

Role of N-Nitro-L-Arginine-Methylester in Neuroprotection of Cerebral Ischemic Preconditioning in Rats

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Abstract

Background and Aim: Ischemic preconditioning (IPC) is a phenomenon in which brief episodes of ischemia protect the brain from subsequent, severe ischemic insult. This study evaluated whether the neuroprotective effect of cerebral IPC is mediated by nitric oxide synthase (NOS) inhibition, using non-selective NOS inhibitor, and N-Nitro-L-Arginine-Methylester (L-NAME). **Materials and Methods:** Fifty adult male Wistar rats divided into five groups ten in each: (1) Sham-operated group (control), (2) ischemia-reperfusion (I/R) group; rats subjected to 30 min of left common carotid artery (CCA) occlusion followed by 24-h of reperfusion, (3) I/R with NOS inhibition group; rats infused with L-NAME intraperitoneally 15 min before the same I/R period, (4) IPC group; rats treated with three 5-min episodes of CCA occlusion (CCAO) with 10 min of reperfusion between stimuli, then 30 min of CCAO followed by 24 h reperfusion, and (5) IPC with NOS inhibition group: Rats were subjected to the same preconditioning stimuli as Group 4 with the infusion of NOS inhibitor (L-NAME) 15 mg/kg, 15 min before CCAO. Neurological assessments were evaluated, enzyme-linked immunosorbent assay used to detect Rho-kinases (ROCK), and nitric oxide metabolites were measured colorimetrically. **Results:** IPC significantly reduces the neurological deficit and lowering the ROCK level with higher nitrite levels. While administration of L-NAME in IPC rats results in a significant enhancement in neurological scoring compared to IPC without NOS inhibition, with significant inhibition of nitrite and ROCK. **Conclusion:** Despite previous evidence that NO involves in neuroprotective mechanism of IPC, the current data suggest the potential of L-NAME as a neuroprotective component of IPC.

Key words: Cerebral, ischemia/reperfusion, ischemic preconditioning, N-nitro-L-arginine-methylester, rho kinase

INTRODUCTION

Cerebral ischemia occurs when there is a sudden disruption to the cerebral blood flow. It can lead to ischemic stroke with dysfunction in the brain.^[1] With a high incidence, prevalence, and mortality, ischemic stroke continues to be a leading cause of death and disability globally.^[2] The instant restoration of blood reperfusion is the most effective way of treating cerebral ischemic stroke; however, ischemia-reperfusion (I/R) can lead to functional impairment and/or neuronal death.^[3] At present, this illness has no effective pharmacotherapy. A broad approach to mitigating ischemic injury is the use of exogenous drugs.^[4] However, there are endogenous approaches to neuroprotection, such as cerebral ischemic preconditioning (IPC). It aims to prepare the brain for a future lesion either by decreasing the intensity of response or by inducing elasticity of the organism toward the biological challenge.^[5]

IPC is a phenomenon in which brief episodes of ischemia protect the brain from subsequent, more severe ischemic insult, it is a technique for producing resistance to the loss of blood supply and does not lead to neuronal death.^[6] Moreover, IPC displays the adaptation of the central nervous system to mild and moderate ischemia, especially enhances ischemic tolerance to prolong subsequent ischemia. The mechanisms of IPC mediated ischemic tolerance have not been precisely known. Nevertheless, two temporal windows of protection have been described for IPC: An early or acute phase that lasts for 1–3 h following the preconditioning stimulus and

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a delayed phase of protection that begins 18–24 h after the initial IPC stimulus and persists for 24–72 h.^[7]

Since the discovery of IPC, research has focused on uncovering the mechanism of IPC-mediated protection, to understand the physiological process of adaptation to ischemia, and developing therapeutics mimicking the protective effects of IPC. Several pharmacological mimetics and biological triggers of delayed IPC, including adenosine, opioids, bradykinin, cytokines, and nitric oxide (NO) have been identified. These agents activate several upstream signaling cascades, of which converges at the point of NO synthase (NOS) activation, suggesting that NO production is a central event in delayed IPC “The NO hypothesis of late preconditioning.”^[8] Moreover, NO has been implicated in several models of cerebral preconditioning.^[9]

There are three types of NOS that produce endogenous NO in the brain: Neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO produced by eNOS was proved to be beneficial during cerebral I/R, due to its vasodilator and antiplatelet effects, as well as enhances angiogenesis after stroke. On contradictory nNOS and iNOS can be neurotoxic.^[10,11]

Rho-kinases are serine/threonine kinases that exist as two isomers Rho-kinase α /ROCK2 and Rho-kinase β /ROCK1. ROCK is implicated in the regulation of vascular smooth muscle cell migration, proliferation, and differentiation.^[12,13] It has been demonstrated that levels of Rho are upregulated in the brains of patients who have died following focal cerebral infarction as well as a mouse model of ischemia.^[14] There is evidence that activation of ROCK could downregulate eNOS expression and worsen the endothelial function.^[15]

It has been reported that an excess of NO associates with the development of cerebral ischemia.^[10] While non-selective inhibition of NOS activity can regulate the content of NO *in vivo* and produces a therapeutic effect on I/R.^[16] Our previous study revealed a potential anti-oxidant effect of nonselective NOS inhibitor, N-Nitro-L-Arginine-Methylester (L-NAME), in rat’s focal cerebral I/R.^[17] Based on these considerations, the present work aimed to study the neuroprotective mechanisms of IPC on Rho-kinase (ROCK II) and NO levels in the brain homogenate of rats as well as the effect of NOS inhibition using L-NAME.

MATERIALS AND METHODS

Experimental animals and groupings

This study was carried on 50 male adult Sprague Dawley rats weighing about 150–250 g. The rats housed at room temperature with free access to standard diet and tap water. They handled following the ethical standards laid down in the US National Institutes of Health (NIH Publication No. 85 - 23) and its later revisions.

The rats were assigned into five groups of ten in each. (1) Sham group: Rats subjected to full surgical preparation without the common carotid artery occlusion (CCAO); and severed as a control group; (2) ischemia/reperfusion (I/R) group: Rats’ exposed to 30 min of left CCAO followed by 24 h reperfusion;^[18] (3) I/R with NOS inhibition group: Rats were received same I/R injury as Group 2 with an infusion with NOS inhibitor (L-NAME) 15 mg/kg intraperitoneally (i.p), 15 min before common carotid artery (CCA) occlusion;^[17] and (4) cerebral IPC group: Rats treated with three 5-min episodes of CCAO with 10 min of reperfusion between stimuli, followed by 30 min of CCAO. Then, the clips were removed to allow reperfusion of the carotid artery for 24 h.^[19] (5) IPC with NOS inhibition group: Rats subjected to the same preconditioning stimuli as Group 4 with the infusion of NOS inhibitor (L-NAME $C_7H_{15}N_5O_4$ purchased from Sigma-Aldrich) 15 mg/kg, 15 min before CCA occlusion (CCAO).^[17]

Induction of cerebral ischemia

The animals fasted overnight before surgery with free access to tap water. Anesthesia was induced by ether inhalation and maintained by thiopental sodium (2.5 mg/kg).^[20] Body temperature was kept constant at $36.5 \pm 0.5^\circ\text{C}$ using a heating pad. A longitudinal cervical incision (2 cm) was made lateral to the midline, and the CCA dissected carefully. Ischemia was induced by placing non-traumatic microvascular clip-on left CCA just before its bifurcation. During ischemia, rats monitored for body temperature and respiration patterns. The clips removed to resume blood flow to the ischemic region for 24 h.^[18] Finally, the incisions sutured, the animal was allowed to recover from anesthesia and returned to a warm cage for recuperation during the reperfusion period. In the case of preconditioning, rats treated with three 5-min episodes of CCA with 10 min of reperfusion between stimuli, followed by 30 min of CCAO. Then, the clips were removed to allow for reperfusion of the carotid artery for 24 h.^[19] The number of animals presented for each group is the number of rats that survived during the 24-h reperfusion period. The collected data of the animals that died during 24 h reperfusion period were excluded from the study.

Neurobehavioral evaluation

Neurobehavioral tests were assessed for all experimental groups to determine the effect of ischemic injury on them. The evaluations performed at three stages: Stage (1) on the day before surgery, Stage (2) on the same day as surgery, and Stage (3) just before killing the rats. Each rat examined in late afternoon hours, as rats that had been operated in the morning would fully recover from the effects of anesthesia by the time of evaluation. The neurobehavioral study consisted of the following six tests: Spontaneous activity, symmetry in the movement of the four limbs, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch. The score given to each rat at the end of the evaluation is the

summation of all six individual test scores. The minimum neurological score was three, and the maximum was 18, Table 1.^[21]

Laboratory investigations

At the end of the experimental period, the rats were sacrificed by decapitation using the same ether inhalation.^[20] Brains were rapidly removed from the skull and washed with cold saline and stored at -20°C for further analysis. A small part of each brain from the affected hemisphere was dissected to approximately 1–2 mm pieces and then homogenized in 7 ml of ice-cold extraction buffer (1% Triton X-100, 10 mmol/l MgSO_4 , 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 0.5 mol/l NaCl, 1% protease inhibitor cocktail, 20 mmol/l HEPES, and pH of 7.5). The homogenate centrifuged and the supernatant were taken and stored at -20°C until being used. A modification of the method of Lowry used for the determination of protein in the brain homogenate.^[22] NO metabolite (nitrite) measured colorimetrically^[23] and Rho-kinase II detected using enzyme-linked immunosorbent assay,^[24] purchased from Cell Biolabs, INC.

Data analysis

Data were expressed as mean \pm S.D. Statistical evaluation was performed using Microsoft Office Excel (Microsoft Office Excel for windows; 2003) and SPSS (SPSS for windows version 25). Screening studied rats' groups for the significant difference in the mean of ROCK and NO was performed using analysis of variance. $P < 0.05$ was considered significant.

RESULTS

L-NAME alleviates neurological deficit in I/R and IPC rats

As shown in Figure 1, it is apparent from the current finding that our IPC model protective evident by significant improvement of the neurological score in preconditioned rats (13.71 ± 0.21) compared with I/R group (12.87 ± 0.71),

Table 1: Correlation between Rho kinase, nitrite level, and neurological scoring in the whole studied rats

Variables	Nitric oxide	Neurological scoring
Rho Kinase		
r	-0.203	0.009
p	0.208	0.955
Nitrite		
r		-0.381*
p		0.015

r: Pearson coefficient, *: Statistically significant at $P \leq 0.05$

I/R with L-NAME (14.88 ± 0.47), and control group (17.5 ± 0.76), where $P < 0.001$. While administration of L-NAME to both I/R and preconditioning group (15.29 ± 0.55) causes a significant increase in neurological scoring as compared to the control group and I/R group, where $P < 0.001$.

L-NAME inhibits NO in IPC rats

Nitrite was estimated in the affected brain homogenate in all five experimental groups. As illustrates in Figure 2, a significant increase in nitrite level in the preconditioning rats ($82.56 \pm 6.45 \mu\text{mol/l}$) as compared to the sham-operated ($53.16 \pm 10.93 \mu\text{mol/l}$) and I/R groups ($60.85 \pm 11.57 \mu\text{mol/l}$) $P < 0.001$. Moreover, there was a significant decrease in nitrite level after administration of L-NAME to preconditioning rats ($66.52 \pm 8.49 \mu\text{mol/l}$) as compared to IPC rats without NOS inhibition ($82.56 \pm 8.49 \mu\text{mol/l}$) $P < 0.001$. Regarding the I/R groups, there was no significant difference observed after L-NAME.

Correlation between neurological scoring and brain nitrite

In the current study, a significant negative correlation found between the degree of neurological deficit and brain nitrite [Table 1], this indicates that more deterioration of neurobehavioral examination is associated with the release of NO from the ischemic cerebral hemisphere.

L-NAME inhibits rho-kinase

Rho-kinase was estimated in the brain homogenate prepared from the affected left hemisphere in all five experimental groups. As shown in Figure 3, the present study revealed that preconditioning rats demonstrate a significant decrease in the mean value of Rho-kinase ($0.009 \pm 0.004 \text{ ng/mg}$) as compared to the control group ($0.021 \pm 0.003 \text{ ng/mg}$) and the I/R group ($0.024 \pm 0.004 \text{ ng/mg}$) $P < 0.001$, while administration of L-NAME to IPC rats ($0.007 \pm 0.002 \text{ ng/mg}$) results in a significant decrease in the mean value of Rho-kinase as compared to the control and I/R group $P < 0.001$. Moreover, the current data demonstrate a significant increase in Rho-kinase level in rats subjected to I/R only ($0.024 \pm 0.003 \text{ ng/mg}$) compared to Groups 3, 4, and 5.

DISCUSSION

Several studies reported the neuroprotective mechanism of IPC in both global and focal cerebral ischemia.^[25,26] It is apparent from the current finding that our IPC model is concurrent with those suggestions evidence by the improvement of the neurological score in preconditioned rats. Nonetheless, unexpectedly and despite the tremendous evidence regarding the involvement of NO in the tolerance

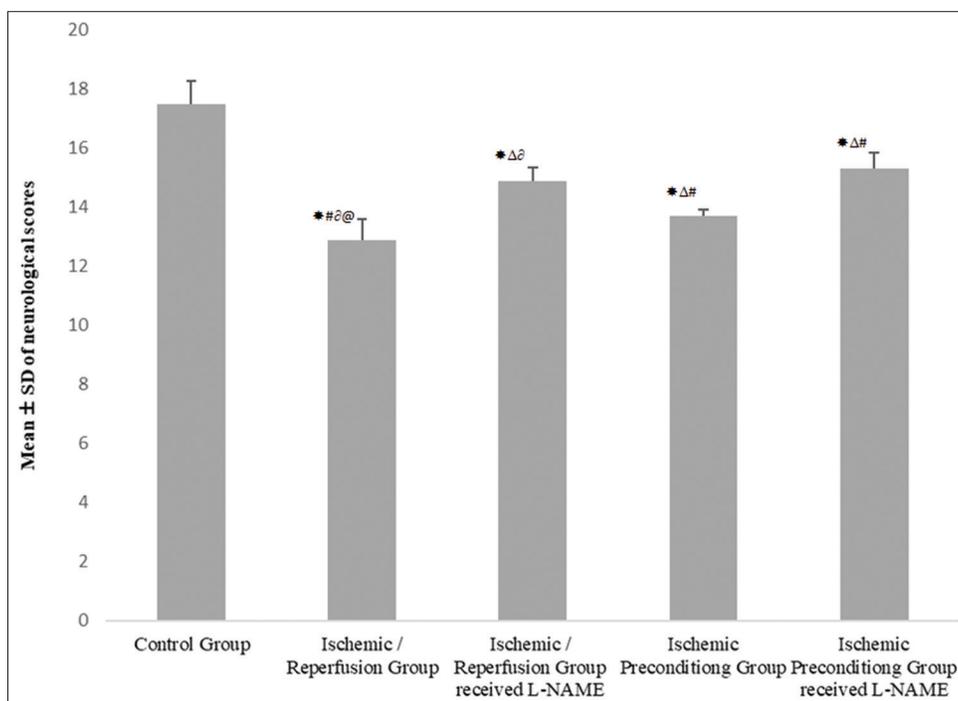


Figure 1: Comparison between the different experimental groups according to mean neurological scoring. F: F test (ANOVA) Statistically significant at $P \leq 0.05$. *Significant with control group, Δ Significant with Group 2 (I/R), #Significant with Group 3 (I/R received L-NAME), \textcircled{c} Significant with Group 4 (IPC), \textcircled{e} Significant with Group 5 (IPC received L-NAME)

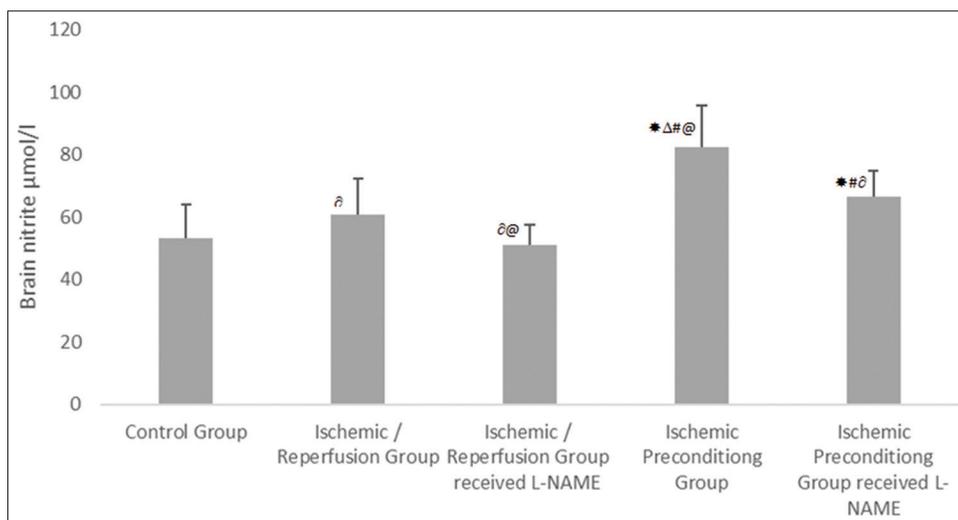


Figure 2: Comparison between the different experimental groups according to mean brain nitrite level. F: F test (ANOVA) Statistically significant at $P \leq 0.05$. *Significant with control group, Δ Significant with Group 2 (I/R), #Significant with Group 3 (I/R received L-NAME), \textcircled{c} Significant with Group 4 (IPC), \textcircled{e} Significant with Group 5 (IPC received L-NAME)

mechanism of IPC, the current study opposes these findings with a probable protective effect due to NO inhibition in IPC model, through the administration of L-NAME. This proposal was supported by further improvement in the neurological score of rats treated by this medication in the ischemic preconditioned group before the onset of severe cerebral I/R injury.

Our finding revealed that IPC rats showed a significant increase in nitrite level when compared both to control

and I/R groups. Other researches with comparable findings reported the elevation of nitrite as a potential mediator of IPC. Jones *et al.* reported an increasing level of nitrite and nitrate after administration of IPC, enhancing the suggested role of NO in delayed IPC tolerance.^[27] In the same manner, several studies reported that nitrite modulates resistance to ischemic stress in IPC models, which was exposed primarily through the measurement of the levels and enzymatic activities of eNOS and iNOS as well as manipulation of these enzymes by knockout or transgenic overexpression strategies

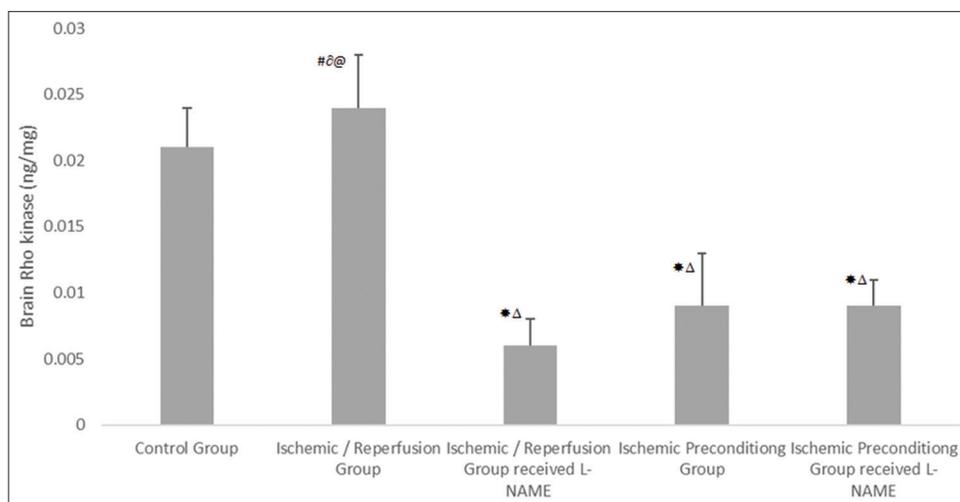


Figure 3: Comparison between the different studied groups according to the brain Rho Kinase level. F: F test (ANOVA) Statistically significant at $P \leq 0.05$. *Significant with control group, Δ Significant with Group 2 (I/R), #Significant with Group 3 (I/R received L-NAME), \circ Significant with Group 4 (IPC), \oplus Significant with Group 5 (IPC received L-NAME)

in animal models of IPC-mediated protection.^[7,28] Moreover, seminal studies have approved that iNOS mRNA and protein expression increase after administration of IPC, resulting in increased iNOS activity as well as tissue nitrite/nitrate levels.^[29,30]

The previous studies carried out to evaluate the effect of L-NAME in rats subjected to cerebral IPC are rare, we reported a possible antioxidant effect of L-NAME in rats' model of cerebral I/R. Hence, we used the same dosing and timing regimen.^[17] The role of NO in cerebral ischemia was examined 24 h after CCAO using a dose of 15 mg/kg L-NAME, 15 min before induction of cerebral ischemia. On the other hand, Puisieux *et al.*^[31] reported that lipopolysaccharide (LPS) administration could result in preconditioning, as LPS was associated with increased eNOS expression and protection from LPS was blocked by L-NAME. However, for unclear reasons, the beneficial effect of IPC was unchanged despite the prior administration of L-NAME. The possible reasons for the discrepancy include differences in species (rat vs. mouse), differences in the model (one episode of 3 min transient MCAO vs. three episodes of 5 min MCAO), and incomplete blockade of eNOS expression by L-NAME. This last possibility is highlighted by the lack of effect of L-NAME on un-preconditioned infarct size in the preceding study.

Moreover, a significant negative correlation found, in the current study, between the degree of neurological deficit and brain nitrite, this indicates that more deterioration of neurobehavioral examination is associated with the release of NO from the ischemic cerebral hemisphere. This can be related to the neurotoxic effect of induced expression of iNOS, in response to locally produced inflammatory cytokines that have been localized in infiltrating phagocytes, vascular cells, and glial cells.^[32] Not be able to measure the enzymatic activity of different NOS isoforms, is a limitation of the current experiment.

The current data demonstrated a significant increase in Rho-kinase level in rats subjected to I/R only, these findings were further supported by several studies proving the involvement of Rho-kinase in neuronal network disturbance and neurological deficits, as well as induce contraction of blood vessels, causing progression of infarction. These hypotheses are consistent with the improvement of the outcome of cerebral infarction by Rho-kinase inhibitors.^[14,33] Studies involving the role of Rho-kinase in cerebral IPC are limited. We found that our IPC rats demonstrated a significant decrease in the Rho-kinase level compared to I/R one. In contrast to this finding, Hu *et al.*^[34] reported increase Rho-kinase after IPC (abdominal aorta occlusion applied 2 h before shock).

In addition, the neuroprotective potential of L-NAME is further supported, in this study, through inhibition of Rho-kinase level in the affected hemisphere as compared to both control and I/R groups. Nevertheless, no significant difference in the Rho-kinase levels in IPC rats that received this medication. Rho-kinase is increasingly implicated in the pathophysiology of cardiovascular diseases such as hypertension, atherosclerosis, and vasospasm.^[12,35] However, the physiological importance of Rho-kinase in the pathogenesis of cerebral infarction is still unclear.

CONCLUSION

IPC affords protection against a subsequent more prolonged ischemic challenge. Our IPC model demonstrates improvement in neurological deficits as compared to ischemia reperfusion. Furthermore, pre-treatment of rats with L-NAME, before I/R and IPC, establish a neuroprotective potential through the improvement of rats' neurological deficits as well as significant decrease Rho kinase levels in the affected cerebral hemisphere.

DECLARATION OF INTEREST

The author reports no conflicts of interest. The author alone is responsible for the content and writing of this article.

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