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Pharmacologic evidence for role of endothelial nitric oxide synthase in neuroprotective mechanism of ischemic postconditioning in mice

Puja Gulati, MPharm, Nirmal Singh, MPharm, PhD, and Arunachalam Muthuraman, MPharm, PhD*

Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, Punjab, India

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ABSTRACT

Background: The present study was conducted to pharmacologically investigate the isoform-specific role of nitric oxide pathway in neuroprotective mechanism of ischemic postconditioning (iPoCo).

Materials and methods: Bilateral carotid artery occlusion of 12 min followed by reperfusion for 24 h was used to produce ischemia- and reperfusion-induced cerebral injury in male Swiss mice. Memory was assessed using Morris water maze test. Degree of motor in-coordination was evaluated using inclined beam-walk test, rotarod test, and lateral push test. Cerebral infarct size was measured using triphenyltetrazolium chloride staining. Brain acetylcholinestrase activity, thiobarbituric acid–reactive species, nitrite/nitrate, and reduced glutathione levels were also estimated. Western blotting was performed to determine endothelial nitric oxide synthase (eNOS) expression.

Results: Bilateral carotid artery occlusion followed by reperfusion produced significant rise in cerebral infarct size, acetylcholinesterase activity, and thiobarbituric acid–reactive species levels along with fall in nitrite/nitrate, and glutathione and eNOS expression levels. A significant impairment of memory and motor coordination was also noted. iPoCo consisting of three episodes of 10-s carotid artery occlusion and reperfusion significantly attenuated infarct size, memory impairment, motor in-coordination, altered biochemicals, and protein expression levels. iPoCo-induced neuroprotective effects were significantly abolished by L-NAME (a nonselective nitric oxide synthase inhibitor) and L-NIO (a selective eNOS inhibitor). However, aminoguanidine (a selective inducible nitric oxide synthase inhibitor) and 7-nitroindazole (a selective neuronal nitric oxide synthase inhibitor) did not modulate beneficial effects of iPoCo.

Conclusions: It may be concluded that nitric oxide pathway probably plays a vital role with specific involvement of eNOS in neuroprotective mechanism of iPoCo.

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* *Corresponding author*. Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, Punjab 147002, India. Tel.: +91 9815129884; fax: +91 175-3046255.

E-mail address: nirmal_puru@rediffmail.com (N. Singh).

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1. Introduction

Ischemic stroke is the acute severe manifestation of cerebrovascular disease. It is a syndrome characterized by rapid onset of neurologic injury due to interruption of blood flow to the brain [1,2]. Although mortality from ischemic stroke has declined over the last decade, it still remains the second leading cause of death because only limited therapeutic strategies exist [3]. Restoration of blood flow to ischemic tissue is the only way to reduce infarct size, but full reperfusion itself results in additional injury known as ischemia-reperfusion (I/R) injury [4]. An attempt to attenuate this injury led to the discovery of phenomenon like ischemic preconditioning (iPreCo) and ischemic postconditioning (iPoCo) [5]. iPreCo is a well-established phenomenon, being used since 1980s to attenuate I/R-induced injury [6,7]. However, inability of preconditioning phenomenon to predict the onset of ischemia in clinical settings [8] led to the discovery of a new concept of iPoCo, whereby brief repetitive cycles of ischemia with intermittent reperfusion immediately after prolonged ischemia elicit tissue protection [5,8,9]. Protective role of iPoCo has been documented in different organs like heart of several animal species [10,11], brain [9,12,13], liver [14], and kidney [15]. iPoCo is also observed to produce cardioprotective effects in clinical settings [16,17].

There is an impressive array of molecular mechanisms contributing to protective effect of iPoCo which include triggers like adenosine, opioid, erythropoietin, endothelial nitric oxide synthase (eNOS), reactive oxygen species, acetylcholine, tissue factors, proinflammatory cytokines, and bradykinin; mediators like RISK pathways including PI3K-Akt, MEK-ERK½, PKG, and PKC; end-effectors like mPTP and mKATP [18].

Nitric oxide (NO), also known as endothelium-derived relaxing factor, is a key biological messenger playing a prominent role in preserving the functions of endothelium. The human genome contains three different genes encoding NO synthases (neuronal nitric oxide synthase [nNOS], inducible nitric oxide synthase [iNOS], and eNOS). A protective effect of NO has also been documented during reperfusion of ischemic myocardium [19]. It has been observed that eNOS plays an important role in mediating cardioprotective effects of iPoCo [20] and both eNOS and iNOS are involved in renal iPoCo [15]. Furthermore, it is also reported that NO is also involved in cerebroprotective effect of delayed preconditioning. However, role of nitric oxide synthase (NOS) in postconditioning mediated neuroprotection still remains to be evaluated.

Therefore, the present study has been designed to pharmacologically investigate the specific role of NOS isoform in iPoCo-induced reversal of global cerebral ischemia- and reperfusion-induced injury in mice.

2. Experimental

2.1. Animals

Male Swiss mice weighing 20 ± 5 g, maintained on standard laboratory diet (Kisan Feeds Ltd, Mumbai, India) and having free access to tap water were used in the present study. They were housed in the departmental animal house and were exposed to 12-h light/dark cycle. All the animals used in the study were naive to Morris water maze (MWM) test. The experiments were conducted in a semisound proof laboratory. The experimental protocol was duly approved by institutional animal ethics committee. The care of the animals was carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No. - 107/1999/CPCSEA).

2.2. Drugs and chemicals

L-NAME (Cayman Chemicals, Ann Arbor, MI), L-NIO (Cayman Chemicals, Ann Arbor, MI), aminoguanidine (Sigma-Aldrich Ltd, St Louis, MO), and chloral hydrate (Riedel-deHaen, Seelze Germany) were dissolved in distilled water, respectively. 7-Nitroindazole (7-NI) (Cayman Chemicals) was dissolved in 5% dimethyl formamide (DMF) solution. Anti-eNOS antibody (EMD chemicals, Darmstadt, Germany) and goat anti-mouse IgG peroxidase conjugate (EMD chemicals) were used for western blotting. All other chemicals used in the present study were of analytical quality. All drug solutions were freshly prepared before use. The drugs were administered 30 min before carotid ligation (ischemia) to the animals. This schedule of drug administration was adapted to ensure maximum drug absorption just before the induction of iPoCo.

2.3. Induction of global cerebral ischemia

Mice were anesthetized using chloral hydrate (400 mg/kg, intraperitoneally [i.p.]) as mortality is negligible at this dose and at the same time cognitive function is not at all affected [3,9,12,21,22]. Global cerebral ischemia in mice was induced surgically according to the methods of Himori *et al.* [23] and as described by Kaur *et al.* [12]. A midline ventral incision was made in the neck to expose right and left common carotid arteries, which were isolated from surrounding tissue and vagus nerve. A cotton thread was passed below each of the carotid arteries. Global cerebral ischemia was induced by occluding the carotid arteries. After 12 min of global cerebral ischemia, reperfusion was allowed for 24 h. The incision was sutured back in layers. The sutured area was cleaned with 70% ethanol and was sprayed with antiseptic dusting powder. The animals were shifted individually to their home cage and were allowed to recover.

2.4. Induction of iPoCo cycles

For the iPoCo episode, the carotid arteries were reoccluded for a period of 10 s followed by 10 s of reperfusion time. Three such cycles of ischemia and reperfusion were allowed immediately after the bilateral carotid artery occlusion (BCAO) performed for 12 min [9].

2.5. Assessment of cerebral infarct size

At the end of 24 h of reperfusion after global cerebral ischemia, animals were killed by spinal dislocation, and the

brains were removed and placed immediately in ice cold saline for 10 min. Brain samples were then sliced into uniform coronal sections of about 1 mm thickness. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C in 0.2 M tris buffer (pH 7.4) for 20 min [24]. TTC is converted to red formazone pigment by nicotinamide adenine dinucleotide and lactate dehydrogenase and thus stained the viable cells deep red. The infarcted cells had lost the enzyme and cofactor and thus remained unstained dull yellow. The infarct size was measured by volume method using National Institutes of Health Image J software (NIH Image version 1.61; National Institutes of Health, Bethesda, MD). In this, the brain slices were placed over glass plate. The images were digitalized. The margins of the infarct area in the captured digital images were manually outlined, and a pixel based area calculation was performed using National Institutes of Health ImageJ software (National Institutes of Health) [25].

2.6. Evaluation of memory using MWM test

MWM has been used to assess learning and memory of the animals [26,27]. MWM procedure is based on the principle where animal is placed in a large pool of water divided into four equal quadrants, as animal dislikes swimming, its tendency to escape is accomplished by finding a hidden escape platform. Each animal was subjected to four consecutive training trials (with an intertrial gap of 5 min) each day for four consecutive days in search for a hidden platform. The d 4 escape latency time (ELT) to locate the hidden platform in water maze was taken as an index of acquisition or learning. On the fifth day, the hidden platform was removed. Each animal was allowed to explore the pool for 120 s. Mean time spent in all the quadrants in search of hidden platform was noted. The mean time spent by the animal in the target quadrant was taken as the index of retrieval or memory.

2.7. Assessment of motor coordination

2.7.1. Rotarod test

Rota rod was used to evaluate motor coordination by testing the ability of mice to remain on revolving rod [28]. The apparatus consisted of horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70-cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted to allow the normal mice to stay on it for 5 min. Each mouse was given five trials before the actual reading was taken. The animals staying on revolving rod for period of 5 min before the surgical procedure were selected and the test was again performed after 12 min of global cerebral ischemia and 24 h of reperfusion.

2.7.2. Inclined beam-walking test

Inclined beam-walking test was used to evaluate fore and hind limb motor coordination [29,30]. Each animal was individually placed on a metallic bar 55 cm long and 1.5 cm wide, inclined at an angle of 60° from ground. The motor performance of mouse was scored on a scale ranging from 0 to 4. A grade of 0 was assigned to animal that could readily traverse the beam, grade 1 was given to animal demonstrating mild impairment, grade 2 was assigned to animal demonstrating moderate impairment, grade 3 was given to animal demonstrating severe impairment, and grade 4 was assigned to animal completely unable to walk on the beam. Inclined beam-walking test was performed before global cerebral ischemia and 24 h after global cerebral ischemia and reperfusion.

2.7.3. Lateral push test

A mouse was placed on a rough surface for firm grip and was evaluated for resistance to lateral push from either side of the shoulder [30,31]. The test was performed before global cerebral ischemia and 24 h after global cerebral ischemia and reperfusion. Mice with increased or decreased resistance to lateral push after global ischemia were assigned + or − score, respectively.

2.8. Biochemical estimations

After behavioral tests, the animals were killed by cervical dislocation, and the brains were removed and homogenized in phosphate buffer (pH = 7.4). The homogenates were than centrifuged at 3000 rpm for 15 min. The supernatant of homogenates were used for biochemical estimations.

2.8.1. Estimation of brain acetylcholinesterase activity

The whole brain acetylcholinesterase (AChE) activity was measured spectrophotometrically (DU 640B spectrophotometer; Beckman Coulter, Inc, Fullerton, CA) at 420 nm by the method of Ellman *et al.* [32] with slight modification by Voss and Sachsse [33]. It was measured on the basis of formation of yellow color due to reaction of thiocholine with dithiobisnitrobenzoate ions. The rate of formation of thiocholine from acetylthiocholineiodide in the presence of brain cholinesterase was measured using a spectrophotometer.

2.8.2. Estimation of brain total protein

The brain total protein was determined spectrophotometrically (DU 640B spectrophotometer; Beckman Coulter, Inc) at 750 nm by the method of Lowry *et al.* [34].

2.8.3. Estimation of brain thiobarbituric acid reactive species

The quantitative measurement of thiobarbituric acid−reactive species (TBARS), an index of lipid peroxidation in brain was determined spectrophotometrically (DU 640B spectrophotometer; Beckman Coulter, Inc) at 532 nm performed according to the method of Ohkawa *et al.* [35].

2.8.4. Estimation of brain reduced glutathione

The reduced glutathione (GSH) content in brain was estimated spectrophotometrically (DU 640B spectrophotometer; Beckman Coulter, Inc) at 412 nm using the method of Beutler *et al.* [36].

2.8.5. Estimation of brain nitrite/nitrate

Brain nitrite/nitrate concentration was measured spectophotometrically (DU 640B Spectrophotometer; Beckman Coulter, Inc) at 542 nm, using the method of Sastry *et al.* [37].

2.9. Immunoblotting

Western blot analysis was done according to the method of Jones *et al.* [38] with slight modifications. For western blot analysis, brain was isolated from mice, weighed, and homogenized in western blot lysis buffer. The samples were centrifuged at 14000 g, 4°C, for 20 min, and protein contents of the supernatants were determined by Lowry method. Samples were prepared in sample buffer and heated at 95° C for 10 min. Samples (25 μg/lane) were resolved by electrophoresis on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h and incubated with anti-eNOS antibody followed by incubation with goat anti-mouse IgG peroxidase conjugate followed by the addition of diaminobenzidine as substrate. Brown colored bands appeared whose intensities were quantified and expressed as a percentage of control band intensity using NIH imageJ software (National Institutes of Health). Constitutively expressed protein β-actin was the internal control.

2.10. Experimental protocol

In total, 21 groups were used and each group comprised of six animals.

Group I (Sham group): Each mouse was subjected to surgical procedure, and a thread was passed below the carotid arteries but they were not occluded. After 12 min, threads were removed, and the animal was sutured back and allowed to recover for 24 h.

Group II (Control group): Each mouse was subjected to 12 min global cerebral ischemia followed by reperfusion for 24 h.

Group III (iPoCo group): Each mouse was subjected to 12 min global cerebral ischemia immediately followed by three episodes of 10 s of ischemia and reperfusion each after which a 24-h reperfusion period was permitted.

Group IV (DMF-Control group): Each mouse was administered 5% DMF, 30 min before surgery. The rest of the procedure was the same as described for group-II.

Group V (DMF-iPoCo group): Each mouse was administered 5% DMF, 30 min before surgery. The rest of the procedure was the same as described for group III.

Groups VI and VII (L-NAME-control group): Each mouse was administered L-NAME (1.5 and 3 mg/kg i.p.) 30 min before carotid artery occlusion. The rest of the procedure was the same as described for group II.

Groups VIII and IX (L-NAME—postconditioning group): Each mouse was administrated L-NAME (1.5 and 3 mg/kg i.p.) 30 min before carotid artery occlusion. The rest of the procedure was the same as described for group III.

Groups X and XI (L-NIO—control group): Each mouse was administered L-NIO (15 and 30 mg/kg i.p.) 30 min before carotid artery occlusion. The rest of the procedure was the same as described for group II.

Groups XII and XIII (L-NIO—postconditioning group): Each mouse was administrated L-NIO (15 and 30 mg/kg i.p.) 30 min before carotid artery occlusion. The rest of the procedure was the same as described for group III.

Groups XIV and XV (7-NI-control group): Each mouse was administered 7-NI (12.5 and 25 mg/kg i.p.) 30 min before carotid artery occlusion. The rest of the procedure was the same as described for group II.

Groups XVI and XVII (7-NI—postconditioning group): Each mouse was administrated 7-NI (12.5 and 25 mg/kg i.p.) 30 min before carotid artery occlusion. The rest of the procedure was the same as described for group III.

Groups XVIII and XIX (aminoguanidine-control group): Each mouse was administered aminoguanidine (200 and 400 mg/kg i.p.) 30 min before carotid artery occlusion. The rest of the procedure was the same as described for group II.

Groups XX and XXI (aminoguanidine-postconditioning group): Each mouse was administrated aminoguanidine (200 and 400 mg/kg i.p.) 30 min before carotid artery occlusion. The rest of the procedure was the same as described for group III.

2.11. Statistical analysis

The results were expressed as mean ± standard error of means. Statistical analysis for all the results was done using one-way analysis of variance followed by Tukey multiple range tests as *post hoc* analysis. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect on cerebral infarct size

Global cerebral ischemia of 12 min followed by reperfusion for 24 h produced significant ($P < 0.05$) increase in cerebral infarct size when compared with sham group. iPoCo significantly ($P < 0.05$) attenuated I/R-induced rise in cerebral infarct size. However, pretreatment of L-NAME and L-NIO significantly attenuated ($P < 0.05$) iPoCo-induced decrease in infarct size, whereas 7-NI and aminoguanidine failed to produce any significant effect. DMF-control and DMF-postconditioning groups did not show any significant modification on cerebral infarct size of control as well as postconditioning groups, so ruling out any effect of vehicle on cerebral infarct size in the present study (Figs. 1 and 2).

3.2. Effect on memory

There was a downward trend in ELT of animals on subsequent water maze exposure during acquisition trial indicating normal learning abilities (data not shown). The sham mice when subjected to retrieval test on d 5 spent significantly ($P < 0.05$) more time in the target quadrant (Q4) in search of the missing platform as compared with time spent in other quadrants (Q1, Q2, and Q3), reflecting normal memory capacity. Global cerebral ischemia followed by reperfusion markedly reduced ($P < 0.05$) d 5 time spent in the target quadrant (TSTQ), when compared with sham group mice indicating memory impairment. On the other hand, iPoCo produced significant increase ($P < 0.05$) in d 5 TSTQ as compared with ischemia control group mice thus attenuating I/R-induced memory impairment. However, pretreatment of L-NAME and L-NIO significantly ($P < 0.05$) abolished iPoCo-mediated rise in day 5

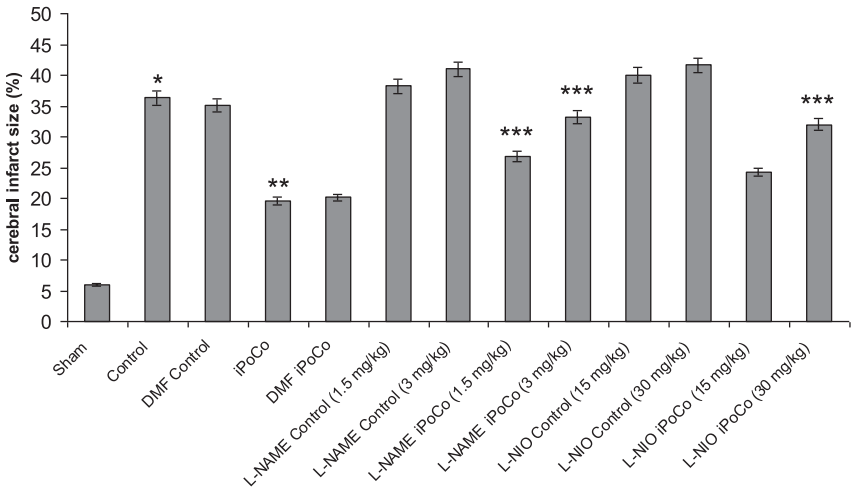

Fig. 1 – Effects of L-NAME and L-NIO on cerebral infarct size using TTC method. L-NIO = N5-(1-iminoethyl)-L-ornithine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control, ***P < 0.05 *versus* iPoCo. One-way ANOVA followed by Tukey multiple range test.

TSTQ, whereas 7-NI and aminoguanidine failed to produce any significant effect. DMF-control and DMF-postconditioning groups did not show any significant effect on ELT of control and postconditioning groups, so ruling out any effect of vehicle on memory in the present study (Figs. 3 and 4).

3.3. *Effect on motor performance*

3.3.1. *Effect on fall down time using rotarod test*

Global cerebral ischemia followed by reperfusion produced significant ($P < 0.05$) reduction in fall down time, as measured by rotarod test, when compared with sham group. iPoCo significantly attenuated I/R-induced reduction in fall down time. However, pretreatment of L-NAME and L-NIO significantly attenuated ($P < 0.05$) iPoCo-induced effect on fall down time, whereas 7-NI and aminoguanidine failed to produce any significant effect. DMF-control and DMF-postconditioning groups did not show any significant effect on fall down time of control and postconditioning groups, so ruling out any effect of vehicle on motor performance in the present study (Figs. 5 and 6).

3.3.2. *Effect on motor in-coordination score using inclined beam-walking test*

Global cerebral ischemia followed by reperfusion produced significant motor in-coordination in mice noted after 24 h of reperfusion when compared with sham group. iPoCo markedly prevented I/R-induced motor in-coordination. However, pretreatment of L-NAME and L-NIO significantly attenuated ($P < 0.05$) iPoCo-induced decrease in motor incoordination, whereas 7-NI and aminoguanidine failed to produce any significant effect. DMF-control and DMF-postconditioning

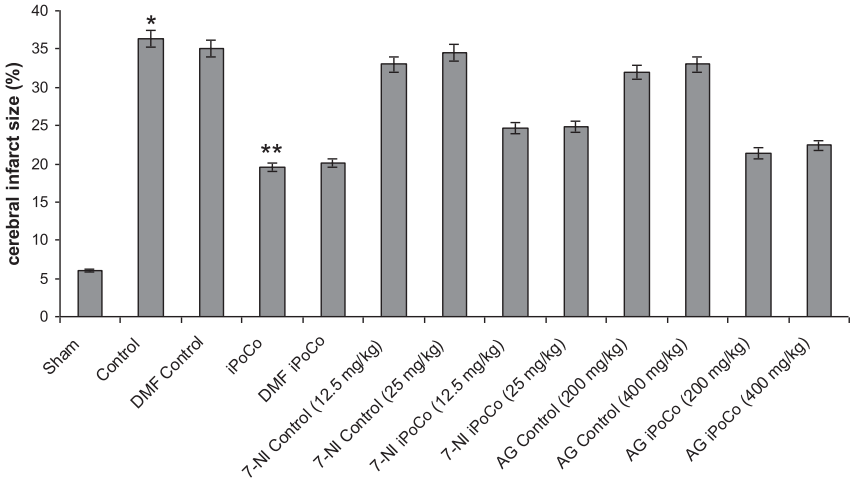

Fig. 2 – Effects of 7-NI and AG on cerebral infarct size using TTC method. AG = aminoguanidine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control. One-way ANOVA followed by Tukey multiple range test.

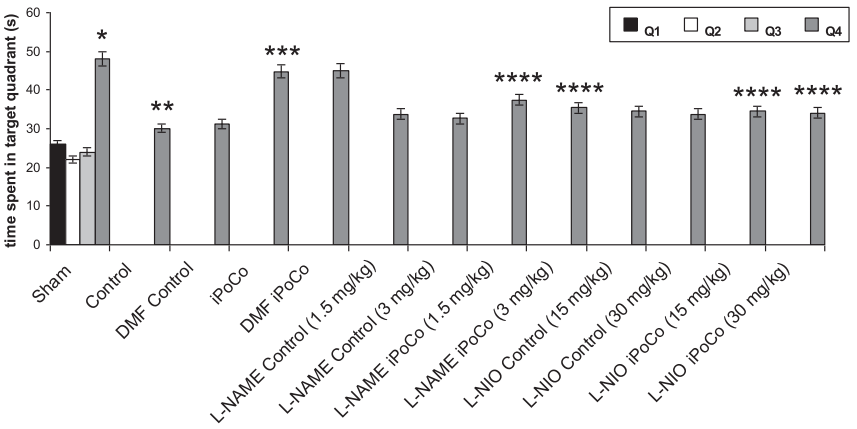

Fig. 3 – Effects of L-NAME and L-NIO on TSTQ using MWM test. L-NIO = N5-(1-iminoethyl)-L-ornithine; iPoCo = ischemic post-conditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* time spent in other quadrants in sham, **P < 0.05 *versus* TSTQ in sham, ***P < 0.05 *versus* TSTQ in control, ****P < 0.05 *versus* TSTQ in iPoCo. One-way ANOVA followed by Tukey's multiple range test.

groups did not show any significant effect on motor incoordination score of control and post-conditioning groups, so ruling out any effect of vehicle on motor performance in the present study (Figs. 7 and 8).

3.3.3. *Effect on lateral push response*

Global cerebral ischemia followed by reperfusion produced a significant decrease in percentage resistance to lateral push noted after 24 h of reperfusion, when compared with sham group. iPoCo significantly prevented I/R-induced decrease in percentage of mice demonstrating resistance to lateral push. However, pretreatment of L-NAME and L-NIO significantly attenuated ($P < 0.05$) iPoCo-induced increase in percentage resistance to lateral push, whereas 7-NI and aminoguanidine failed to produce any such significant effect. DMF-control and DMF-postconditioning groups did not show any significant effect on lateral push response of control and postconditioning groups, so ruling out any effect of vehicle on motor performance in the present study (Figs. 9 and 10).

3.4. *Effect on various biochemical parameters*

Global cerebral ischemia followed by reperfusion produced significant ($P < 0.05$) increase in brain AChE activity and TBARS and significantly decreased brain reduced GSH and nitrite/nitrate level when compared with sham group. iPoCo significantly attenuated I/R-induced increase in brain AChE activity and TBARS level and decrease in GSH and nitrite/nitrate level. However, pretreatment of L-NAME and L-NIO significantly attenuated ($P < 0.05$) iPoCo-induced effect on brain biochemicals, whereas aminoguanidine and 7-NI failed to produce any significant effect (Table 1 and Table 2).

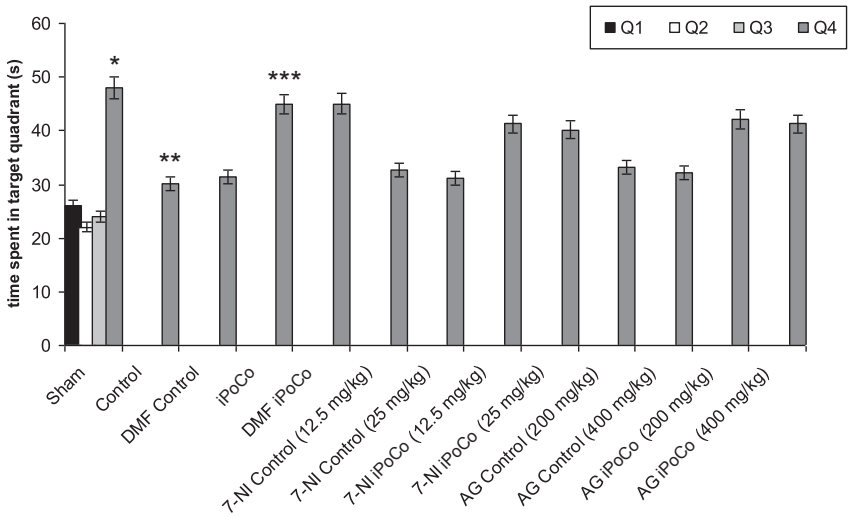

Fig. 4 – Effects of 7-NI and AG on TSTQ using MWM test. AG = aminoguanidine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* time spent in other quadrants in sham, **P < 0.05 *versus* TSTQ in sham, ***P < 0.05 *versus* TSTQ in control. One-way ANOVA followed by Tukey multiple range test.

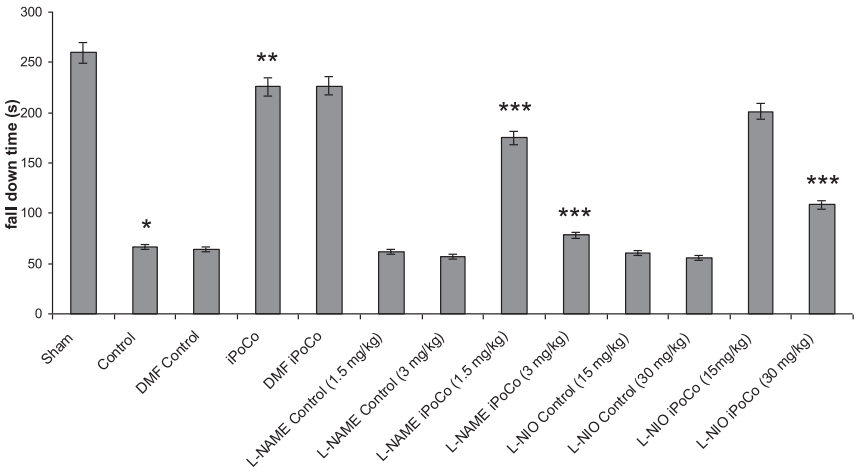

Fig. 5 – Effects of L-NAME and L-NIO on motor performance (fall down time) using rotarod test. L-NIO-N5-(1-iminoethyl)-L-ornithine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control, ***P < 0.05 *versus* iPoCo. One-way ANOVA followed by Tukey multiple range test.

3.5. *Effect on protein expression level*

Global cerebral ischemia followed by reperfusion produced a significant decrease in % eNOS protein expression after 24 h of reperfusion, when compared with sham group. iPoCo significantly prevented I/R-induced decrease in eNOS expression of mice. Furthermore, pretreatment of L-NAME and L-NIO significantly attenuated ($P < 0.05$) the effect of iPoCo on eNOS expression (Fig. 11).

4. Discussion

The results of present investigation indicate that iPoCo significantly attenuated I/R injury-induced deleterious effects on cerebral infarct size, memory, various biochemical parameters, and eNOS expression. These neuroprotective effects of iPoCo are significantly abolished by pretreatment of L-NAME, a nonselective NOS inhibitor, as well as L-NIO, a selective eNOS inhibitor.

Male mice were used in the study because it has been reported that high estrogen level itself exerts protection in cerebral I/R injury [39]. Global cerebral ischemia used in this investigation is well reported to simulate the clinical situation of cerebral ischemia [40]. Cerebral ischemia has been reported to impair memory because hippocampal neurons are susceptible to the deleterious effects of I/R [41] and the hippocampus is involved in regulation of memory. Cerebral ischemia is further documented to impair sensorimotor ability as well [42]. Therefore, in the present study, we used MWM test to assess memory and rotarod test, lateral push test, and inclined beam-walk test for the evaluation of motor coordination. Global ischemia primarily leads to injury of hippocampus, but in addition, it also leads to injury of other sensitive areas of brain in a diffuse manner, due to this reason, whole brain is used for measuring extent of I/R-

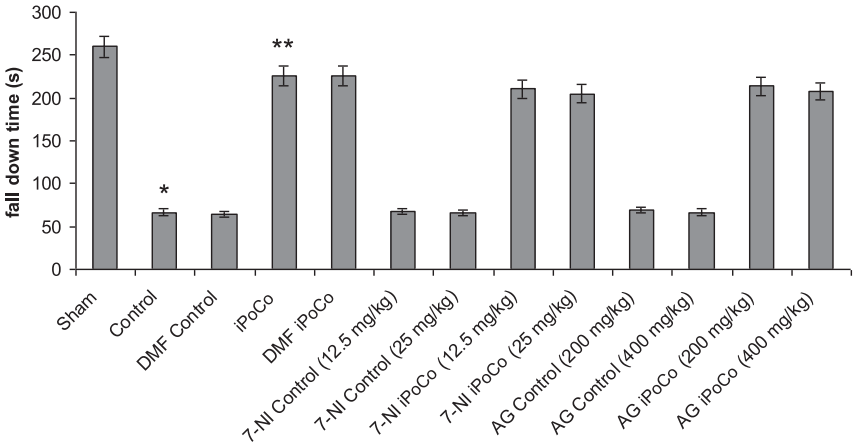

Fig. 6 – Effects of 7-NI and AG on motor performance (fall down time) using rotarod test. AG = aminoguanidine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control. One-way ANOVA followed by Tukey multiple range test.

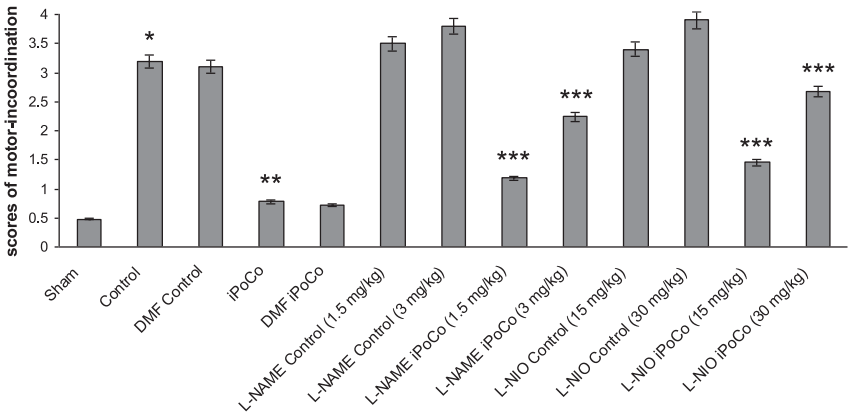

Fig. 7 – Effects of L-NAME and L-NIO on motor performance (score) using inclined beam-walking test. L-NIO = N5-(1-iminoethyl)-L-ornithine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control, ***P < 0.05 *versus* iPoCo. One-way ANOVA followed by Tukey multiple range test.

induced neuronal injury, in term of cerebral infarction by TTC staining [3,12,24]. This has been well established in our earlier published reports [3,12]. The infarct size was measured by volume method using NIH imageJ software (National Institutes of Health) [25]. The protein expression was done using western blotting.

In our study, global cerebral ischemia induced by BCAO for 12 min, followed by prolonged reperfusion of 24 h produced a significant rise in infarct size along with the impairment of memory and motor-coordination. These results are in line with our earlier reports [3,43] and reports from other laboratories [23,44].

One of the most prominent contributors of I/R injury is oxidative stress. Cerebral I/R injury is well documented to produce oxidative stress [3,45]. This is further supported by our observations showing enhanced oxidative stress (increased brain TBARS and decreased GSH) level in the present study. Interestingly, in our previous finding, we have noticed that enhanced brain oxidative stress is somehow related to increased brain AChE activity [3]. However, the exact reason for observed enhancement of brain AChE activity remains to be determined. Cerebral I/R resulted in significant decrease in brain nitrite/nitrate levels. This is also in line with the earlier studies, which clearly document massive fall in NO in I/R injury [46]. This fall in NO may be due to damage to vascular endothelium responsible for producing eNOS. This event coupled with NO inactivation due to reactions with abundant reactive oxygen species, such as $O2^{-\bullet}$, results in reduced NO bioavailability. The reduced NO bioavailability leads to increased oxidative stress, increased apoptosis, increased leukocyte adhesion, increased microcirculatory tone, and perturbed mitochondrial function [47]. Moreover, it has also been reported that NO is involved in the regulation of cerebral blood flow and inhibiting NO pathway by various inhibitors impair cerebral vasodilation, which is one of the primary factors responsible for I/R injury [48,49]. A significant fall in eNOS expression noted in I/R group of our study supports the above statements.

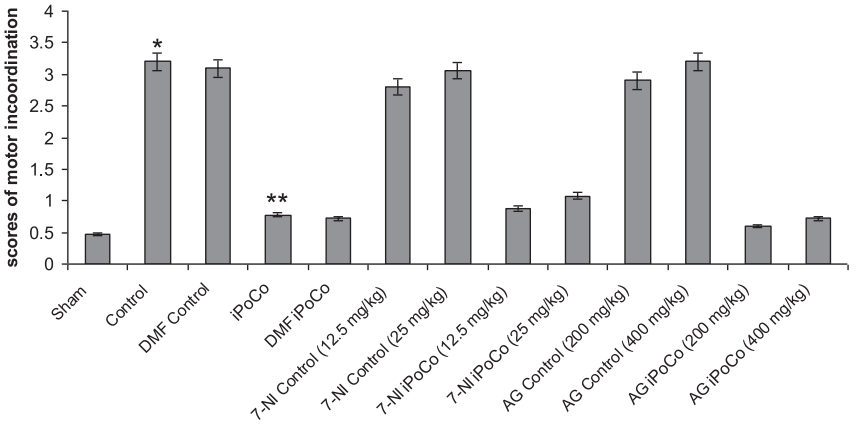

Fig. 8 – Effects of 7-NI and aminoguanidine on motor performance (score) using inclined beam-walking test. AG = aminoguanidine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control. One-way ANOVA followed by Tukey multiple range test.

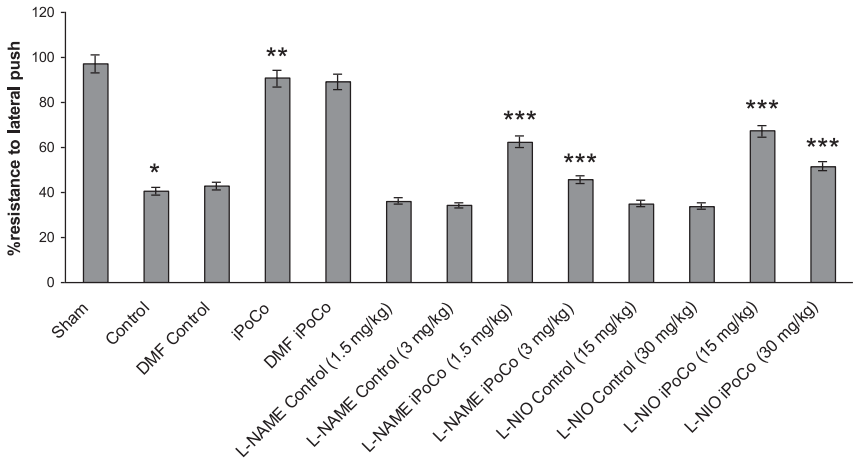

Fig. 9 – Effects of L-NAME and L-NIO on motor performance (% resistance to lateral push) using lateral push test. L-NIO = N5-(1-iminoethyl)-L-ornithine; iPoCo = ischemic post-conditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control, ***P < 0.05 *versus* iPoCo. One-way ANOVA followed by Tukey multiple range test.

In the present investigation, iPoCo comprising of three preceding episodes each of 10 s ischemia and 10 s reperfusion significantly prevented I/R-induced rise in cerebral infarct size, impairment of memory, and motor-coordination, thereby indicating a neuroprotective effect. This observation complies with our earlier findings, whereby similar protocol of iPoCo has been shown to produce neuroprotection [9,50]. iPoCo also attenuated BCAO-induced rise in brain oxidative stress (indicated by a fall in TBARS and a rise in GHS levels), brain AChE activity and fall in brain nitrite/nitrate, and eNOS expression levels. Postconditionings in earlier studies have been reported to alleviate oxidative stress [14] and fall in nitrite/nitrate levels [14] induced by I/R injury. Furthermore, studies conducted by Inserte *et al.* [51] showed enhanced eNOS expression in postconditioned myocardium. Therefore, the results of our study are in line with previous reports.

The neuroprotective effect of iPoCo was significantly abolished by pretreatment of L-NAME, a nonselective NOS inhibitor, and L-NIO, a selective eNOS inhibitor. Whereas, aminoguanidine, a selective iNOS inhibitor, and 7-NI, a selective nNOS inhibitor, did not modulate neuroprotective effect of iPoCo.

In the present investigation, treatment of L-NAME (3 mg/kg, i.p.), a nonselective NOS inhibitor, given 30 min before ischemia, has attenuated the neuroprotective effects of iPoCo. Earlier studies have reported the implication of NO pathway in iPoCo-mediated cardioprotection [52], renal protection [15], and hepatic protection [14]. Therefore, it may be possible to

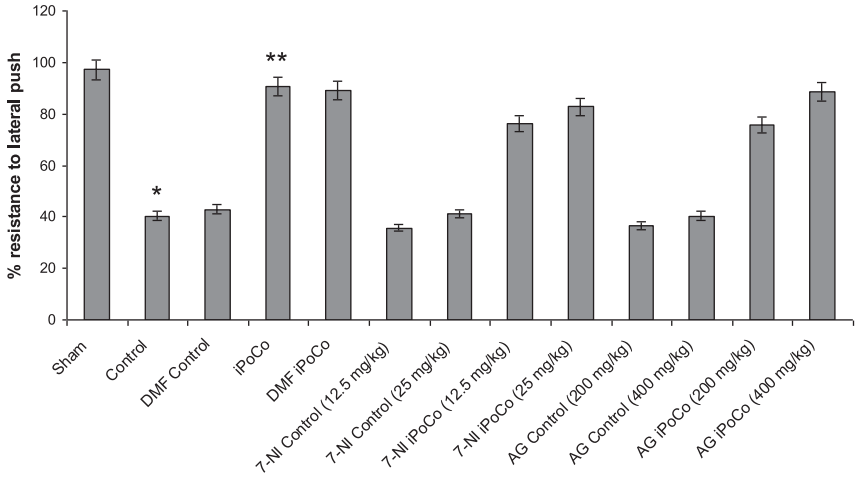

Fig. 10 – Effects of 7-NI and AG on motor performance (% resistance to lateral push) using lateral push test. AG = aminoguanidine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control. One-way ANOVA followed by Tukey multiple range test.

Table 1 – Effect of various interventions on nitrite/nitrate; oxidative stress (TBARS and GSH) levels and AChE activity.

Group	Name of the group	Brain nitrite/nitrate (μg/mg of protein)	Brain TBARS (nM/mg of protein)	Brain GSH (nM/mg of protein)	Brain AChE activity (nM/min/mg protein)
I	Sham	35.25 ± 1.2	19.25 ± 0.6	32.25 ± 0.4	120.08 ± 0.5
II	Control	17.15 ± 1.1*	75.25 ± 0.4*	14.75 ± 0.5*	226.78 ± 0.7*
III	DMF Control	17.95 ± 1.1	76.33 ± 0.4	15.33 ± 0.4	228.15 ± 0.4
IV	iPoCo	31.50 ± 0.8†	23.45 ± 2.1†	29.50 ± 0.6†	130.75 ± 2.1†
V	DMF iPoCo	30.98 ± 0.6	22.35 ± 0.5	28.95 ± 0.5	131.38 ± 0.6
VI	L-NAME (1.5 mg/kg; i.p.) Control	14.25 ± 1.3	77.25 ± 0.5	12.75 ± 0.8	230.78 ± 0.4
VII	L-NAME (3 mg/kg; i.p.) Control	12.35 ± 0.6	79.25 ± 0.5	10.88 ± 0.8	232.71 ± 1.1
VIII	L-NAME (1.5 mg/kg; i.p.) iPoCo	22.75 ± 1.1‡	55.75 ± 0.8‡	22.25 ± 0.6‡	195.75 ± 1.8‡
IX	L-NAME (3 mg/kg; i.p.) iPoCo	20.40 ± 1.4‡	60.78 ± 0.8‡	19.25 ± 0.6‡	200.25 ± 0.9‡
X.	L-NIO (15 mg/kg; i.p.) Control	13.75 ± 1.5	77.75 ± 0.4	11.22 ± 0.4	231.25 ± 0.6
XI	L-NIO (30 mg/kg; i.p.) Control	11.0 ± 1.2	79.78 ± 0.9	10.05 ± 0.5	233.15 ± 1.5
XII	L-NIO (15 mg/kg; i.p.) iPoCo	21.75 ± 1.4‡	62.28 ± 1.2‡	22.78 ± 0.8‡	196.78 ± 1.2‡
XIII	L-NIO (30 mg/kg; i.p.) iPoCo	20.05 ± 1.3‡	65.25 ± 0.4‡	18.25 ± 0.7‡	201.38 ± 0.6‡

L-NIO = N5-(1-iminoethyl)-L-ornithine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean.
One-way ANOVA followed by Tukey multiple range test.
* $P < 0.05$ *versus* sham.
† $P < 0.05$ *versus* control.
‡ $P < 0.05$ *versus* iPoCo.

suggest here that iPoCo-induced neuroprotection also involves NO pathway.

It is well known that pharmacologic role of NO is isoform dependent. Three structurally distinct isoforms of NOS have been identified in mammals including eNOS, iNOS, and nNOS. In the present study, we tried to explore isoform specific involvement of NO in neuroprotective mechanism of iPoCo by using selective inhibitors of all the three isoforms of NOS. Administration of L-NIO (a selective eNOS inhibitor) attenuated iPoCo-induced neuroprotection in a significant manner, whereas aminoguanidine (a selective iNOS inhibitor) and 7-NI (a selective nNOS inhibitor) failed to modulate iPoCo-induced neuroprotective effects. These results indicate that perhaps eNOS but not iNOS or nNOS probably is involved in postconditioning mediated neuroprotection. This is further confirmed by our western blotting analysis showing a significant fall in eNOS expression levels of iPoCo groups pretreated with L-NAME and L-NIO.

Therefore, with support from literature and data in hand, it may be suggested that neuroprotective mechanism of iPoCo involves NO-mediated pathway with eNOS playing a critical role.

Table 2 – Effect of various interventions on nitrite/nitrate; oxidative stress (TBARS and GSH) levels and AChE activity.

Group	Name of the group	Brain nitrite/nitrate (μg/mg of protein)	Brain TBARS (nM/mg of protein)	Brain GSH (nM/mg of protein)	Brain AChE activity (nM/min/mg protein)
I	Sham	35.25 ± 1.2	19.25 ± 0.6	32.25 ± 0.4	120.08 ± 0.5
II	Control	17.15 ± 1.1*	75.25 ± 0.4*	14.75 ± 0.5*	226.78 ± 0.7*
III	DMF Control	17.95 ± 1.1	76.33 ± 0.4	15.33 ± 0.4	228.15 ± 0.4
IV	iPoCo	31.50 ± 0.8†	23.45 ± 2.1†	29.50 ± 0.6†	130.75 ± 2.1†
V	DMF iPoCo	30.98 ± 0.6	22.35 ± 0.5	28.95 ± 0.5	131.38 ± 0.6
VI	7-NI (12.5 mg/kg; i.p.) Control	14.25 ± 1.2	76.75 ± 0.7	13.17 ± 0.3	231.75 ± 0.9
VII	7-NI (25 mg/kg; i.p.) Control	12.35±1.1	77.78 ± 0.7	12.25 ± 0.5	233.75 ± 0.9
VIII	7-NI (12.5 mg/kg; i.p.) iPoCo	29.75±1.2	26.31 ± 0.8	26.25 ± 0.6	140.25 ± 0.7
IX	7-NI (25 mg/kg; i.p.) iPoCo	28.95±1.4	28.15 ± 0.9	27.15 ± 0.5	145.25 ± 1.1
X	AG (200 mg/kg; i.p.) Control	15.75 ± 1.0	73.75 ± 0.9	12.98 ± 0.8	229.78 ± 1.1
XI	AG (400 mg/kg; i.p.) Control	15.01 ± 1.4	78.25 ± 1.0	11.78 ± 0.9	230.75 ± 0.9
XII	AG (200 mg/kg; i.p.) iPoCo	28.95 ± 1.3	27.18 ± 1.1	27.78 ± 1.1	136.27 ± 0.8
XIII	AG (400 mg/kg; i.p.) iPoCo	27.75 ± 1.2	28.75 ± 0.8	28.85 ± 1.2	137.75 ± 0.9

AG = aminoguanidine; iPoCo = ischemic postconditioning. Each group represents mean ± standard error of the mean.
One-way ANOVA followed by Tukey multiple range test.
* $P < 0.05$ *versus* sham.
† $P < 0.05$ *versus* control.

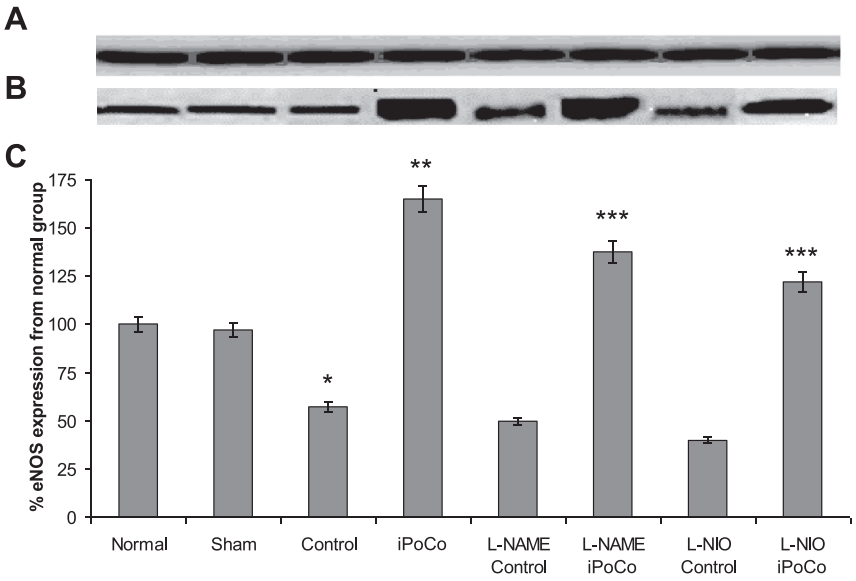

Fig. 11 – Effects of various interventions on eNOS protein expression using western blotting. (A) Loading control (B) representative western blotting and (C) densitometric analysis of eNOS protein expression. iPoCo = ischemic postconditioning; L-NIO = N5-(1-iminoethyl)-L-ornithine. Each group represents mean ± standard error of the mean. *P < 0.05 *versus* sham, **P < 0.05 *versus* control, ***P < 0.05 *versus* iPoCo. One-way ANOVA followed by Tukey multiple range test.

5. Conclusion

From the above discussion, it can be concluded that it is neither iNOS nor nNOS but eNOS isoform, which is probably responsible for neuroprotective mechanism of iPoCo. Nevertheless, in depth, further studies involving eNOS knock-out mice are needed to substantiate these findings.

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