sevoflurane post-conditioning improved HO-1 and HIF- α

6-shogaol treatment was found to enhance expressions levels of HO-1 and HIF- α both at mRNA and protein levels following I/R (Fig. 5 a-d). The substantially increased ($p < 0.05$) mRNA expressions of HO-1 and HIF- α in I/R control was further enhanced on 6-shogaol treatment at 25 mg and 50 mg doses. The mRNA levels of HIF- α and HO-1 increased to 1.9 and 2.23 folds respectively in rats treated with 6-shogaol and sevoflurane vs. 1.35 and 1.23 folds in IR control. Post-conditioning with sevoflurane following 6-shogaol pre-treatment was noticed to significantly ($p < 0.05$) elevate protein expressions of HO-1 and HIF- α vs. I/R control. Post-conditioning with sevoflurane alone enhanced the translation of HIF- α and HO-1, as seen in the raise of the protein levels from 114% and 109% in IR control to 147.76% and 141.82%. While 6-shogaol treatment along with sevoflurane post-conditioning up-regulated the expressions of HO-1 and HIF- α to 171.2% and 169% respectively vs. 136% and 139.8% in 6-shogaol alone treated group. The results suggest 6-shogaol, and sevoflurane

post-conditioning was more effective in regulating the expressions HIF- α and HO-1 in comparison with sevoflurane or 6-shogaol administered alone.

6-shogaol and sevoflurane post-conditioning activated the PI3K/Akt/ mTOR/s6K signaling

Analysis of the protein expression by western blotting showed up-regulation in the phosphorylated forms of PI3K, Akt, mTOR, s6K phosphorylation in I/R vs. normal control. 6-shogaol treatment was found to enhance the phosphorylation levels of PI3K, Akt, mTOR and s6K proteins (Fig. 6 a-e) vs. I/R control. Interestingly, 6-shogaol treatment and sevoflurane post conditioning were found to be more effective in the stimulation of PI3K/Akt/ mTOR/s6K signaling pathway in comparison to sevoflurane post-conditioning alone. p-PI3K expressions were noticed to increase to 2.1 fold from 1.4 fold in rats treated with 6-shogaol and exposed to sevoflurane vs. sevoflurane alone exposed rats (Fig. 6 a and b). p-mTOR and p-Akt expression enhanced to 2.5 and 2.7 folds (Fig. 6 a, c-d). p-s6K levels increased from 1.58 fold in I/R control to 3.6 folds on treatment with 6-shogaol and sevoflurane post-conditioning (Fig. 6 a and e). Further, 6-shogaol (50 mg/kg) administered to rats that were not induced with I/R did not affect the expression of proteins in comparison to normal control. The results demonstrate the protective effects of 6-shogaol and also that 6-shogaol enhanced the neuroprotection of sevoflurane post-conditioning.

DISCUSSION

I/R-induced brain injury, a pathological complication resulting from various neurological and cardiovascular procedures may source many clinical concerns. Consequently, the identification of more effective strategies to prevent IR-induced brain injury is inevitable for cerebral protection [37]. Volatile anaesthetic, Sevoflurane, is widely employed in surgeries owing to its negligible pungency, low solubility and toxicity. Studies have revealed that sevoflurane post-conditioning alleviated I/R induced brain injury [38, 10]. Preconditioning with sevoflurane has been shown to enhance spatial learning and also improve memory in rodents following I/R [39].

Prior treatment with 6-shogaol was found to exert significant neuroprotective effects, as evidenced by a substantial decrease in the brain water content and cerebral infarct volume along with improved neuromuscular coordination. Sevoflurane post-conditioning following reperfusion also resulted in a noticeably decreased infarct volume and edema. Sevoflurane also alleviated I/R-induced neurobehavioral deficits. Interestingly the infarct volume and edema were reduced more significantly, and muscular coordinations were found to be effectively near the normal in animals that were pre-treatment with 6-shogaol and exposed to sevoflurane compared to animals administered with sevoflurane or shogaol alone. These observations illustrate that 6-shogaol markedly increased the neuroprotective efficacy of sevoflurane.

Apoptosis is well documented as a major mechanism resulting in neuronal cell death in I/R [40]. Neuronal cell apoptosis at 24h following I/R was analysed by TUNEL assay. Significantly ($p < 0.05$) lesser neuroapoptotic cell counts were noticed in rats administered with 6-shogaol and/or post-conditioned with sevoflurane. Nevertheless, the results were more pronounced in sevoflurane post conditioned rats that were prior treated with 6-shogaol.

Also, the effects of 6-shogaol and sevoflurane were assessed on the expressions of proteins associated with cell apoptosis. Previous investigations have illustrated that the neuroprotective effects of volatile anaesthetics following cerebral IR injury involve anti-apoptotic mechanisms [19, 41]. Our results revealed significantly down-regulated expressions of pro-apoptotic factors, cleaved caspase 3, Bax and Bad along with substantially up-regulated anti-apoptotic protein- Bcl-2 on treatment with 6-shogaol and post-conditioning with sevoflurane. Similar to the results of TTC staining and TUNEL assay, administration of both 6-shogaol prior I/R and post-conditioning with sevoflurane more effectually regulated the expressions of apoptosis-associated proteins. Studies by Bedirli *et al.* [1] showed that pre-conditioning with sevoflurane caused inhibition of apoptosis via downregulation of pro-apoptotic protein as Tp53 and through elevated anti-apoptotic proteins as Bcl-2. The data obtained demonstrate the effectiveness of 6-shogaol in potentiating the neuroprotective effects of sevoflurane post-conditioning following I/R.

We further explored the effects of 6-shogaol and sevoflurane post-conditioning on the PI3K/Akt /mTOR/s6K signaling. The PI3K/Akt signal is critically regulated neuronal survival. The pathway is articulated extensively in the CNS and is stimulated by several growth factors as the nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [42].

The PI3K/Akt/mTOR pathway is the major pathway that involves in cell survival and is well documented to exert protective roles in ischemic brain injury [14, 15, 43]. Akt/ Protein kinase B (PKB) exerts anti-apoptotic roles upon activation by phosphorylation in a PI3K-dependent-manner [44, 45]. The previous study by Maiese *et al.* [46] illustrated that suppression of the PI3K/AKT/mTOR pathway was associated with the pathogenesis of myocardial I/R injury.

The results revealed, improved levels of phosphorylation of proteins-PI3K, Akt and mTOR, on 6-shogaol pre-treatment suggesting activation of the pathway. Sevoflurane post-conditioning following reperfusion resulted in marked activation of PI3K, Akt, mTOR and s6K proteins. Similar observations were presented by Wang *et al.* [38].

The PI3K/Akt pathway upon activation, induces the activity of the anti-apoptotic proteins and deactivates the pro-apoptotic factors [47]. Activation of the signal by sevoflurane and 6-shogaol could have contributed to the increased expressions of Bcl-2 in line with down-regulated Bad and Bax levels. Activated Akt exerts anti-apoptotic effects by phosphorylating and inactivating Bad and inducing the release of Bcl-xL, an anti-apoptotic protein, that inhibits pro-apoptotic- Bax [48-51].

Sevoflurane-induced up-regulation of p-Akt as observed in the present suggests inhibition of pro-apoptotic proteins. Isoflurane pre-conditioning was shown to exert neuroprotective effects via up-regulation of the Akt/mTOR/s6K signaling pathway [52]. In line with the observations reported by Yan *et al.* [52] the data obtained from the present study also revealed activation of the pathway by sevoflurane and 6-shogaol. The activation of the PI3K/Akt/mTOR/s6K signal was more pronounced upon 6-shogaol and sevoflurane post-conditioning in comparison to either sevoflurane or 6-shogaol administered alone.

The findings our study also revealed a significantly ($p < 0.05$) increased mRNA and HIF-1 α and HO-1 protein levels on treatment with sevoflurane and/or 6-shogaol. Further, prior treatment with 6-shogaol followed by sevoflurane post-conditioning more effectively enhanced HIF-1 α and HO-1 expressions at transcriptional and translational levels as well. HIF-1 α , activated under hypoxic conditions, plays a critical role in ischemia [53, 54]. HIF-1 regulates many genes associated with vital events of the cells including cell survival, proliferation, and iron metabolism [55-57]. HO-1 is one of the major protein that is regulated by HIF-1 α that is induced under ischemic condition [23]. Protective effects of HO-1 in

ischemia is well documented [58]. Neuroprotective effects of HO-1 could be attributed to the effective regulation of intracellular heme levels and conversion of heme to antioxidant bilirubin by HO-1 [59]. Thus, markedly improved HIF-1 α and HO-1 noticed on 6-shogaol and/or sevoflurane post-conditioning in part could have contributed to the decrease in neuronal apoptosis as observed. However, the administration of 6-shogaol before induction of I/R injury demonstrated to substantially potentiate the neuroprotective effects of sevoflurane-post-conditioning. Furthermore, 6-shogaol at 50 mg/kg dose when administered to rats not induced with I/R did not alter the expressions of tested proteins, suggesting, its cytoprotective properties.

CONCLUSION

The results illustrate that 6-shogaol supplementation prior to induction of I/R exhibited neuroprotective effects and also effectually potentiated the protective efficiency of sevoflurane-post-conditioning, via activating PI3K/Akt/mTOR/s6K signaling and HIF-1 α and HO-1 expressions. The findings indicate 6-shogaol, as a new therapeutic candidate in I/R brain injury.

DECLARATIONS

Conflict of interest

No conflict of interest was declared by the authors of the study.

Contributions of Authors statement

The authors have equally contributed in the study design, layout, conduction of the experiments and in the presentation of this study.

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FIGURE LEGENDS

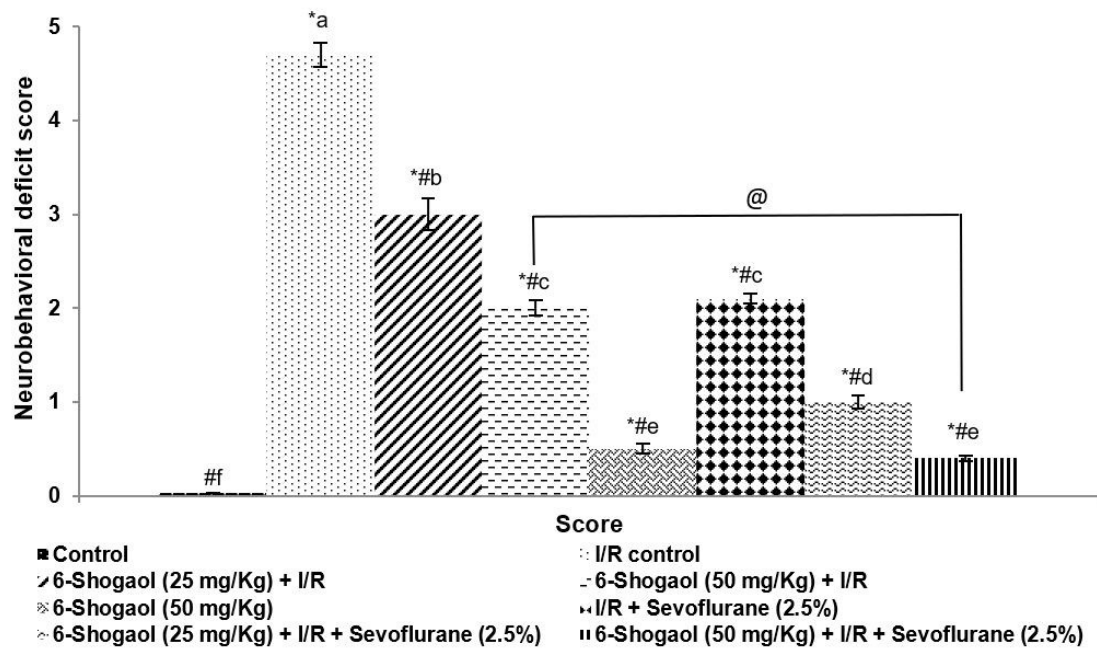


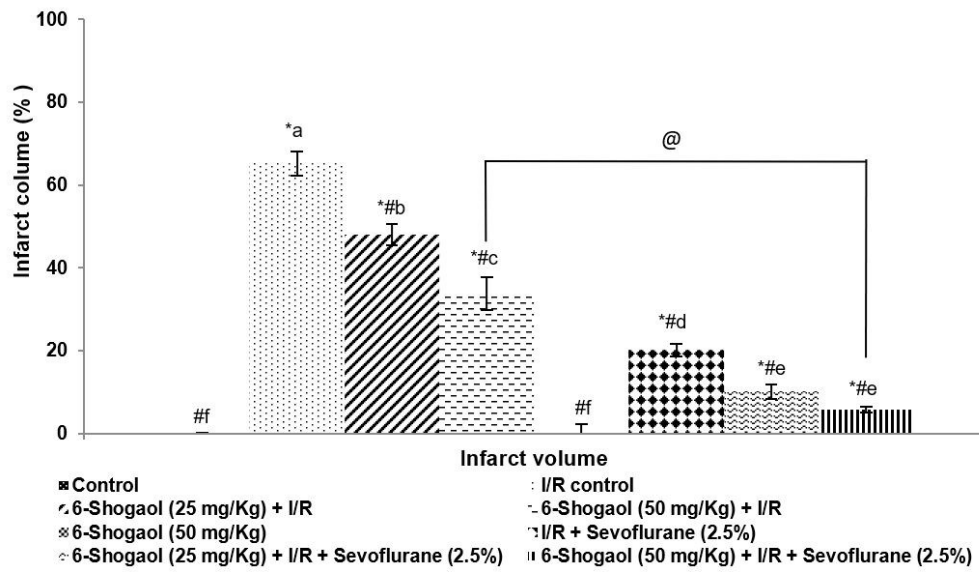
Figure 1. Effects of 6-shogaol and sevoflurane on I/R induced neurobehavioural deficits

The data are represented as mean \pm SD, $n = 6$. $p < 0.05$ as obtained by one-way ANOVA and DMRT analysis.

* represents $p < 0.05$ vs. control; # represents $p < 0.05$ vs. I/R control; @ represents 6-shogaol (50 mg/Kg) + I/R vs. 6-shogaol (50 mg/Kg) + I/R+ sevoflurane (2.5%) at $p < 0.05$

a-f represents means from various study groups that differ at $p < 0.05$

A



B

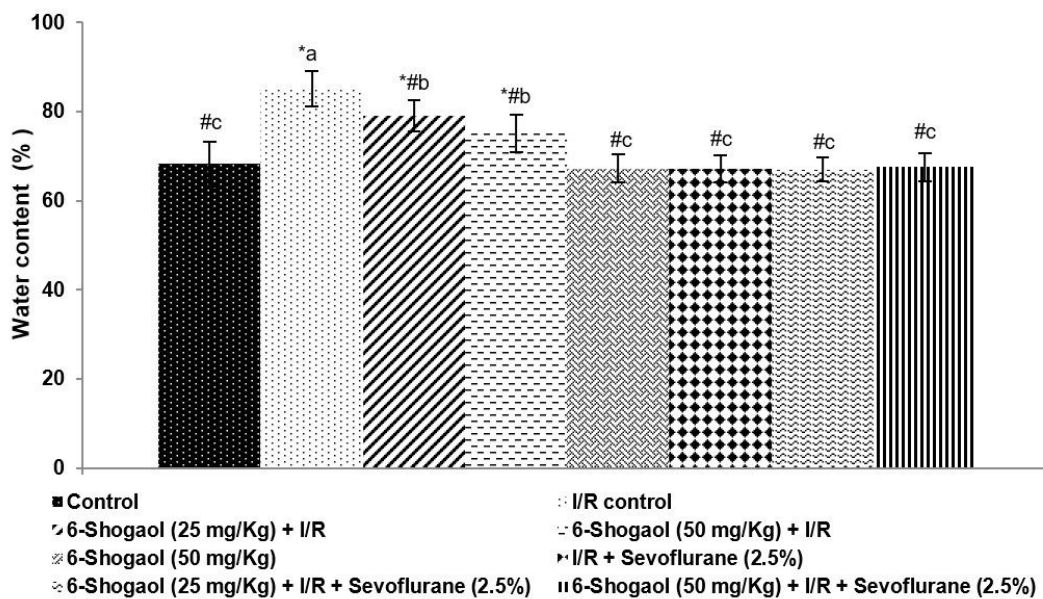


Figure 2. Effect of 6-shogaol and sevoflurane on brain edema and infarct area

6-shogaol and sevoflurane reduced cerebral infarct area (a) 6-shogaol and sevoflurane reduced brain edema following I/R injury (b)

The data are represented as mean \pm SD, $n = 6$. $p < 0.05$ as obtained by one-way ANOVA and DMRT analysis.

* represents $p < 0.05$ vs. control; # represents $p < 0.05$ vs. I/R control; @ represents 6-shogaol (50 mg/Kg) + I/R vs. 6-shogaol (50 mg/Kg) + I/R+ sevoflurane (2.5%) at $p < 0.05$

a-f represents means from various study groups differing at $p < 0.05$

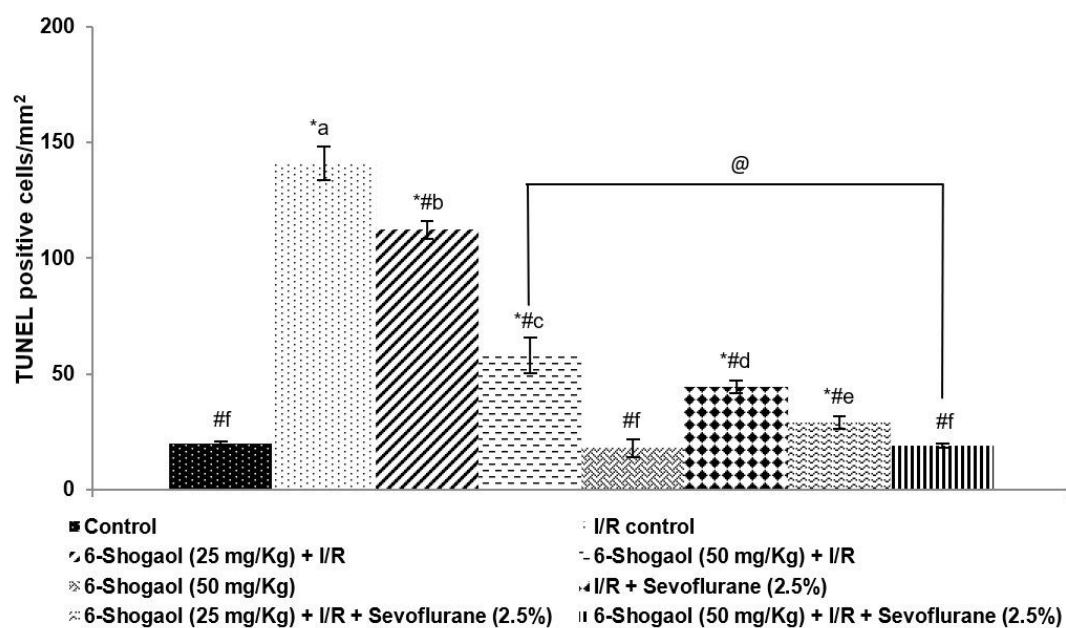


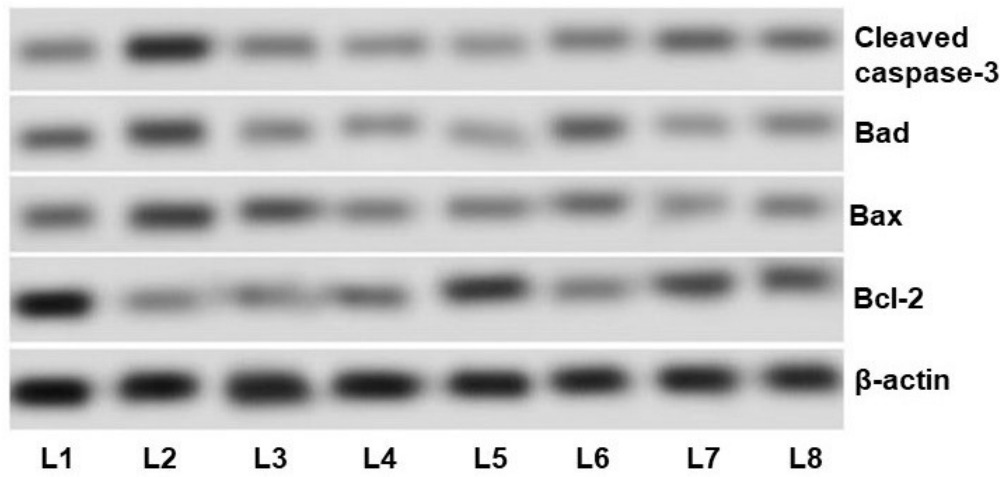
Figure 3. Effects of 6-shogaol and sevoflurane post-conditioning on neuronal apoptosis

The data are represented as mean \pm SD, $n = 6$. $p < 0.05$ as obtained by one-way ANOVA and DMRT analysis..

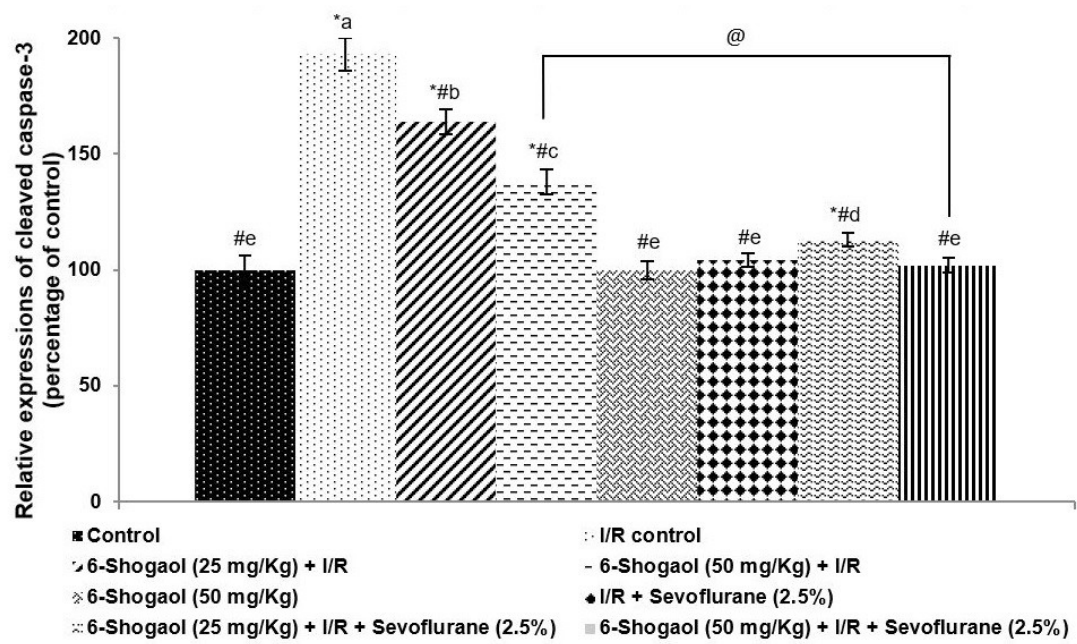
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a-f represents means of the various study groups that differ at $p < 0.05$.

A



B



C

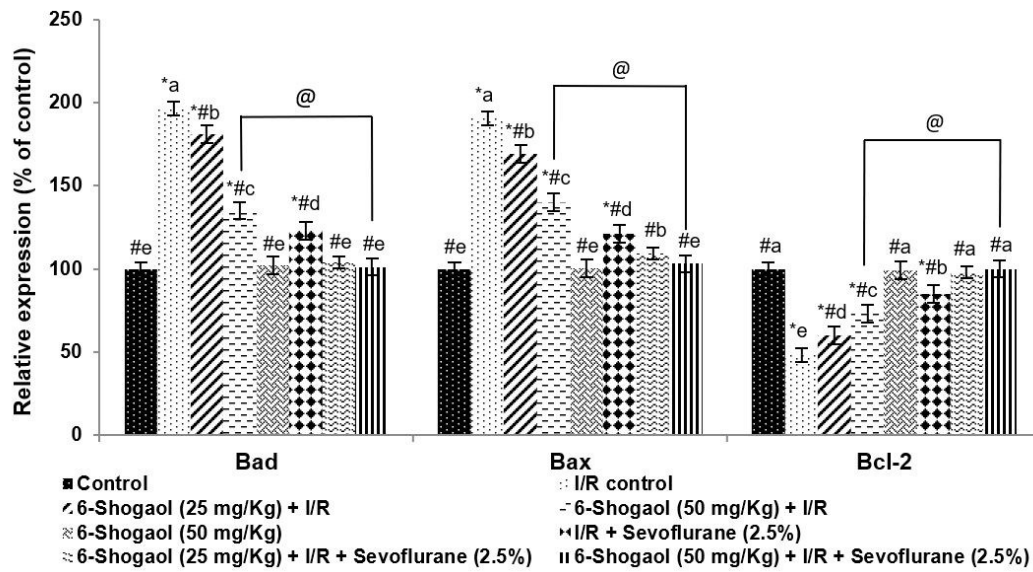


Figure 4. Effects of 6-shogaol and sevoflurane post-conditioning on the expressions of apoptotic protein following ischemia/reperfusion injury

Representative immunoblot (A) Expressions of test proteins relative to control expressions set at 100% (B and C).

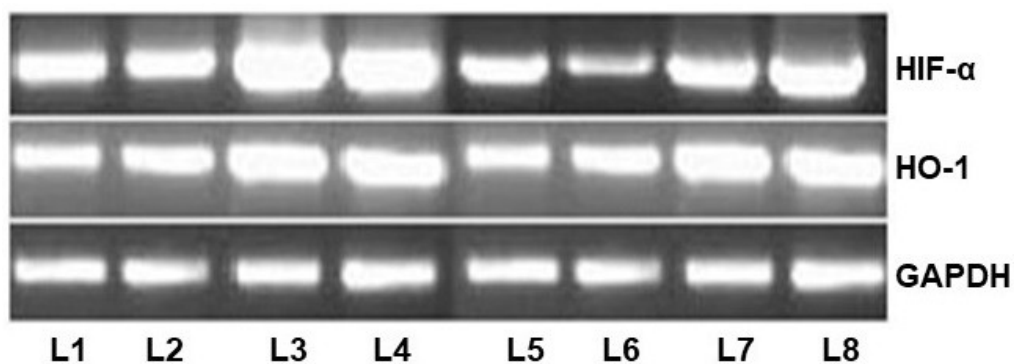
The data are represented as mean \pm SD, $n = 6$. $p < 0.05$ as obtained by one-way ANOVA and DMRT analysis.

* represents $p < 0.05$ vs. control; # represents $p < 0.05$ vs. I/R control; @ represents 6-shogaol (50 mg/Kg) + I/R vs. 6-shogaol (50 mg/Kg) + I/R+ sevoflurane (2.5%) at $p < 0.05$

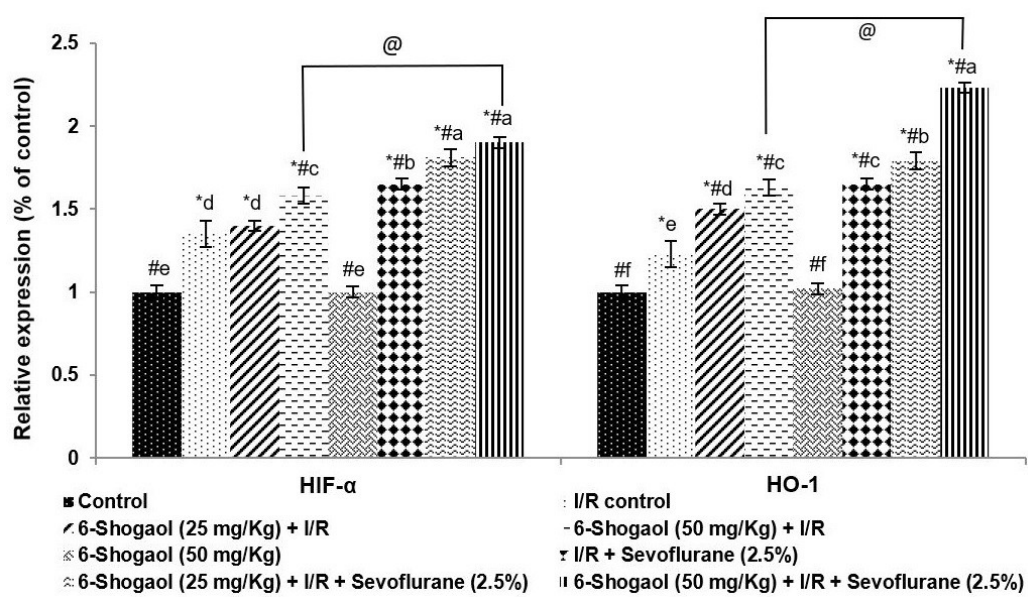
a-f represents means from various study groups differing at $p < 0.05$

[L1-Control; L2-I/R control; L3-6-shogaol (25 mg/Kg) + I/R; L4-6-shogaol (50 mg/Kg) + I/R; L5-6-shogaol (50 mg/Kg); L6-I/R + sevoflurane (2.5%); L7-6-shogaol (25 mg/Kg) + I/R + sevoflurane (2.5%); L8-6-shogaol (50 mg/Kg) + I/R + sevoflurane (2.5%)]

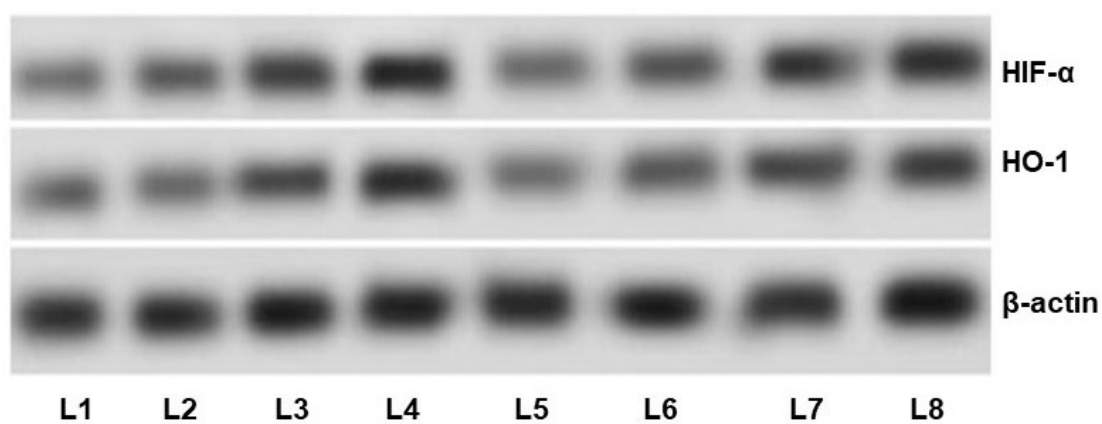
A



B



C



D

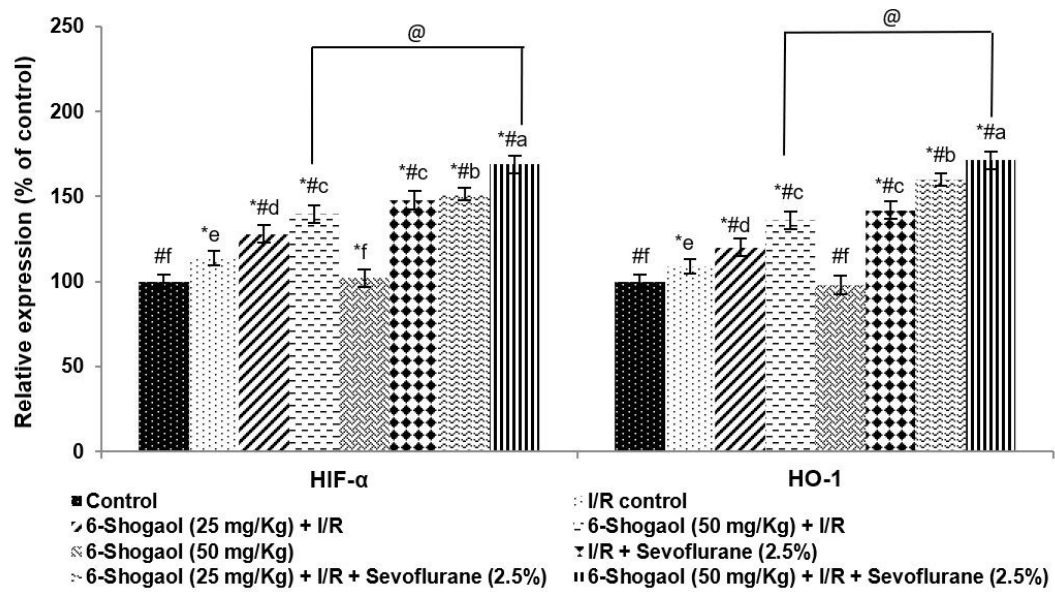


Figure 5. 6-shogaol and sevoflurane regulated HIF- α and HO-1 expressions following ischemia/reperfusion injury

Representative gel of RT-PCR analysis (A) Relative expressions of HIF- α and HO-1mRNA (B) Representative immunoblot (C) Protein expressions relative to control set at 100% (D)

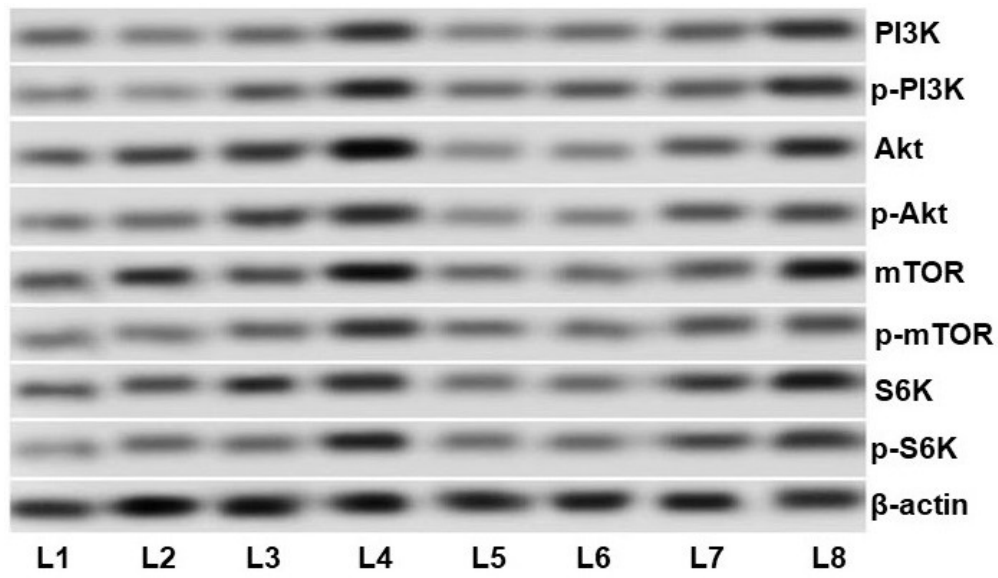
The data are represented as mean \pm SD, $n = 6$. $p < 0.05$ as obtained by one-way ANOVA and DMRT analysis.

* represents $p < 0.05$ vs. control; # represents $p < 0.05$ vs. I/R control; @ represents 6-shogaol (50 mg/Kg) + I/R vs. 6-shogaol (50 mg/Kg) + I/R+ sevoflurane (2.5%) at $p < 0.05$

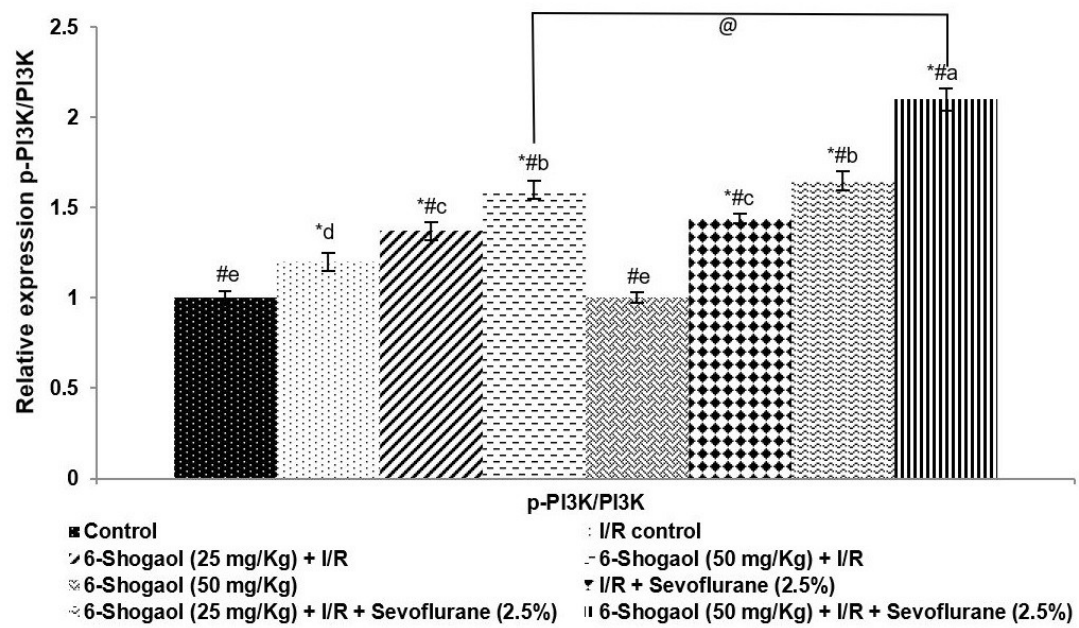
a-f represents means from various study groups differing at $p < 0.05$

[L1-Control; L2-I/R control; L3-6-shogaol (25 mg/Kg) + I/R; L4-6-shogaol (50 mg/Kg) + I/R; L5-6-shogaol (50 mg/Kg); L6-I/R + sevoflurane (2.5%); L7-6-shogaol (25 mg/Kg) + I/R + sevoflurane (2.5%); L8-6-shogaol (50 mg/Kg) + I/R + sevoflurane (2.5%)]

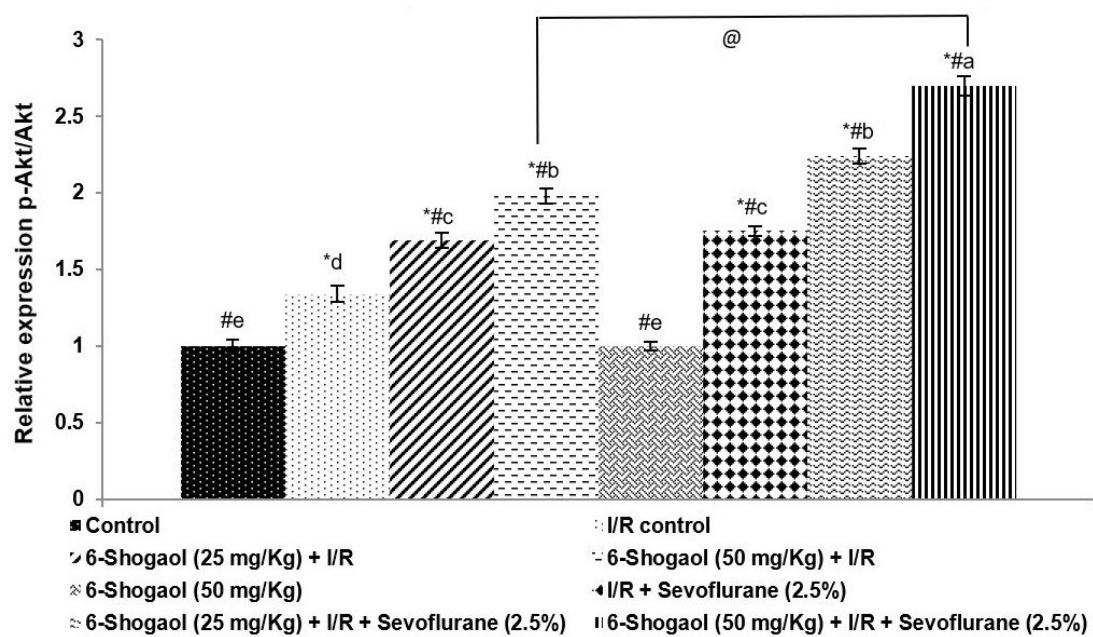
A



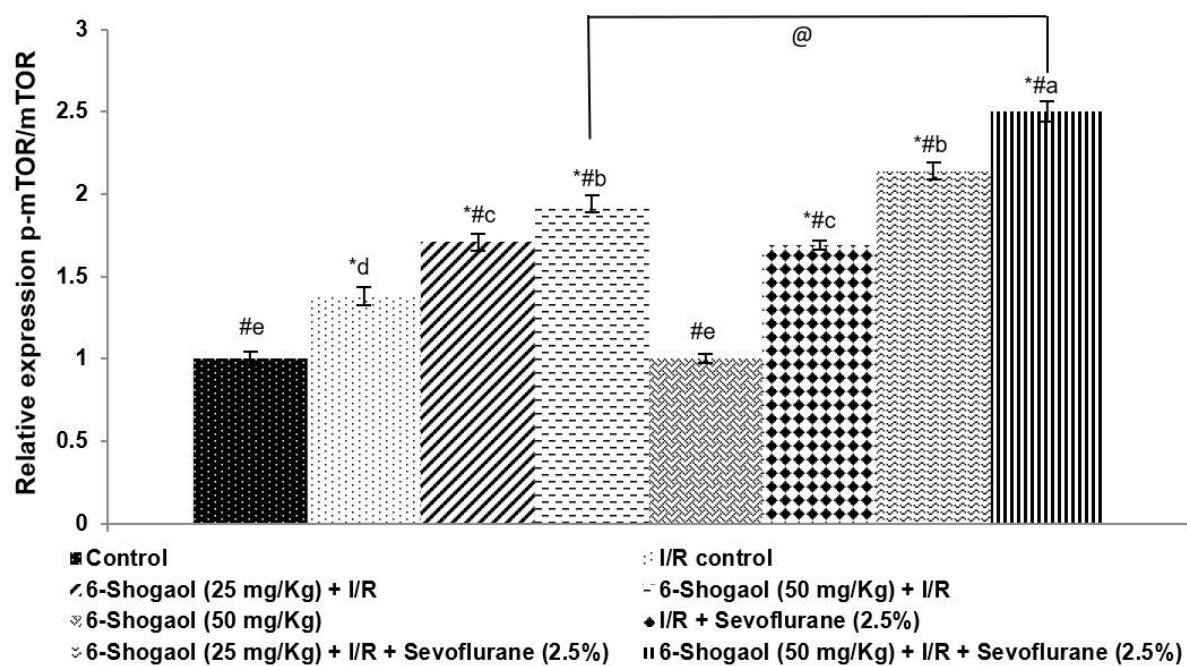
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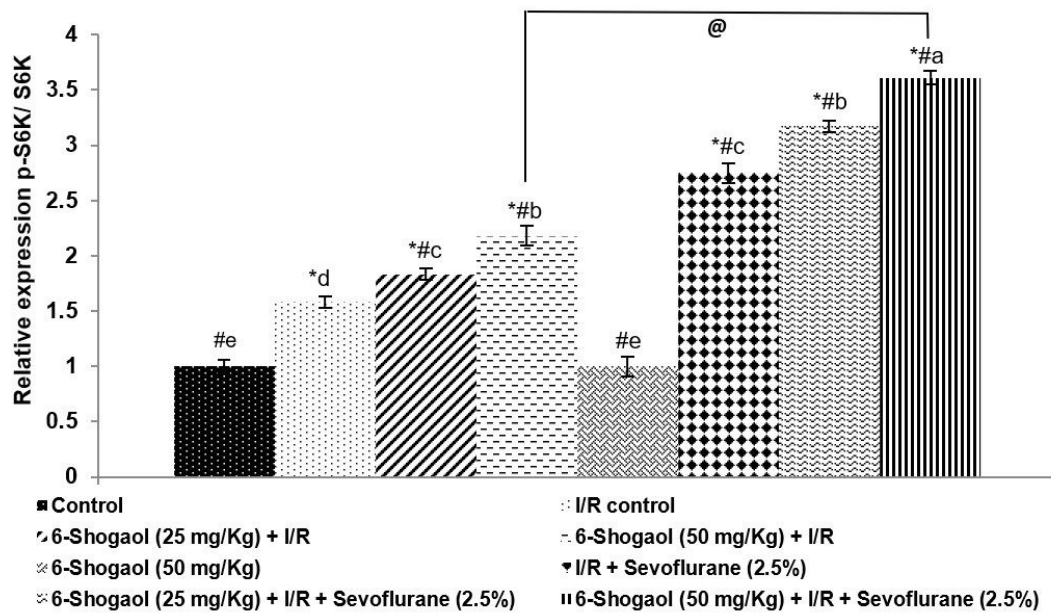


Figure 6. Effects of 6-shogaol and sevoflurane post-conditioning on the PI3K/Akt/mTOR/s6K signaling following ischemic/reperfusion injury.

Representative immunoblot of protein expressions (A) Phosphorylated protein levels to respective total protein levels (B-D).

The data are represented as mean \pm SD, $n = 6$. $p < 0.05$ as obtained by one-way ANOVA and DMRT analysis.

* represents $p < 0.05$ vs. control; # represents $p < 0.05$ vs. I/R control; @ represents 6-shogaol (50 mg/Kg) + I/R vs. 6-shogaol (50 mg/Kg) + I/R+ sevoflurane (2.5%) at $p < 0.05$

a-f represents means from various study groups differing at $p < 0.05$.

[L1-Control; L2-I/R control; L3-6-shogaol (25 mg/Kg) + I/R; L4-6-shogaol (50 mg/Kg) + I/R; L5-6-shogaol (50 mg/Kg); L6-I/R + sevoflurane (2.5%); L7-6-shogaol (25 mg/Kg) + I/R + sevoflurane (2.5%); L8-6-shogaol (50 mg/Kg) + I/R + sevoflurane (2.5%)]