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ORIGINAL RESEARCH

Involvement of Endothelin-1, H_2S and Nrf2 in Beneficial Effects of Remote Ischemic Preconditioning in Global Cerebral Ischemia-Induced Vascular Dementia in Mice

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Abstract

The present study explored the role of endothelin-1, H_2S, and Nrf2 in remote preconditioning (RIPC)-induced beneficial effects in ischemia–reperfusion (I/R)-induced vascular dementia. Mice were subjected to 20 min of global ischemia by occluding both carotid arteries to develop vascular dementia, which was assessed using Morris water maze test on 7th day. RIPC was given by subjecting hind limb to four cycles of ischemia (5 min) and reperfusion (5 min) and it significantly restored I/R-induced locomotor impairment, neurological severity score, cerebral infarction, apoptosis markers along with deficits in learning and memory. Biochemically, there was increase in the plasma levels of endothelin-1 along with increase in the brain levels of H_2S and its biosynthetic enzymes viz., cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CLS). There was also an increase in the expression of Nrf2 and glutathione reductase in the brain in response to RIPC. Pretreatment with bosentan (dual blocker of ET_A and ET_B receptors), amino-oxyacetic acid (CBS synthase inhibitor), and DL-propargylglycine (CLS inhibitor) significantly attenuated RIPC-mediated beneficial effects and biochemical alterations. The effects of bosentan on behavioral and biochemical parameters were more significant than individual treatments with CBS or CLS inhibitors. Moreover, CBS and CLS inhibitors did not alter the endothelin-1 levels possibly suggesting that endothelin-1 may act as upstream mediator of H_2S. It is concluded that RIPC may stimulate the release endothelin-1, which may activate CBS and CLS to increase the levels of H_2S and latter may increase the expression of Nrf2 to decrease oxidative stress and prevent vascular dementia.

Keywords Cerebral ischemia · Dementia · Remote preconditioning · Remote postconditioning

Introduction

Ischemic preconditioning is the phenomenon in which delivery of short episodes of ischemia and reperfusion to a particular organ prior to onset of sustained ischemia prevents the subsequent deleterious effects of ischemia–reperfusion injury on that organ. This concept was initially discovered in heart and it was found to protect heart from prolonged ischemia–reperfusion injury (Hausenloy et al. 2016). Later this phenomenon was also found to exert protection in patients suffering from ischemic damage in clinical settings (Cabrera-Fuentes et al. 2016a, b). The phenomenon of ischemic preconditioning was extrapolated to develop the concept of remote ischemic preconditioning. Remote ischemic preconditioning is a novel intervention to prevent ischemia–reperfusion injury in which short cycles of ischemia and reperfusion to a remote organ affords protection to a target organ from sustained ischemic injury.

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Initially, Przyklenk et al. described that remote preconditioning is capable of inducing protection to heart from ischemic injury (Przyklenk et al. 1993). Later, this was also found to confer protection to other organs including kidney, liver, and intestine from ischemic injury (Gomes et al. 2018; Wojciechowska et al. 2018). Moreover, studies have also documented the beneficial effects of remote preconditioning in attenuating ischemic damage in the brain (Chen et al. 2018; Yang et al. 2018). Nevertheless, the mechanisms involved in remote preconditioning-induced neuroprotection are not fully explored. Vascular dementia is a state of impairment in the memory functioning secondary to problems related to blood supply to the brain. Ischemia–reperfusion-induced cerebral injury is one of more commonly employed models to induce vascular dementia in animals (Wu et al. 2015; Wan et al. 2015). In the present study, a state of cerebral ischemia reperfusion was induced by occlusion of both carotid arteries. In other words, a state of global ischemia was induced and this model of global ischemia–reperfusion injury in rodents mimics the clinically conditions of cardiac arrest, cardiac surgery, and severe hypotension (Harukuni and Bhardwaj 2006; Wiklund et al. 2012).

Scientists have attempted to occlude different arteries to deliver remote preconditioning stimulus to target organs including remote renal preconditioning (by occlusion of renal arteries) (Khaksari et al. 2017), remote mesenteric preconditioning (by occlusion of mesenteric arteries) (Teng et al. 2015), remote aortic preconditioning (by occlusion of aorta) (Emontzpohl et al. 2018), and remote hind limb preconditioning (occlusion of femoral arteries by applying pressure on the hind limbs) (Kim et al. 2019). In comparison to other methods, remote hind limb preconditioning has the advantage that the femoral arteries of the hind limbs can be occluded non-invasively by applying pressure more than systolic pressure through blood pressure cuff. Moreover, remote hind limb preconditioning has other advantage that short period of ischemia during occlusion of femoral arteries induces less damage to skeletal muscles as these are more resistant to ischemia. On the other hand, short periods of occlusion of renal arteries, mesenteric arteries or aorta may induce relatively more injury to body organs (Zhang et al. 2012; Randhawa and Jaggi 2016).

Hydrogen sulfide (H_2S) is a gaseous neurotransmitter in different tissues and organs including the brain (Kimura 2015). It is found to produce a large number of physiological effects including synaptic transmission, maintenance of vascular tone, angiogenesis (Kimura 2014), cardiovascular functions (Kuksis et al. 2014), and mitochondrial bioenergetic functions (Szabo et al. 2014). There have been studies suggesting that H_2S produces beneficial effects in a large number of diseases including cardiovascular diseases (Shen et al. 2015), gastrointestinal diseases (Chan and Wallace 2013), and renal diseases (Song et al. 2014). Cystathionine γ lyase and cystathionine β synthase are two main enzymes, which are involved in the biosynthesis of hydrogen sulfide (Panza et al. 2015). There have been a large number of studies documenting the protective role of H_2S and its biosynthetic enzymes in different models of learning and memory deficits (Liu et al. 2015; Li et al. 2016). N2rf is a transcriptional factor and it has been suggested that H_2S may produce beneficial effect in preventing cognitive decline through Nrf2 (Kumar and Sandhir 2018). However, the role of H_2S and Nrf2 in ischemia-reperfusion-induced vascular dementia has not been explored.

Endothelin is one of the major factors released from the endothelium (Pernow and Wang 1997) and its family comprises of three 21-amino-acid long isopeptides, endothelin-1, endothelin-2, and endothelin-3. These endothelins produce their actions by activating endothelin receptors, ET_A and ET_B (Rubanyi and Polokoff 1994). Studies have also shown that exogenous administration of endothelin produces preconditioning like effects in heart (Bugge and Ytrehus 1996; Gourine et al. 2005). A recent study has shown that remote preconditioning-induced cardioprotection involves release of endothelin-1 (Zhang et al. 2018). However, the role of endothelins in remote preconditioning-induced protective effects particularly in vascular dementia has not been explored. Therefore, the present study was designed to investigate the role of endothelin, H_2S, and Nrf2 in beneficial effects of remote ischemic preconditioning in global cerebral ischemia-induced vascular dementia in mice. To meet the aim and objectives of this study, amino-oxyacetic acid as a cystathionine β synthase inhibitor, DL-propargylglycine as irreversible inhibitor of cystathionine γ-lyase (Donovan et al. 2011; Szabo and Papapetropoulos 2017; Lertratanangkoon et al. 1999), and bosentan as a dual blocker of ET_A and ET_B receptors were employed.

Materials and Methods

In the present study, Swiss albino male mice of 20–25 g were employed and the experimental protocol was duly approved by China-Japan Union Hospital of Jilin University Ethics Committee with approval number AF-IRB-180239. All experiments were performed as per ethical guidelines. The drugs and chemicals including bosentan, amino-oxyacetic acid hemi hydrochloride, DL-propargylglycine, triphenyl tetrazolium chloride (TTC), thiopental, and others were procured from Sigma–Aldrich, USA. The ELISA kits for the quantitative estimation of endothelins, caspase-3, bcl-2, Nrf2, and glutathione reductase were procured from LifeSpan Biosciences, USA and ELISA kit for quantification of cystathionine-γ-lyase was procured from Aviva Systems Biology, USA. The fluorometric assay kit of cystathionine-β-synthase was procured from BioVision, Inc USA. The

doses of propargylglycine (Han et al. 2015; Lin et al. 2017), amino-oxyacetic acid (Xiao et al. 2016), and bosentan (Serafim et al. 2015) were selected on the basis of literature.

Global Cerebral Ischemia Reperfusion Model

Animals were anesthetized using thiopental sodium (45 mg/kg *i.p.*) and mice were subjected to global cerebral ischemia by occluding both carotid arteries for 20 min. Thereafter, occlusion was removed and blood flow was restored (Kim and Lee 2014; Ya et al. 2012).

Behavioral Tests to Assess Locomotor and Motor coordination Activities

The behavioral tests to assess the functionality of locomotion and motor coordination were performed on different days i.e., 24 h after ischemia and 7 days after ischemia.

Locomotor Activity Test

The locomotor activity was evaluated using an actophotometer test. The total score was noted for 10 min in an actophotometer test.

Inclined Beam Walking Test

The forelimb and hind limb motor coordination was assessed using an inclined beam walking test. Mice were allowed to walk on a metallic bar of 60 cm length and 1.5 cm width and inclined at an angle of 60°. The motor coordination was assessed by giving scores between 0 and 4. 0: No foot fault; 1: Foot faults between 45 and 60 cm; 2: foot faults between 30 and 60 cm; 3: foot faults between 15 and 60 cm, and 4: unable to walk on the beam (Ya et al. 2017).

Neurological Deficits Scoring

Mice were assigned scores from 0 (normal) to 10 (the most severe form) on the basis of total neurological deficits (Rodriguez et al. 2005; Ya et al. 2017).

Memory Testing on Morris Water Maze

On the 7th day after global ischemia, mice were subjected to memory testing on Morris Water maze. The animals were subjected to acquisition trials on 4 days (7th, 8th, 9th, and 10th day after ischemia) and it was followed by test for retrieval of memory on 11th day after ischemia. The escape latency time measured on first 4 days (acquisition trials) served as index of learning. The final reading was taken on 11th day after ischemia and escape latency time on this day depicted as index of retrieval (index of memory or retention) (Vorhees and Williams 2006).

Cerebral Infarct Size

On 11th day after memory testing, animals were sacrificed to remove brain. The half of brain portion was homogenized to form supernatant for biochemical estimations. The other half portion was kept in refrigerated for freezing. The frozen brain was cut in slices and these slices were stained using TTC. The viable portions are stained red, while non-viable portions are not stained and remain unstained. The extent of cerebral infarction was expressed in terms percentage (Okuno et al. 2001).

Caspase-3 Activity and Bcl-2 Expression

The apoptosis markers viz, caspase-3 activity and Bcl-2 expression were determined in the brain homogenate using ELISA kits as per manufacturer instructions.

Determination of H_2S

The levels of H_2S were measured in the brain homogenate colorimetrically at 670 nm using sodium hydrosulfide hydrate as standard (Stipanuk and Beck 1982; Xu et al. 2009; Hwang et al. 2013).

Expression of Cystathionine-β-Synthase, Cystathionine-γ-Lyase, Nrf2 and Glutathione Reductase

The activity of cystathionine-β-synthase was determined in the brain homogenate using fluorometric activity assay kits. The cystathionine-γ-lyase activity along with expressions of Nrf2 and glutathione reductase was determined in the brain supernatants using ELISA kit as per manufacture instructions.

Determination of Endothelin Levels

The levels of ET-1 were determined in the plasma using ELISA kits as per manufacturer's instructions.

Experimental Protocol

Eleven groups were employed and each group comprised of six animals (Table 1):

Normal Control

Animals were not subjected to ischemia and reperfusion. The different behavioral and biochemical tests were performed on different days as described above.

Table 1 Brief description of experimental protocol employed in the present study

S. No	Name of the groups	Protocol	Remote preconditioning stimulus	Pharmacological intervention
1	Normal control	No ischemia	–	–
2	Ischemia–reperfusion injury	Ischemia to brain for 20 min	–	–
3	Remote ischemic preconditioning	Ischemia to brain for 20 min	4 cycles of occlusion and de-occlusion of 5 min each on the hind limb before brain ischemia	–
4	Bosentan (50 mg/kg) in remote ischemic preconditioning	Ischemia to brain for 20 min	4 cycles of occlusion and de-occlusion of 5 min each on the hind limb before brain ischemia	Bosentan (50 mg/kg) 30 min before remote preconditioning
5	Bosentan (100 mg/kg) in remote ischemic preconditioning	Ischemia to brain for 20 min	4 cycles of occlusion and de-occlusion of 5 min each on the hind limb before brain ischemia	Bosentan (100 mg/kg) 30 min before remote preconditioning
6	Propargylglycine (25 mg/kg) in remote ischemic preconditioning	Ischemia to brain for 20 min	4 cycles of occlusion and de-occlusion of 5 min each on the hind limb before brain ischemia	Propargylglycine (25 mg/kg) 30 min before remote preconditioning
7	Propargylglycine (50 mg/kg) in remote ischemic preconditioning	Ischemia to brain for 20 min	4 cycles of occlusion and de-occlusion of 5 min each on the hind limb before brain ischemia	Propargylglycine (50 mg/kg) 30 min before remote preconditioning
8	Amino-oxyacetic acid (25 mg/kg) in remote ischemic preconditioning	Ischemia to brain for 20 min	4 cycles of occlusion and de-occlusion of 5 min each on the hind limb before brain ischemia	Amino-oxyacetic acid (25 mg/kg) 30 min before remote preconditioning
9	Amino-oxyacetic acid (50 mg/kg) in remote ischemic preconditioning	Ischemia to brain for 20 min	4 cycles of occlusion and de-occlusion of 5 min each on the hind limb before brain ischemia	Amino-oxyacetic acid (50 mg/kg) 30 min before remote preconditioning
10	Propargylglycine (50 mg/kg) and amino-oxyacetic acid (50 mg/kg) in Remote Ischemic preconditioning	Ischemia to brain for 20 min	4 cycles of occlusion and de-occlusion of 5 min each on the hind limb before brain ischemia	Propargylglycine and amino-oxyacetic acid (50 mg/kg) 30 min before remote preconditioning

Ischemia–Reperfusion Injury

After surgery, a thread was passed beneath the carotid arteries and the blood vessels were occluded for 20 min. Afterwards, the thread was removed and wound was incised. The different behavioral and biochemical tests were performed as described above.

Remote Ischemic Preconditioning

In anesthetized mice, the left hind limb was subjected to four alternate cycles of occlusion (5 min duration to induce ischemia) and de-occlusion (5 min duration to induce reperfusion) using a neonatal blood pressure cuff. Thereafter, animals were subjected to cerebral ischemia of 20 min duration. The different behavioral and biochemical tests were performed as described above.

Bosentan (50 mg/kg i.p.) in Remote Ischemic Preconditioning

Bosentan was administered 30 min before subjecting to remote ischemic preconditioning. Thereafter, animals were subjected to cerebral ischemia of 20 min duration. The different behavioral and biochemical tests were performed as described above.

Bosentan (100 mg/kg i.p.) in Remote Ischemic Preconditioning

*Bosentan was ad*ministered 30 min before subjecting to remote ischemic preconditioning. Thereafter, animals were subjected to cerebral ischemia of 20 min duration. The different behavioral and biochemical tests were performed as described above.

Propargylglycine (25 mg/kg i.p.) in Remote Ischemic Preconditioning

Propargylglycine was administered 30 min before subjecting to remote ischemic preconditioning. Thereafter, animals were subjected to cerebral ischemia of 20 min duration. The different behavioral and biochemical tests were performed as described above.

Propargylglycine (50 mg/kg i.p.) in Remote Ischemic Preconditioning

Propargylglycine was administered 30 min before subjecting to remote ischemic preconditioning. Thereafter, animals were subjected to cerebral ischemia of 20 min duration. The different behavioral and biochemical tests were performed as described above.

Amino-Oxyacetic Acid (25 mg/kg i.p.) in Remote Ischemic Preconditioning

Amino-oxyacetic acid was administered 30 min before subjecting to remote ischemic preconditioning. Thereafter, animals were subjected to cerebral ischemia of 20 min duration. The different behavioral and biochemical tests were performed as described above.

Amino-Oxyacetic Acid (50 mg/kg i.p.) in Remote Ischemic Preconditioning

Amino-oxyacetic acid was administered 30 min before subjecting to remote ischemic preconditioning. Thereafter, animals were subjected to cerebral ischemia of 20 min duration. The different behavioral and biochemical tests were performed as described above.

Propargylglycine (50 mg/kg i.p.) and Amino-Oxyacetic Acid (50 mg/kg i.p.) in Remote Ischemic Preconditioning

Both propargylglycine and amino-oxyacetic acid was administered 30 min before subjecting to remote ischemic preconditioning. Thereafter, animals were subjected to cerebral ischemia of 20 min duration. The different behavioral and biochemical tests were performed as described above.

Vehicle (DMSO) in Remote Ischemic Preconditioning

DMSO (vehicle of bosentan) was administered 30 min before subjecting to remote ischemic preconditioning. Thereafter, the different behavioral and biochemical tests were performed as described above.

Statistical Analysis

The results were expressed as mean S.D. The data were analyzed using Two Way ANOVA followed by Bonferroni *post hoc* test. The value of $p < 0.05$ was considered to statistically significant.

Results

Global Cerebral Ischemia–Reperfusion Produces Injury, Apoptosis, and Behavioral Deficits

Male mice subjected to global cerebral ischemia–reperfusion developed significant injury in comparison to mice belonging to normal group. There were significant behavioral alterations in term of locomotor deficits evaluated on actophotometer test (Fig. 1a), motor in-coordination evaluated

on the inclined beam walking test (Fig. 1b), and neurological severity score (Fig. 2a) assessed on 24 h following cerebral ischemia. However, there was significant restoration of these behavioral alterations on 7th day following cerebral ischemia.

The assessment of learning and memory in ischemia-subjected animals was started on 7th day following ischemia and continued for 5 days (4 days of acquisition and 1 day of retrieval). In ischemia-subjected mice, there was deficit in learning as depicted by no significant change in escape latency time on 10th day (fourth day of acquisition trial) in comparison to 7th day (first day of trial) (Fig. 3a). Furthermore, the parameter to assess the memory i.e., time spent in target quadrant was significantly less on 11th day in ischemia-subjected mice in comparison to normal group mice (Fig. 3b). There was

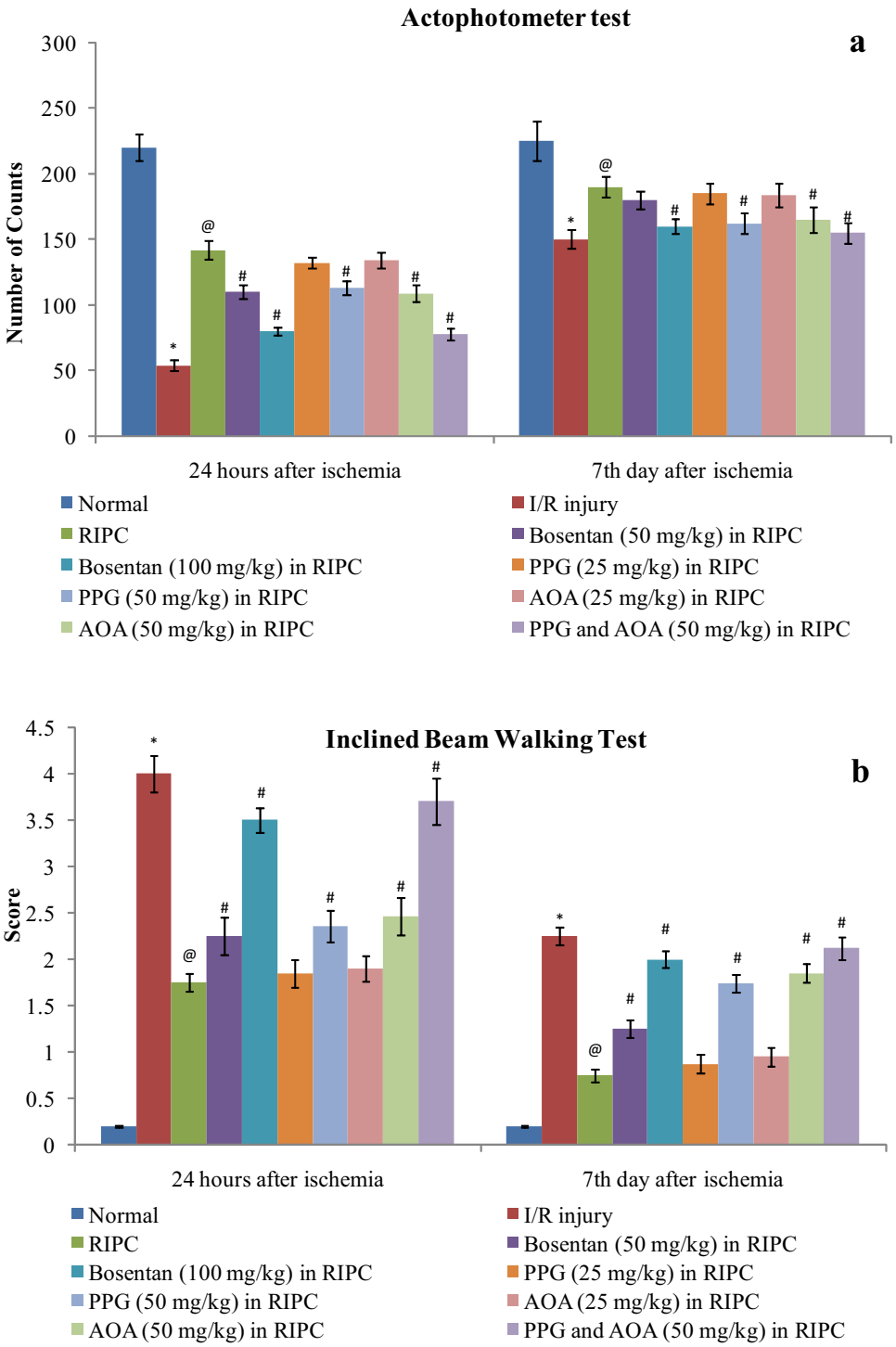
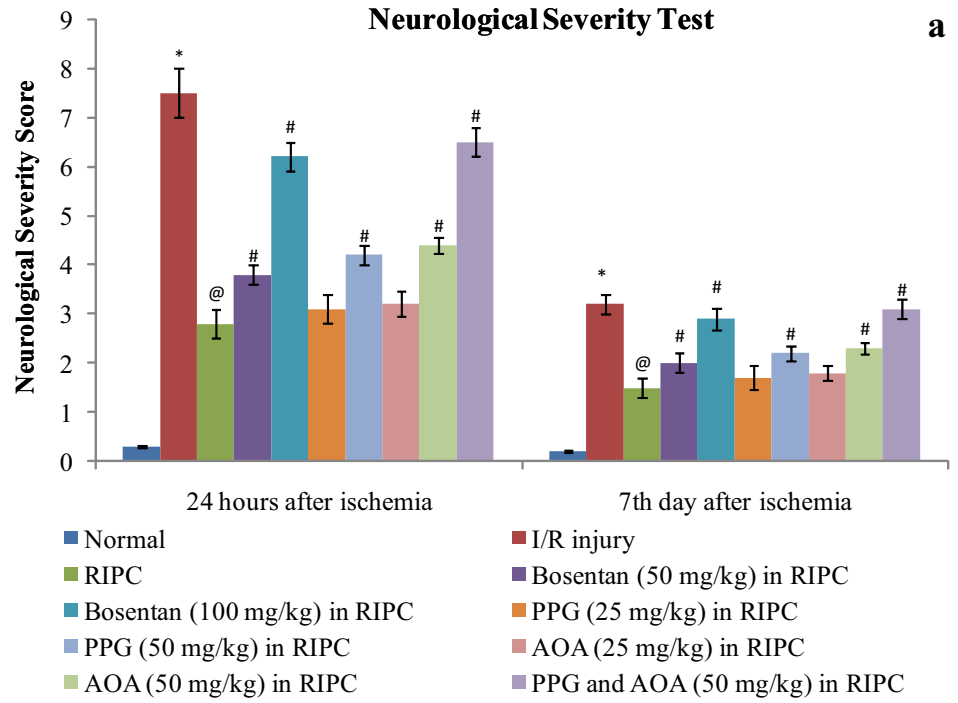


Fig. 1 The results of actophotometer test depicting the changes in locomotor activity (**a**) and inclined beam walking test depicting motor in-coordination (**b**) in mice due to ischemia-reperfusion-induced cerebral injury in different experimental groups. $^{*}p < 0.05$ vs. normal group; $^{@}p < 0.05$ vs. I/R injury; $^{\#}p < 0.05$ vs. RIPC. *I/R* Ischemia reperfusion, *RIPC* Remote ischemic preconditioning



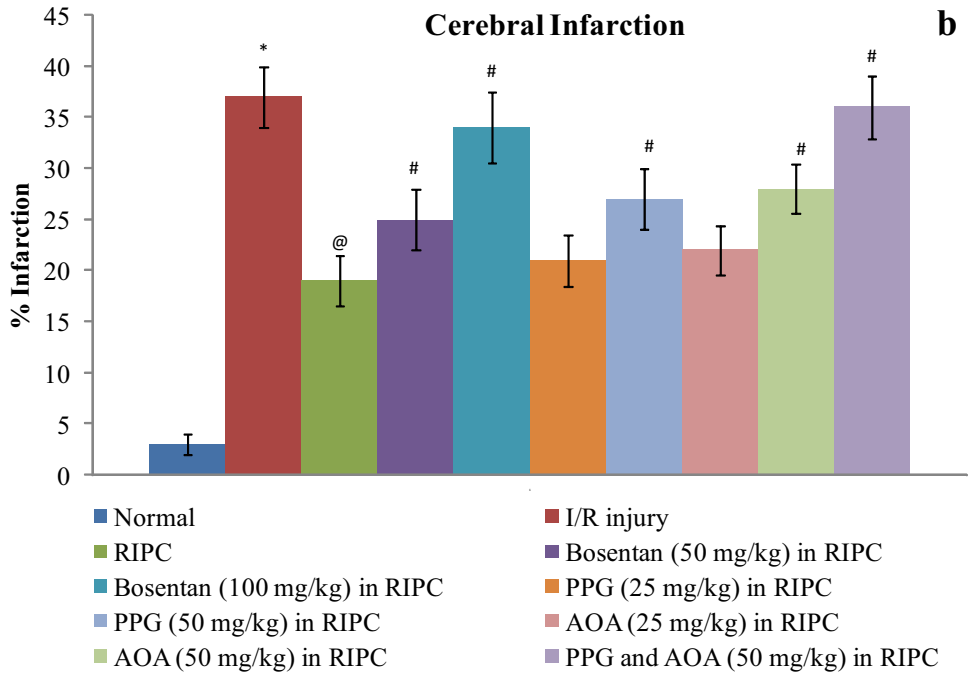


Fig. 2 The results of neurological severity score (**a**) and cerebral infarction (**b**) in mice due to ischemia-reperfusion-induced cerebral injury in different experimental groups. $^{*}p < 0.05$ vs. normal group; $^{@}p < 0.05$ vs. I/R injury; $^{\#}p < 0.05$ vs. RIPC. *I/R* Ischemia reperfusion, *RIPC* remote ischemic preconditioning

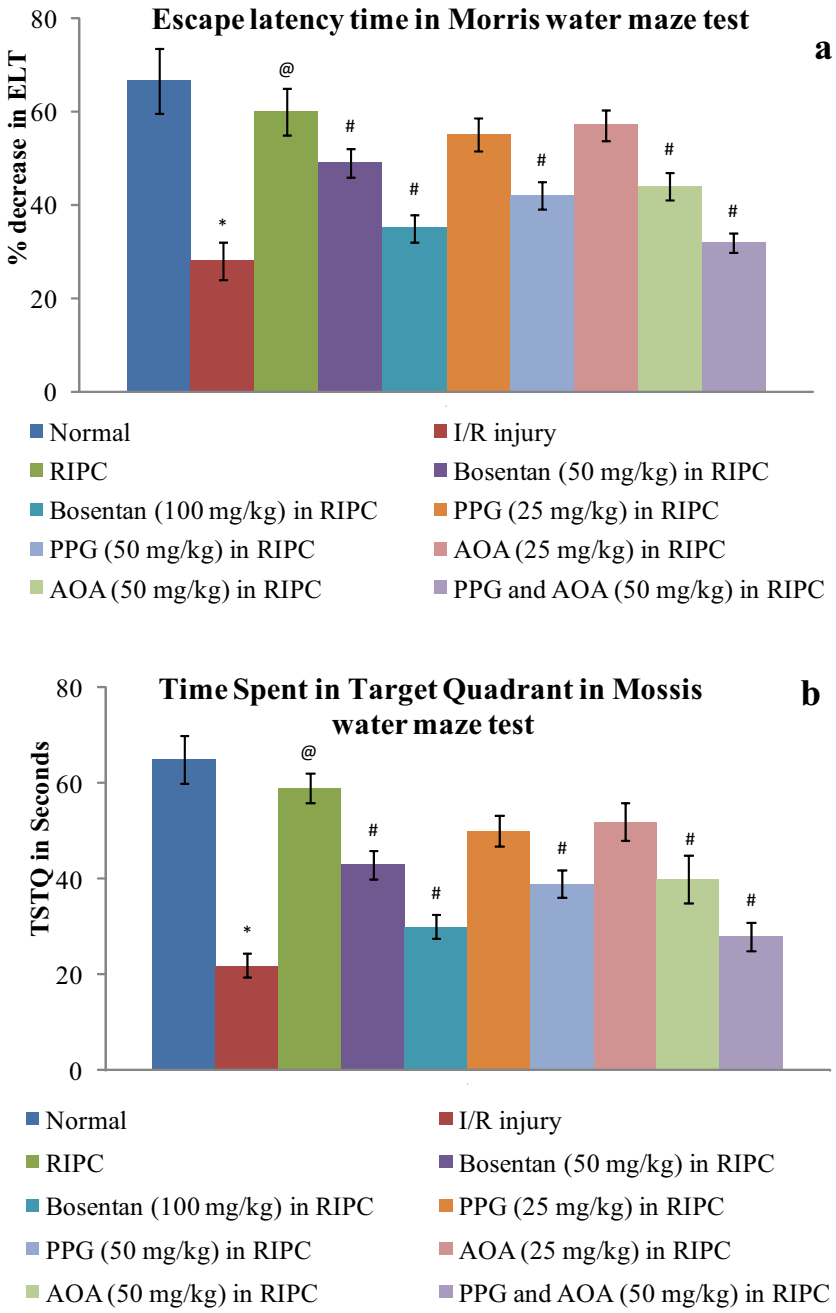


Fig. 3 The results of Morris water maze test in terms of percentage decrease in escape latency time (ELT) (**a**) and time spent in target quadrant (TSTQ) in seconds (**b**) in mice due to ischemia-reperfusion-induced cerebral injury in different experimental groups. $^{*}p < 0.05$ vs. normal group; $^{@}p < 0.05$ vs. I/R injury; $^{\#}p < 0.05$ vs. RIPC. *I/R* Ischemia reperfusion, *RIPC* remote ischemic preconditioning

a significant increase in cerebral infarction (Fig. 2b) and apoptosis markers including caspase-3 (Fig. 4a) and Bcl-2 (Fig. 4b) in ischemia–reperfusion subjected mice in comparison to normal group mice.

Remote Ischemic Preconditioning Restores Global Cerebral Ischemia–Reperfusion Injury Induced Injury and Behavioral Changes

Four short cycles of ischemia and reperfusion of 5 min each significantly attenuated global ischemia-reperfusion-induced locomotor deficits (Fig. 1a), motor in-coordination (Fig. 1b), and neurological severity score (Fig. 2a) assessed on 24 h following cerebral ischemia. Moreover, the extent of restoration in these parameters was relatively more complete on 7th day in remote preconditioning subjected mice. Furthermore in these animals, there was a significant decrease in escape latency time on 10th day (fourth day of acquisition trial) in comparison to 7th day (first day of trial) (Fig. 3a). The time spent in target quadrant was also significantly more on 11th day in these mice in comparison to ischemia reperfusion group mice (Fig. 3b). Apart from these, there was a significant increase in cerebral infarction (Fig. 2b) and apoptosis

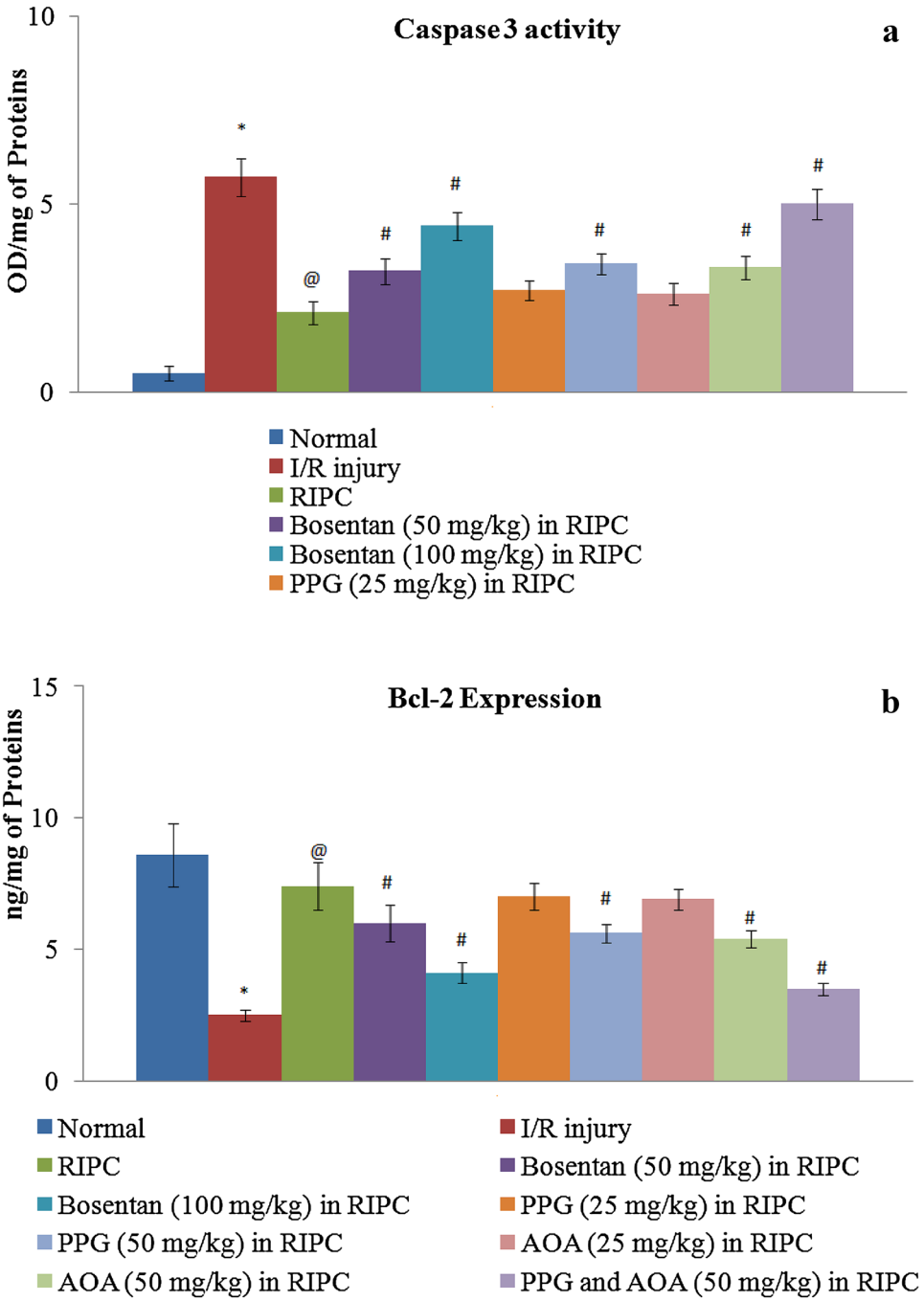


Fig. 4 The effects of different interventions on the markers of apoptosis i.e., caspase-3 activity (**a**) and Bcl-2 expression (**b**) in the brain homogenate of mice in different experimental groups.*$p < 0.05$ vs. normal group; $^{@}p < 0.05$ vs. I/R injury; $^{\#}p < 0.05$ vs. RIPC. *I/R* Ischemia reperfusion, *RIPC* remote ischemic preconditioning

markers including caspase-3 (Fig. 4a) and Bcl-2 (Fig. 4b) in remote preconditioning subjected mice.

Effect of Global Ischemia and Remote Preconditioning on Biochemical Parameters

In global ischemia-subjected mice, there was a significant decrease in the levels of H_2S along with decrease in the activity of cystathionine-β-synthase (Fig. 5a) and cystathionine-γ-lyase (Fig. 5b), the enzymes responsible for the synthesis of H_2S (Fig. 6a). Moreover, there was also a decrease in the expression of Nrf-2 (Fig. 7a) and glutathione reductase (Fig. 7b) in response to ischemia reperfusion injury. However, remote preconditioning led to significant increase in the levels of H_2S, activity of cystathionine-β-synthase, cystathionine-γ-lyase, and expression of Nrf-2 and glutathione reductase. There was a significant increase in the levels of ET-1 in the plasma after 40 min of remote preconditioning stimulus in comparison to normal group (Fig. 6b).

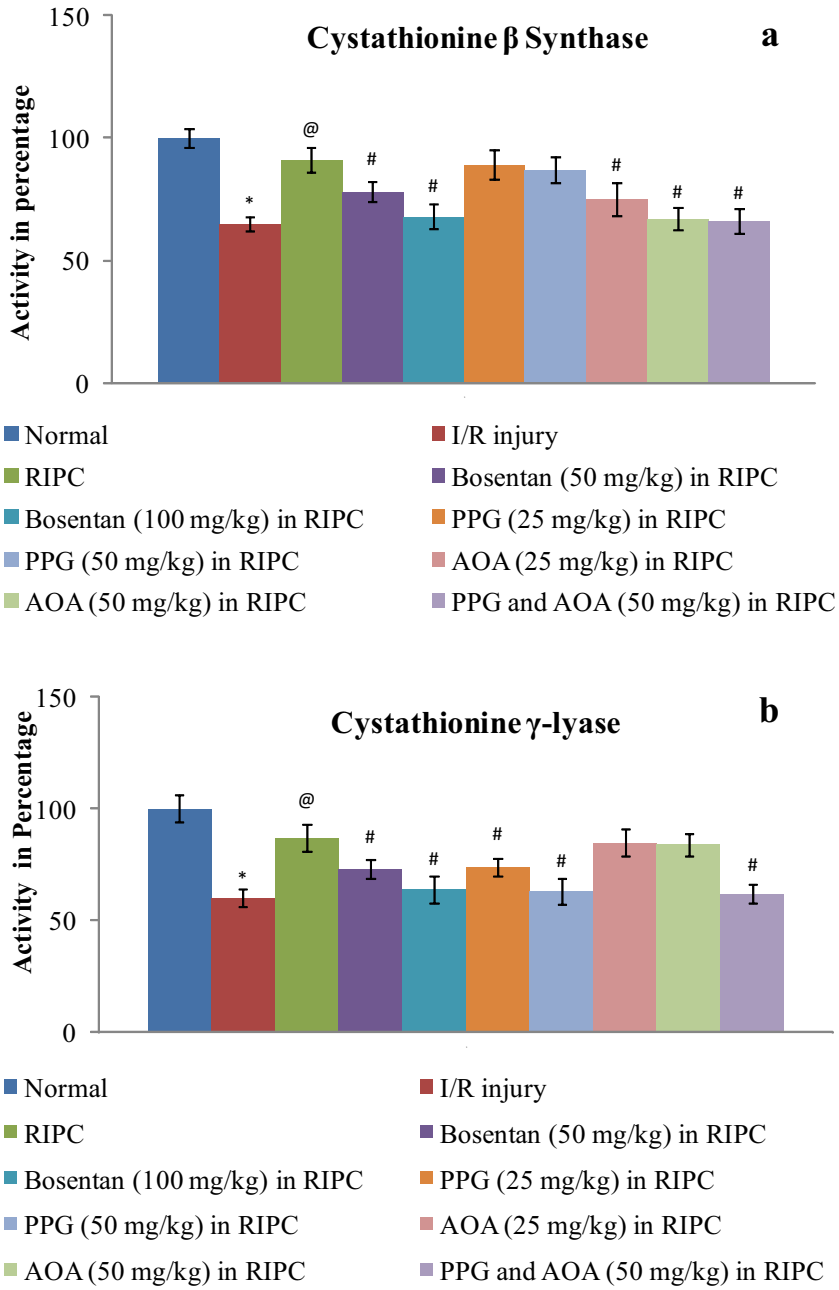


Fig. 5 The results of changes in cystathionine-β-synthase (**a**) and cystathionine-γ-lyase (**b**) in the brain of the mice due to ischemia-reperfusion-induced cerebral injury in different experimental groups. $^{*}p < 0.05$ vs. normal group; $^{@}p < 0.05$ vs. I/R injury; $^{\#}p < 0.05$ vs. RIPC. *I/R* Ischemia reperfusion, *RIPC* Remote ischemic preconditioning

Influence of Pharmacological Agents on Remote Preconditioning-Mediated Effects on Behavior, Cerebral Injury, and Biochemical Parameters

Administration of bosentan before remote preconditioning significantly attenuated its protective effects on locomotor activity (Fig. 1a), motor coordination (Fig. 1b), neurological severity (Fig. 2a), learning and memory (Fig. 3a, b), cerebral infarction (Figs. 2b, 8), and apoptosis markers (Fig. 4a, b). Furthermore, it also abolished the effects of remote preconditioning on H_2S (Fig. 6a), cystathionine-β-synthase (Fig. 5a), cystathionine-γ-lyase (Fig. 5b), Nrf-2 (Fig. 7a), and glutathione reductase (Fig. 7b). However, it did not modulate remote preconditioning-mediated increase in the plasma levels of ET-1 (Fig. 6b). Administration of propargylglycine and amino-oxyacetic acid also abolished the various effects of remote preconditioning (Fig. 1, 2, 3, 4, 5) without any significant effect on plasma ET-1 levels (Fig. 6b). However, the notable difference was that effects of individual drugs were comparatively less in comparison to bosentan. However, the combined administration of these two drugs produced effects equivalent to bosentan. Furthermore, the inhibitory effects of propargylglycine and amino-oxyacetic acid were limited to their respective enzymes and

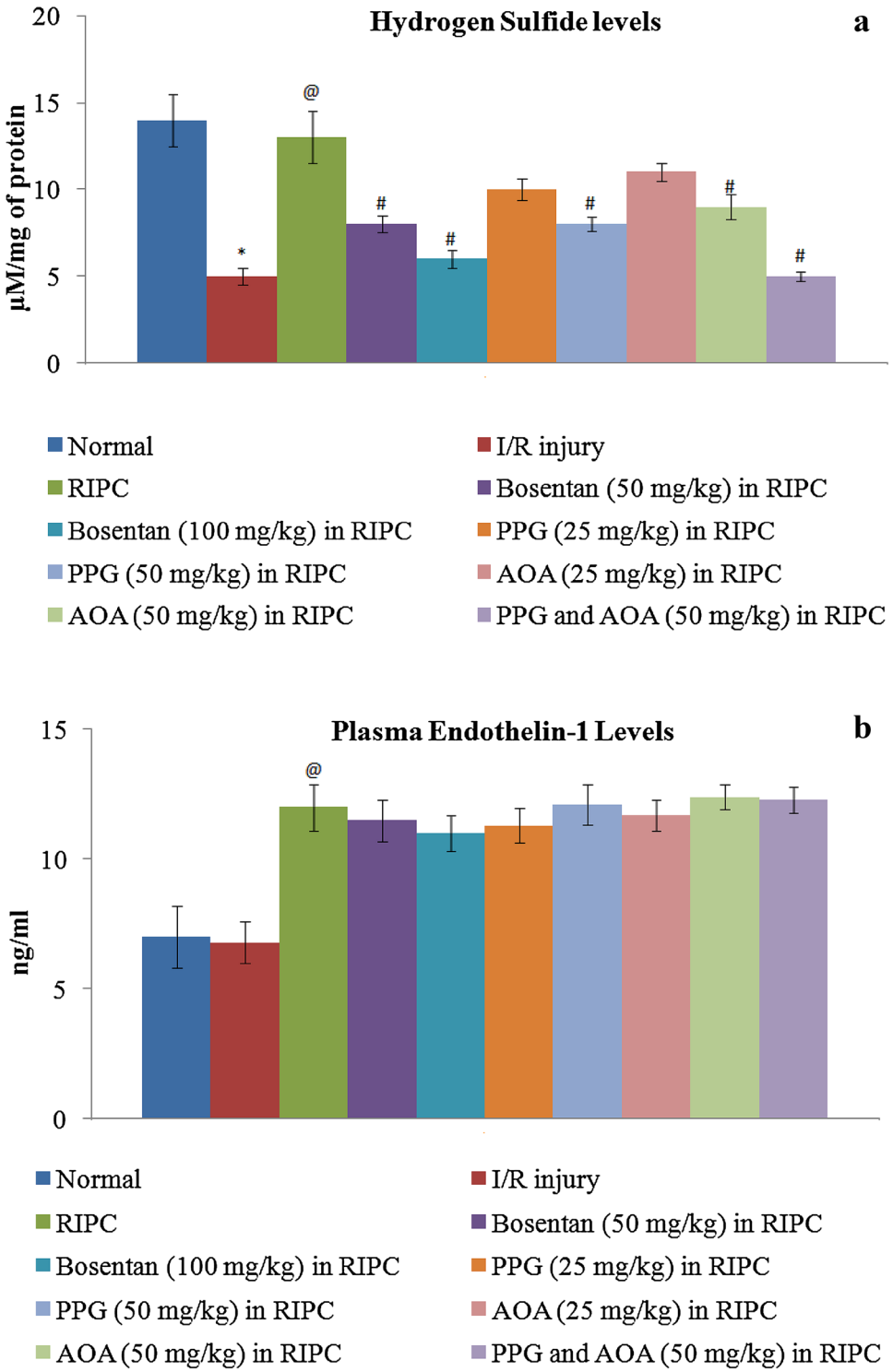


Fig. 6 The results of changes in hydrogen sulfide levels in the brain (**a**) and plasma endothelin-1 levels (**b**) of the mice due to ischemia-reperfusion-induced cerebral injury in different experimental groups. $^{*}p<0.05$ vs. normal group; $^{@}p<0.05$ vs. I/R injury; $^{\#}p<0.05$ vs. RIPC. *I/R* Ischemia reperfusion, *RIPC* remote ischemic preconditioning

thus, propargylglycine selectively attenuated the activity of cystathionine-γ-lyase, while amino-oxyacetic acid selectively attenuated the activity of cystathionine-β-synthase.

Discussion

In the present study, global ischemia and reperfusion produced significant cerebral injury in male mice, which was assessed by different parameters including behavioral deficits after 24 h of global ischemia, which included locomotor

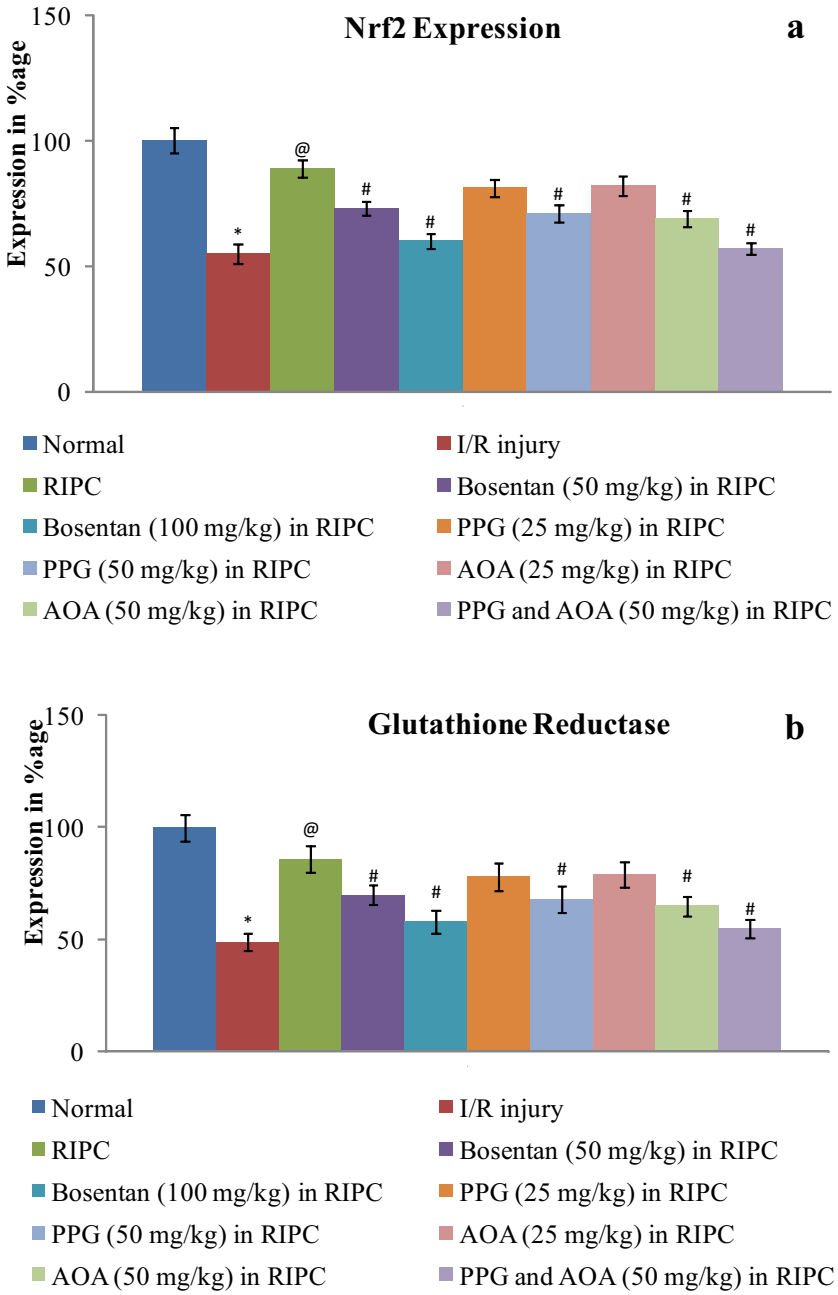


Fig. 7 The results of changes in Nrf2 expression (**a**) and glutathione reductase (**b**) in the brain of the mice due to ischemia-reperfusion-induced cerebral injury in different experimental groups. $^{*}p < 0.05$ vs. normal group; $^{@}p < 0.05$ vs. I/R injury; $^{\#}p < 0.05$ vs. RIPC. *I/R* Ischemia reperfusion, *RIPC* remote ischemic preconditioning

activity, motor in-coordination, and neurological severity score. However, these locomotor-related parameters were significantly restored on 7th day after ischemia. Ischemia-subjected mice displayed poor learning as well as retention (memory) ability in Morris water maze test, assessed from 7th to 11th day after ischemia. Moreover, there was a significant increase in cerebral infarct size, assessed using TTC staining, and increase in apoptosis markers, increase in caspase-3 activity, and decrease in Bcl-2 expression. Male mice were employed in the present study to avoid the influence of variations in circulating estrogen and progesterone levels. Remote ischemic preconditioning in the form of short cycles of ischemia and reperfusion significantly attenuated global cerebral ischemia-reperfusion-induced alterations in locomotor-related parameters, restored learning and memory deficits, and prevented cerebral infarction and apoptosis. Thiopental was employed as an anesthetic agent in the present study. There have been studies documenting that thiopental increases the ischemic brain injury in spontaneously hypertensive rats (Duan et al. 2009). However, certain studies have also documented the neuroprotective effects of thiopental (Cao et al. 2003). Since thiopental was administered in all the experimental groups in this study, therefore, any interference imparted by thiopental shall be evenly

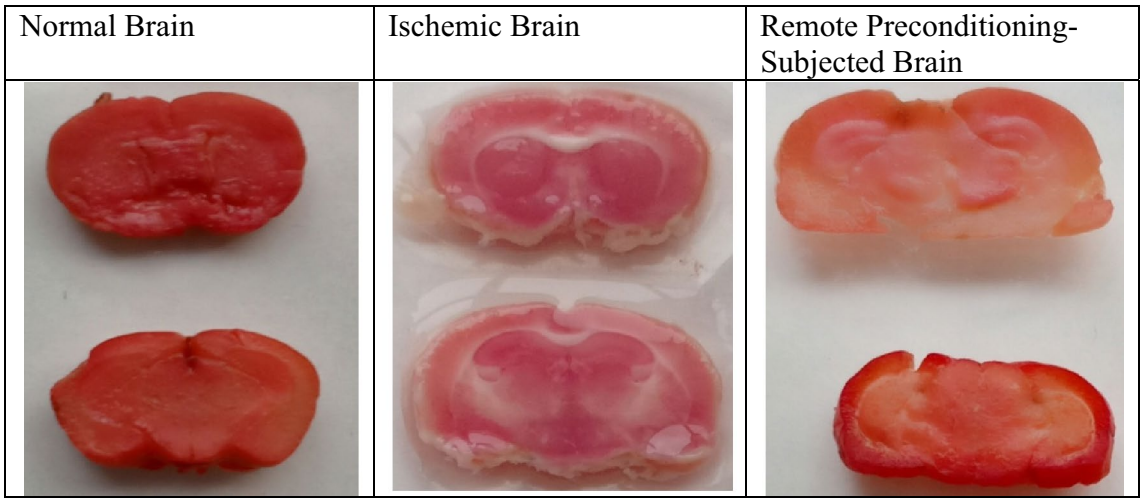


Fig. 8 Representative pictures of TTC stained brain portions for cerebral infarction

distributed in all groups and relative difference observed in these experimental groups may be possibly attributed to other interventions. Moreover, studies have also shown that thiopental does not interfere with the process of ischemic preconditioning (Diwan et al. 2008; Mullenheim et al. 2001).

In this study, pretreatment with bosentan (nonselective ET_A and ET_B receptor antagonist) significantly attenuated the beneficial effects of remote preconditioning against ischemia reperfusion injury. Moreover, there was a significant increase in the plasma levels of endothelin-1 in response to remote preconditioning. Therefore, it may be proposed that during remote ischemic preconditioning an increase in endothelin-1 may activate ETA and ETB receptors to protect brain from ischemic damage and may be responsible for preserved learning and memory. Endothelins are endothelium derived factors and it is shown to produce preconditioning like effects on heart (Bugge and Ytrehus 1996; Gourine et al. 2005). A recent study has shown the critical role of endothelin 1 in remote ischemic preconditioning-induced cardioprotection (Zhang et al. 2018). However, it is the first report suggesting the crucial role of endothelin-1 in remote preconditioning-induced beneficial effects in a model of vascular dementia.

To further explore the biochemical mechanisms responsible for remote preconditioning-mediated protection against ischemic damage, the levels of hydrogen sulfide along with enzyme activities of cystathionine-β-synthase and cystathionine-γ-lyase (enzymes responsible for H_2S production) were measured in the brain samples. The levels of hydrogen sulfide along with enzyme activities of cystathionine-β-synthase and cystathionine-γ-lyase were significantly increased in the brain samples in remote preconditioning subjected mice in comparison to ischemia–reperfusion subjected mice. Accordingly, it is possible to propose that a relative increase in the H_2S levels due to increase in enzyme activities of cystathionine-β-synthase and cystathionine-γ-lyase may contribute in decreasing the cerebral ischemic susceptibility in mice. Moreover, pretreatment with amino-oxyacetic acid (cystathionine β synthase inhibitor) and DL-propargylglycine (inhibitor of cystathionine γ-lyase) significantly attenuated the neuroprotective effects of remote preconditioning including on learning and memory. These pharmacological interventions also attenuated the levels of H_2S and decreased the activities of their respective enzymes. Moreover, combined pretreatment with amino-oxyacetic acid and DL-propargylglycine more completely attenuated the brain levels of H_2S and the activities of its biosynthetic enzymes. The attenuating effects on remote preconditioning were also more significant with combined administration of both enzyme inhibitors. It indicates that both enzymes are crucial in increasing the levels of H_2S during remote preconditioning. There have been earlier studies showing that increase in hydrogen sulfide and its biosynthetic enzymes may contribute in producing beneficial effects in a number of diseases (Kumar and Sandhir 2018; Zheng et al. 2018). An increase in H_2S has been shown to restore learning and memory in different animal models (Giuliani et al. 2013; Wei et al. 2014). However, it is the first report documenting that increase in hydrogen sulfide and its biosynthetic enzymes viz., cystathionine-β-synthase and cystathionine-γ-lyase may contribute in remote preconditioning-mediated protective effects in vascular dementia.

The interrelationship between H_2S and endothelin-1 may be deduced by the observations of the present study documenting that pretreatment with bosentan led to decrease in H_2S levels and enzyme activities of cystathionine β synthase and cystathionine γ-lyase in remote preconditioning subjected mice. Bosentan-mediated decrease in H_2S levels

and its biosynthetic enzymes was more significant than observed with individual treatments with amino-oxyacetic acid and DL-propargylglycine. However, the combined treatment with amino-oxyacetic acid and DL-propargylglycine produced equivalent decrease in H_2S and its biosynthetic enzymes. It probably suggests that endothelin-1 acts as a trigger for the synthesis of H_2S, which may be possibly due to increased activities of cystathionine β synthase and cystathionine γ-lyase. This contention that endothelin-1 acts as a upstream mediator of H_2S is supported by another observation of this study documenting that treatment with amino-oxyacetic acid or DL-propargylglycine did not affect remote preconditioning-induced increase in the endothelin-1 levels. A very recent study has shown that the effects of endothelin-1 in the brain may be mediated through increase in H_2S production and inhibitor of cystathionine γ-lyase is shown to attenuate the effects of ET-1 (Patel et al. 2018). Therefore, it may be possible to suggest that endothelin-1 may increase the enzyme activities of cystathionine β synthase and cystathionine γ-lyase to increase the brain levels of H_2S.

In the present study, pretreatment with bosentan, amino-oxyacetic acid, or DL-propargylglycine significantly attenuated remote preconditioning-induced increase in the expression of Nrf2 and glutathione reductase. Nrf2is a transcriptional factor, which is well reported to regulate the expression of antioxidants in the cells to protect against free radicals (Kim et al. 2010) and glutathione reductase in well documented antioxidant enzyme. A very recent study has shown that H_2S donor produces beneficial effect in homocysteine-induced cognitive decline model by increasing the expression of Nrf2 and glutathione reductase (Kumar and Sandhir 2018). Therefore, it may be postulated that remote preconditioning episodes stimulate the endothelium to release endothelin-1, which may stimulate the enzyme activities of cystathionine β synthase and cystathionine γ-lyase to increase the levels of H_2S and latter may trigger signaling cascade to increase the expression of Nrf2 to decrease oxidative stress and prevent vascular dementia.

Conclusion

It is concluded that RIPC may stimulate the release endothelin-1, which may activate CBS and CLS to increase the levels of H_2S and latter may increase the expression of Nrf2 to decrease oxidative stress and prevent vascular dementia.

Author Contributions JTH did the experiments; HL helped JTH in experimental work and participated in writing; LY helped in writing the manuscript; KLC conceived the idea and edited the manuscript.

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Compliance with Ethical Standards

Conflict of interest There is no conflict exists among the authors.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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