L-NAME and 7-Nitroindazole Reduces Brain Injuries in Transient Focal Cerebral Ischemia in Rat

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Abstract

Background: The role of nitric oxide (NO) of endothelial or neuronal origins in cerebral ischemia and reperfusion injuries are far from being settled, extending from being important to not having any role at all.

Objective: To investigate the role of NO of endothelial and neuronal origins in ischemia/reperfusion injuries in focal cerebral ischemia, L-NAME, a non selective NO synthase inhibitor (NOS), and 7-nitroindazole (7-NI), a selective neuronal NOS were used.

Methods: Transient focal cerebral ischemia was induced in rats by 90 min occlusion of middle cerebral artery, followed by 24 hr reperfusion. Vehicle (saline, DMSO), L-NAME (1 mg/kg) or 7-NI (50 mg/kg) was administered ip at 30 min before or 60 min after the onset of ischemia. At the end of reperfusion period, neurological deficit score (NDS) test was performed. Then under deep anesthesia the brain removed and prepared for the evaluation of cortical and striatal infarct volumes using Triphenyltetrazolium chloride staining.

Results: Pre-ischemic administration of L-NAME significantly lowered cortical (-66±6%) and striatal (-45±12%) infarct volumes with a concomitant improved NDS (-38±10%). A significant decrement in cortical (-39±7%) and striatal (-26±5%) infarct volumes occurred during post-ischemic administration of L-NAME without an improvement in NDS. Pre-ischemic administration of 7-NI also significantly reduced cortical (-37±10%) and striatal (-37±13%) infarct volumes, but did not change NDS significantly. Whereas, post-ischemic administrations of 7-NI neither changed cortical and striatal infarct volumes nor changed NDS.

Conclusion: Our presumptive conclusion is that, in the rat model of transient focal cerebral ischemia, NO of neuronal origin is involved in ischemic and that of endothelial origin participates in reperfusion injuries.
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Keywords • Cerebral ischemia • 7-Nitroindazole • L-NAME • Nitric Oxide • Rat

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Introduction

Cessation or severe reduction, below threshold of cerebral blood flow to any part of the brain, leads to an ischemic core surrounded by a poorly-perfused area, called penumbra. Damages to the ischemic core are irreversible and neurons die rapidly. In penumbra, which receives collateral blood supply, the neuronal cell death occurs at a much slower rate.[1] Rapid restoration of blood flow to the ischemic region and inhibition of neurotoxic cascades might be important in reducing neural damage in penumbra.[2] However, restoration of blood flow to ischemic area, if not done at a proper time, is associated with side effects, namely reperfusion injuries.[3]

Various mechanisms have been proposed to account for ischemia/reperfusion injuries including calcium overloading, glutamate excitotoxicity, and toxic free-radicals.[4] Among these mechanisms, however, the role of nitric oxide (NO) is not agreed upon. It has been demonstrated that NO might decrease,[5,6] or increase,[7,8] and or have no effect,[9,10] on the cerebral infarct size in rat, mice or cat model of focal cerebral ischemia. Moreover, it has been reported that NO either did not improve,[5] or improved,[11] ischemia/reperfusion-induced neurological deficits score (NDS).

In the light of controversies surrounding the role of NO in the ischemia/reperfusion injuries, this study was designed to investigate the role of NO of endothelial and neuronal origins in rat model of transient focal cerebral ischemia using 7-nitroindazole (7-NI), a selective neuronal NO synthase (NOS) inhibitor, and N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor.

Materials and Methods

Experiments were performed in conformity with the university research council guidelines for conducting animal studies. Male Sprague-Dawley rats (Razi institute, Shiraz Iran) were housed in standard cages in room temperature (22-24ºC), humidity (40-60%), and light period (07.00-19.00 h) controlled environment with food and water ad libitum.

Instrumentation and surgical procedures

Rats (300-370g) were anesthetized with an intraperitoneal (ip) injection of chloral hydrate (400mg/kg; Sigma, UK). The right femoral artery was cannulated in about 50% of the animals of each group for continuous recording of mean arterial pressure (MAP) and taking 0.2 ml blood for evaluation of blood gases and pH.
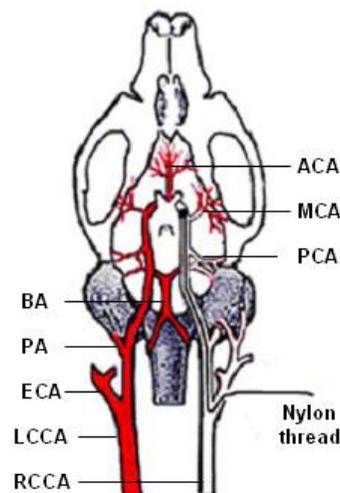

Fig1: Schematic representation of rat brain arteries and nylon thread used to make middle cerebral artery occlusion. LCCA= Left common carotid artery, RCCA= Right common carotid artery, ECA=external carotid artery, ICA=internal carotid artery, PA= Pterygopalatine artery, MCA= middle cerebral artery, BA=Basilar artery

Under an operating microscope, right common carotid artery (CCA) and external carotid artery (ECA) were exposed through a midline incision (2 cm in length) in front of the neck by putting a retractor between the digastrics and sterno-mastoid muscles and dividing omohyiod muscle. Then CCA was carefully dissected free from surrounding fascia and vagus nerve to reach its bifurcation to external (ECA) and internal carotid (ICA) arteries. The occipital artery and superior thyroid branches of the ECA were then isolated and coagulated. Subsequently, ECA was ligated permanently at about 8 mm form the CCA bifurcation. The internal carotid artery (ICA) was dissected to the level of pterygopalatine artery (PA). Afterwards, PA was ligated, a silk thread was placed loosely around the ECA stump, and CCA and ICA were clamped temporarily using microvascular clips. Then a small incision was made on ECA, and a Poly-L-Lysine-coated nylon thread (3-0), prepared in advance, and was inserted through. While holding the thread tightly around ECA to prevent bleeding, the microvascular clip on ICA was removed, and the nylon thread was carefully and slowly pushed forward through ICA. Depending on weight of the animal, an advance for 20-22 mm of nylon thread from CCA bifurcation would place its tip at the beginning of anterior cerebral artery, resulting in the occlusion of middle cerebral artery (MCA; Fig 1).

Reperfusion phase started by gently pulling the intraluminal nylon thread out of ICA. Then

the above-mentioned loose silk thread around ECA stump was tightened to prevent bleeding. After 30 min reperfusion, the femoral arterial catheter was removed, the artery ligated, and after suturing the incisions the animal was allowed to recover from anesthesia and returned to a warm cage. Body temperature was maintained during the experiment at 37±1ºC using a rectal thermometer and a heating lamp.

Experimental design
Rats (n=75) were randomly assigned to 10 groups. Groups 1 (n=6) and 6 (n=7) were sham, in which MCA was not occluded, and vehicle or drug were not applied. Groups 2 (n=6) and 3 (n=9) were control groups receiving 0.1 ml dimethyl sulfoxide %10 (DMSO, Sigma, Germany) or saline (0.1 ml) at 30 min before MCA occlusion (MCAO), respectively. Group 4 (n=7) and group 5 (n=6) were control groups receiving DMSO or saline, at 60 min after MCAO. Groups 7 (n=8) and 8 (n=10) received 1 mg/kg L-NAME,[12] or 50 mg/kg 7-NI,[11] at 30 min before MCAO,[11] respectively. Groups 9 (n=8) and 10 (n=8) received L-NAME (1 mg/kg) or 7-NI (50mg/kg) at 60 min after MCAO, 30 min before reperfusion,[13] respectively.

Experimental protocol
Experiments were performed in two series. In the first series, in which animals received pre-ischemic treatment, after 30 min recuperation from surgical stress, animals were given ip injections of vehicle (saline or DMSO), L-NAME (Fluka, Switzerland), or 7-NI, (Sigma, Germany) and 30 min later 90 min MCA occlusion followed by 24-hr reperfusion. At 10 min before MCAO and at 10 min after the end of MACO, a blood sample was taken for the determination of blood gases. At 24 hrs of reperfusion neurological deficit score test was performed, then the animal was sacrificed using an overdose of anesthesia, the brain was re-

moved and prepared for determination of infarct volumes. In the second series, in which animals received post-ischemic treatment, experiments were performed using a similar protocol to that of first series, except that ip injections of vehicle (saline, DMSO), L-NAME, or 7-NI were given 60 min after the onset of ischemia. The mortality rates of MCAO animals during ischemia/reperfusion in pre-ischemic and in post-ischemic treated series were 55% (40 out of 73) and 60% (43 out of 72) and their data were excluded from the analysis.

Evaluation of neurological deficits:
Evaluation of NDS was performed using five point scoring system as described by Plesnila and colleagues.[14] Accordingly, the scoring are as follows: 0=normal motor function, 1=failure to extend forepaw when suspend vertically by its tail, 2=circling to the contralateral side but have normal posture at rest when put on the table, 3=loss of righting reflex, and 4=no spontaneous motor activity.

Measurement of infarct volumes
Rats' brain were removed, cleaned carefully and immersed in 4ºC cold saline for 5 minutes. They were then sectioned coronally into six 2-mm thick slices using a Brain Matrix. Afterwards, slices were immersed in 2% Triphenyltetrazolium chloride (TTC; Sigma Germany) solution, and kept at 37ºC in a water bath for 15 minutes. The slices were then transferred to 10% buffered formalin for 24 hrs. These slices were then photographed separately using a digital camera (Cannon-Japan) connected to a computer and their cortical and striatal infarct areas were measured using an Image Analyzer Software (NIH Image Analyzer). The infarct volume of each slice was then calculated by multiplying infarct area by its thickness (2 mm). The cortical and striatal infarct volume of each brain was then calculated as the sum of the infarct volume of

Table 1: Values are mean ± SEM. Data obtained at 10 min before and 10 min after MCAO., pH, PaCO₂ (mmHg), PaO₂ (mmHg) and mean arterial pressure (MAP, mmHg) in sham group and groups receiving saline, DMSO, L-NAME or 7-NI.

|		Pre-ischemic treatment							
		10 min. before MCAO				10 min. after reperfusion			
Variable	Sham (n=4)	Saline (n=4)	DMSO (n=4)	L-NAME (n=4)	7-NI (n=6)	Saline (n=4)	DMSO (n=4)	L-NAME (n=4)	7-NI (n=6)
pH	7.35±0.02	7.34±0.03	7.29±0.03	7.27±0.02	7.31±0.01	7.28±0.06	7.33±0.01	7.29±0.04	7.28±0.02
P$_a$CO2	35±6	36±7	35±5	36±4	32±8	36±4	35±7	37±5	34±6
P$_a$O2	78±7	82 ±4	73±3	80±7	72±7	78±4	83±4	75±3	70±6
MAP	94±5	91±7	94±11	115±4	102±10	90±9	102±5	111±2	94±9
		Post-ischemic treatment							
	(n=4)	(n=4)	(n=4)	(n=4)	(n=5)	(n=4)	(n=4)	(n=4)	(n=5)
pH	7.32±0.2	7.31±0.02	7.27±0.02	7.34±0.02	7.32±0.02	7.33±0.01	7.29±0.04	7.29±0.02	7.29±0.03
P$_a$CO2	36±7	35±5	34±8	34±3	38±5	37±4	36±8	34±4	38±6
P$_a$O2	76±6	77±4	79±9	80±3	75±5	76±8	81±4	78±4	72±3
MAP	91±6	93±8	93±4	98±3	107±9	95±11	97±5	119±6	94±9
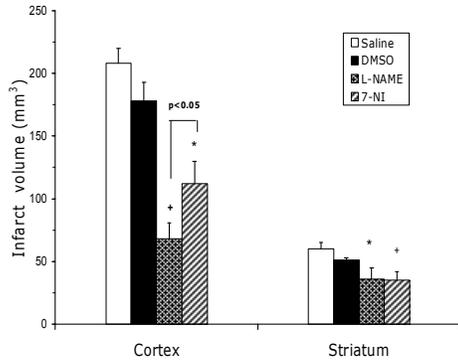
Fig 2: Infarct volumes in the cortex and striatum of rats receiving pre-ischemic administrations of saline (0.1 ml, n=9), DMSO, (0.1 ml, n=6), L-NAME (1mg/kg, n=8), or 7-NI (50 mg/kg, n=10). * denotes significant difference (P<0.05) from saline-treated rats. + denotes significant difference (P<0.05) from DMSO-treated rats.
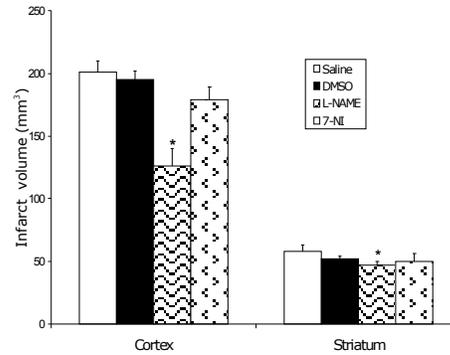

Fig 3: Infarct volumes in the cortex and striatum of rats receiving post-ischemic administrations of saline (0.1 ml, n=6), DMSO (0.1 ml, n=7), L-NAME (1mg/kg, n=8) or 7-NI (50 mg/kg, n=8). * denotes significantly difference (P<0.05) from saline-treated rats.

the six brain slices. Since brain edema might significantly affect the accuracy of the estimation of infarct volumes[15, 16] the calculated infarct volumes were then corrected for brain edema using the formula described by Swanson and colleagues.[16]

Statistical analysis

Data are presented as Mean ± SEM. Infarct volumes and physiological variables are compared using one-way analysis of variance (ANOVA). Where a significant difference was found with ANOVA, the source of difference was located with Tukey tests. Neurological deficit scores were analyzed using Kruskal-Wallis followed by Dunn's test for pair wise comparison or Mann-Whitney-U tests. The probability of type one error (α value) was chosen at $p<0.05$.

Results

Physiological parameters

The values of mean arterial pressure (MAP), arterial carbon dioxide and oxygen pressures ($PaCO_2$, PaO_2) and pH were not significantly different among, pre-ischemic or post-ischemic series at 10 min before induction of ischemia and 10 min after reperfusion. Furthermore, there were no significant differences between these physiological variables of sham-operated, control and experimental groups (Table 1).

Effect of pre-ischemic administration of L-NAME and 7-NI:

Cortical and striatal infarct volumes as well as NDS of sham group were zero. The cortical infarct and striatal infarct volumes of control

saline and DMSO groups were not significantly different from each other (Fig 2). Relative to control saline group, administration of L-NAME significantly reduced cortical and striatal infarct volumes by 66±6% and 45±12% respectively (Fig 2). Pre-ischemic administration of 7-NI did also significantly reduce cortical and striatal infarct volumes by 37±10% and 37±13% respectively as compared to of DMSO group (Fig 2). As shown in Fig 2 the cortical infarct volume of L-NAME-treated group was significantly lower than of 7-NI-treated group. Whereas, striatal infarct volumes of both L-NAME and 7-NI treated groups were statistically the same.

There was no significant difference between the NDS of control saline (2.78±0.15) and DMSO (2.67±0.21) groups. Administration of L-NAME resulted in a significantly lower NDS (1.57±0.29) as compared to its control saline group, whereas the NDS of 7-NI-treated group (2.0±0.21) was not statistically different from that of its control DMSO group.

Effect of post-ischemic administration of L-NAME and 7-NI

Similarly, to the pre-ischemic-treated series, in sham group the cortical and striatal infarct volumes, as well as NDS were zero.

There were no significant differences between cortical and striatal infarct volumes in saline and DMSO-treated groups. The respective values for cortical and striatal infarct volumes of L-NAME-treated group were lower (39±7% and 26±5%) as compared with saline-treated group. However, no statistically significant differences were found between the infarct volumes of cortex and striatum of 7-NI and DMSO-treated control groups (Fig 3).

There was no significant difference between the values of NDS of control groups receiving saline (2.66±0.15) or DMSO (2.43±0.20) and of experimental groups receiving L-NAME (2.62±0.18) or 7-NI (2.50±0.19).

Discussion

Cerebral ischemia/reperfusion injuries in temporary focal cerebral ischemia have been attributed to a number of mechanisms including calcium overload, glutamate excitotoxicity, free radicals, cytokines as well as NO.[4] However, the role of NO of endothelial or neuronal origins in ischemia/reperfusion injuries is not universally agreed upon. Moreover, the role of NO has mainly been investigated in the whole brain ischemia/reperfusion injuries, and rarely in specific areas such as cortex or striatum. As well, the effects of selective and non-selective NOS inhibitors in such injuries have not been investigated. Therefore, the present study was designed to investigate the role of NO in ischemia/reperfusion injuries in transient focal cerebral ischemia on cortex and striatum using L-NAME, a nonselective and 7-NI, selective neuronal inhibitors of NOS.

Reports have indicated that hypotension (low arterial blood pressure), hypoxemia (low PaO2) and hypercapnia (high PaCO2) exacerbated the ischemic injuries,[17] independently of NOS activity. In this study, therefore, 50% of the rats of each group randomly selected to cannulate their femoral artery and measure these parameters. It is noteworthy of mentioning that, these parameters in all groups, before and after induction of ischemia; all were in normal physiological range and were not statistically different from each other, hence they did not influence the ischemic injuries (Table 1). Furthermore, since sham operation did not lead to cerebral infarction or neurological deficits, the ischemic injuries were mainly due to MCA occlusion.

Pre-ischemic administration of L-NAME or 7-NI did reduce the cortical and striatal infarct volumes. These findings are in accordance with those of some investigations,[11,18] but not others who showed that L-NAME or 7-NI did not change the cerebral infarct volumes in rat and mice.[9, 13,18]

NDS is a good indication of cortical motor activity.[14] Low NDS usually is associated with low cortical injuries and vice versa. While pre-ischemic treatment with L-NAME was able to reduce the ischemia/reperfusion-induced NDS, in 7-NI-treated group it was as high as DMSO-treated group and failed to reach a statistical significance. This is in accordance with the fact that in L-NAME treated group the infarct volume of the cortex decreased by 66% whereas in 7-NI-treated group it only decreased by 37%.

Post-ischemic administrations of drugs usually are taken as their effects on reperfusion injuries.[11] The present study are suggesting that post-ischemic administrations of L-NAME did reduce both cortical and striatal infarct volumes. Although, our findings, regarding L-NAME, are supported by the studies of Ding-Zhou and Margaill's colleauges,[12,19] they are inconsistent with other findings.[9,6] Such differences might be due to animal species and the protocols,[19,20] model of ischemia,[21] timing and doses of inhibitors,[19] severity of ischemia,[21] and types of anesthetics.[22] However, in spite of L-NAME, 7-NI did not change the cortical or striatal infarct volumes indicating that the source of NO contributing to reperfusion injuries might be of endothelial rather than neuronal origins. Moreover, neuronal NOS was shown to be short-lived, increases 10 min after induction of focal ischemia and returns to low normal level, at 60 min ischemia.[23,24] Therefore, it is possible that during the first 60 min period of ischemia high NOS activity has accomplished cerebral injuries such that the prevention of minimal activity of neuronal NOS by 7-NI during the remaining 30 min of ischemia and reperfusion could not change the infarct volumes of cortex and striatum. A similar explanation might justify the lack of improvement on NDS of post-ischemic administered 7-NI.

This study indicated that pre-ischemic administration of selective or non-selective inhibitors of NOS were more efficient in reducing ischemia/reperfusion injuries and NDS. Despite reducing the cortical and striatal infarct volumes, post-ischemic administration of L-NAME did not improve NDS. Although, the techniques employed here could not explain such a discrepancy, one might speculate upon the fact that the neurons that controlling the motor functions are mainly located in the ischemic core that has mostly suffered by irreversible damages. This is so, because the major ischemic damages are developed prior to administration of NOS inhibitors. This might justify the rationale, as stated by others too,[25] for the inability of post-ischemic administered NOS inhibitors in preventing the early ischemic injuries.

In summary, the findings of the present study indicated that, pre-ischemic administration of selective neuronal or non-selective inhibitors of NOS were able to reduce ischemia/reperfusion injuries is suggestive of the participation of NO of endothelial and neuronal origins in ischemia injuries in rat model of tran-

sient focal ischemia. It also showed that NO of neuronal origin may not be involved in reperfusion injuries. Moreover, the findings indicated that pre-ischemic treatment with NOS inhibitors was more effective in reducing ischemia/reperfusion injuries than post-ischemic administrations.

Acknowledgments

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