

Neuroprotective Effect of L-Norvaline against Ischemic Brain Damage in Rats

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Abstract

Stroke is the second leading cause of death and permanent disability in adults worldwide. The treatment options for ischemic are very limited and new therapies are still required.

Arginase is an endogenous competitor of Nitric acid Synthase (NOS) for the common substrate L-arginine, and upregulation of arginase during Ischemia/Reperfusion (I/R) compromises NO-mediated effects.

In this work, we focused on the inflammatory response to brain ischemia and the potential role of L-norvaline as arginase inhibitor against ischemic brain injury.

Rats were subjected to Middle Cerebral Artery Occlusion (MCAO) and the effects of L-Norvaline (50 mg/kg/day) on infarct volume, serum Advanced Glycation End products (AGEs), behaviour (gait score and ability to grasp), expression of Arginase (I & II), iNOS, eNOS, TNF- α and IL-1 β level was compared to the standard Cerebrolysin (0.15 mg/kg, ip).

The results of the current study support the involvement of inflammation in ischemic brain injury and point out a possible role of arginase in this process.

Keywords: Arginase Inhibitor; L-Norvaline; MCAO; Rats; Neuroprotective

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Introduction

Stroke is the second leading cause of death in industrialized countries and the most frequent cause of permanent disability in adults worldwide [1]. The WHO has estimated that there were,

worldwide, 16 million first-ever strokes and 5.7 million stroke deaths in 2005. By 2020, they predict that, worldwide, the number of years lost to disability resulting from stroke will reach 61 million.

Most routinely used management of patients in stroke care unit is thrombolytic therapy with recombinant tissue Plasminogen Activator (rtPA) within 3 hours and the administration of oral aspirin within 48 h of ischemic stroke [2].

Arginase is a manganese metalloenzyme that hydrolyses L-arginine to urea and ornithine [3, 4]. Increased expression and/or activity of arginase has been demonstrated in ischemia/ reperfusion (I/R) models [5, 6].

Arginase is an endogenous competitor of NOS for their common substrate L-arginine, and upregulation of arginase during I/R compromises NO-mediated vasodilatory function [7]. So, arginase inhibitors can increase the production of NO and thus prevent the development of endothelial dysfunction [8-11]. In addition, arginase inhibition decreases oxidative stress and vascular inflammation [12] and decreases leukocyte binding to endothelial cells [13].

Oxidative stress is a major inducer of arginase activity [14-16]. In addition, inflammatory mediators including Lipopolysaccharide (LPS), TNF- α , interferon- γ , IL-4, IL-10, and IL-13 [3], oxidized LDL [17], glucose [11], thrombin [18], hypoxia [19], angiotensin II [20], reactive oxygen and nitrogen species (ROS and RNS) including H₂O₂ [16] and peroxynitrite derived from NOS [21] all increase arginase activity in a wide variety of cells [5, 22].

In an animal model of subarachnoid haemorrhage, increased arginase resulted in impaired availability of L-arginine and NO production [7, 23].

Arginase II deletion significantly improves neuronal survival and function through the regulation of mitochondrial membrane permeability mediated apoptosis [24]. Increased arginase II mRNA level and cytosolic distribution after hypoxia was accompanied by a reduction in NOS activity [19]. In addition, excessive arginase activity can increase the formation of polyamines which are involved in excitotoxic neuronal death [25] and in the pathogenesis of ischemic brain damage [26, 27]. Glutamate that has known harmful role in I/R [28] by stimulating NMDA receptors is a product of the arginase/ ornithine pathway [29]. Glutamate causes an influx of calcium and sodium and depolarization of postsynaptic neurons, and increased production of ROS, RNS and mitochondrial dysfunction all of which contribute to cell death [30, 31].

The current study was designed to investigate effect of an arginase inhibitor; L-norvaline in ameliorating CNS complications in ischemic rats. This was done through evaluation of behaviour changes, serum AGEs level, arginase I, II, eNOS, iNOS and TNF- α mRNA expression. Also, IL-1 β was measured by ELISA. The effects of the tested drugs were compared to the effects of the standard neuroprotective drug; Cerebrolysin.

Materials and Methods

Animals

Adult male Wistar rats (200 $\pm$ 20 g) were purchased from Egyptian Organization for Biological products and Vaccines (Cairo, Egypt). The rats were kept under standard environmental and nutritional conditions throughout the investigation. This study was performed in accordance with the guidelines of the Ethical Committee for Animal Handling at Zagazig University (ECAHZU).

Rats were randomly distributed into 4 major groups (n=10) as follows: group (1): Sham operated rats, group (2): Ischemic rats, group (3): Ischemic rats treated daily with oral L-nornalline (50 mg/kg) [10] for 8 days starting one week before surgery and group (4): Ischemic rats treated with Cerebrolysin (0.15 mg/kg, ip) [32] 30 min. before and 24 h after surgery [1].

L-nornalline, Formaldehyde, rat IL-1 β ELISA kit and TTC (2,3,5-triphenyltetrazolium chloride) were purchased from Sigma Aldrich (Munich, Germany) while Thiopental was purchased from SANDOZ (GmbH, Kundl -Austria). RT-PCR kit (Stratagene) was purchased from Sigma Aldrich (Germany).

Induction of Ischemia

Rats were subjected to Middle Cerebral Artery Occlusion (MCAO) in the left hemisphere according to the method described previously [33, 34] using silicone coated nylon filament (size 4-0, diameter 0.19 mm, length 30 mm, tip diameter 0.35 $\pm$ 0.02 mm and coating length 5-6 mm, Doccol Corporation, USA). After 48 h, the animals were anesthetized and perfused with 200 ml Ringer's solution through the heart and the brain was carefully isolated and stored at -80 °C.

Serum AGEs Analysis

Blood was collected from the retro-orbital plexus using heparinized microcapillary tube. Serum was obtained by centrifugation at 3000 g at 4 °C for 20 min (HERMLE Z326K, Germany). AGEs level was determined fluorometrically in serum diluted 50 fold with phosphate-buffered saline (PBS) [35] at 370 nm excitation wavelength and 445 nm emission [36-38] using (LS45 fluorescence spectrophotometer, PerkinElmer, USA).

Behavioural Test

Animal gait was used to assess motor coordination as described previously [39, 40] as follows: (0): normal, (1): open circling, (2): open but persistent circling and (3): closed and persistent circling.

Unilateral grasp strength test was used to assess motor function which is a modification of the previously described test [41]. Scores were given as follows: (0): normal (catch the stick firmly), (1): try to catch the stick and (2): no response (no trial to catch the stick).

Determination of the Infarct Volume by TTC

TTC was used to quantify the dead brain tissue as described [39]. The sections were scanned using Scanner (HPScanjetG2710). The areas of infarction were calculated using Image J® programme (Scion Corporation, Frederick, MD, USA). Infarct volume (mm³) was measured using the following equation:

$$\text{Volume (mm}^3\text{)} = \frac{\sum [\text{area of sections (mm}^2\text{) per side}]}{\text{No.of sides analyzed}} \times \text{maximal extent of infarct}$$

Determination of Arginase (I, II), eNOS, iNOS, and TNF- α expression in brain tissues by qRT-PCR

Total RNA was extracted from ischemic hemisphere homogenate using SV Total RNA Isolation system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The extracted RNA was reverse transcribed into cDNA using RT-PCR kit (Stratagene, USA) according to the manufacturer's protocol.

A real-time PCR reaction mixture was composed of 25 μ l SYBR Green Mix (2x), 0.5 μ l cDNA, 2 μ l of each primer pair mix (5 pmol/ μ l each primer), and dH₂O to 50 μ l.

The primers used are listed in table 1. The PCR program used to amplify cDNA consisted of 120 sec. at 50 °C, 10 min. at 95 °C and 40 cycles of 15 sec. at 94 °C, 30 sec. at 60 °C and 30 sec. at 72 °C, followed by 10 min. at 72 °C. The RT-PCR result was analyzed with the step PE Applied Biosystems (Perkin Elmer) software and the data were expressed in Cycle threshold (Ct). Target gene expression was assessed and related to reference gene (GAPDH) [42-44].

Table 1: Sequence of the primers used for RT-PCR

Genes	Forward	Reverse
Arginase I	AAAGCCCATAGAGATTATCGGAGCG	AGACAAGGTCAACGGCA
Arginase II	TTAGTAGAGCTGTGTCAGGTGGC	ACTTGAAGCAATCACATCCACTGC
eNOS	CTG CGG TGA TGT CAC TAT GG	AAA TGT CCT CGT GGT AGC GT
iNOS	GGG CCA CCT TTA TGT TTG TG	CCT CAA CCT GCT CCT CAC TC
TNF-α	GAAAAGCAAGCAGCCAACCA	CGGATCATGCTTTCTGTGCTC
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCATGTTGCTGTA

Determination of IL-1 β by ELISA

The level of IL-1 β was measured in ischemic hemisphere homogenates using ELISA kit according to the manufacturer's protocol (R & D systems, USA).

Statistical Analysis

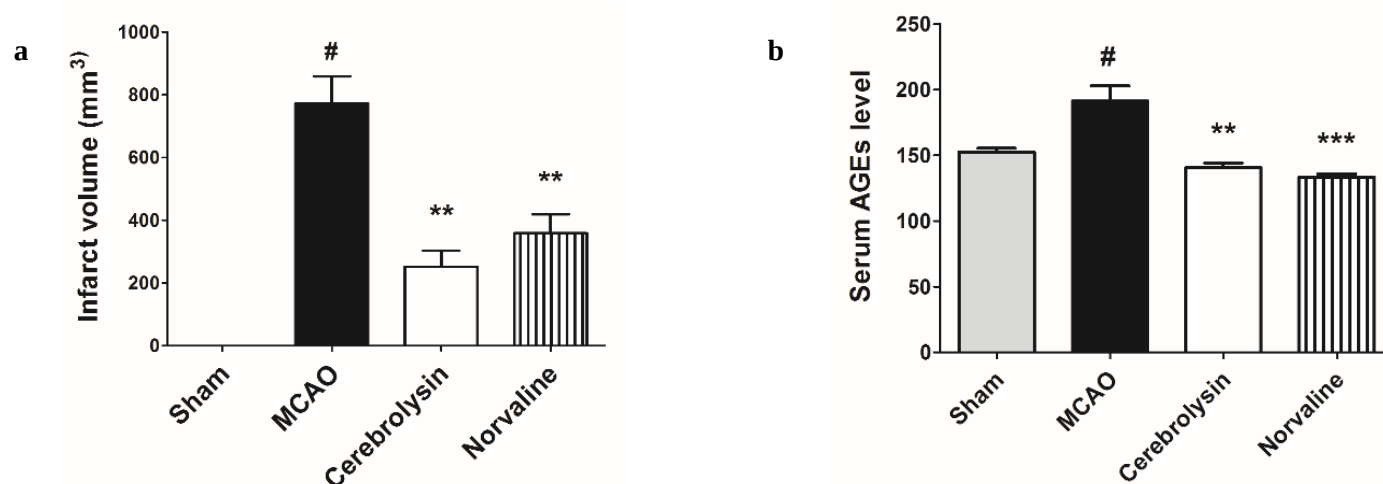
Data are expressed as mean $\pm$ standard error of mean. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's post Hoc test using Graph pad Prism software version 5. For all analysis, the level of statistical significance was set at P < 0.05.

Results

The current study has shown that MCAO resulted in obvious infarction while the sham-operated rats had no infarct (772 vs 0 mm³). Treatment with L-norvaline (50 mg/kg) and cerebrolysin (0.15 mg/kg, ip) significantly reduced the infarct volume compared to MCAO group (358 and 252 vs 772 mm³ respectively) (Figure 1a)

Although MCAO did not cause a significant change in serum AGEs level, however, treatment of ischemic rats with L-norvaline (50 mg/kg) and cerebrolysin (0.15 mg/kg, ip) significantly reduced AGEs serum levels by 33 and 31 % respectively compared to MCAO group (Figure1b).

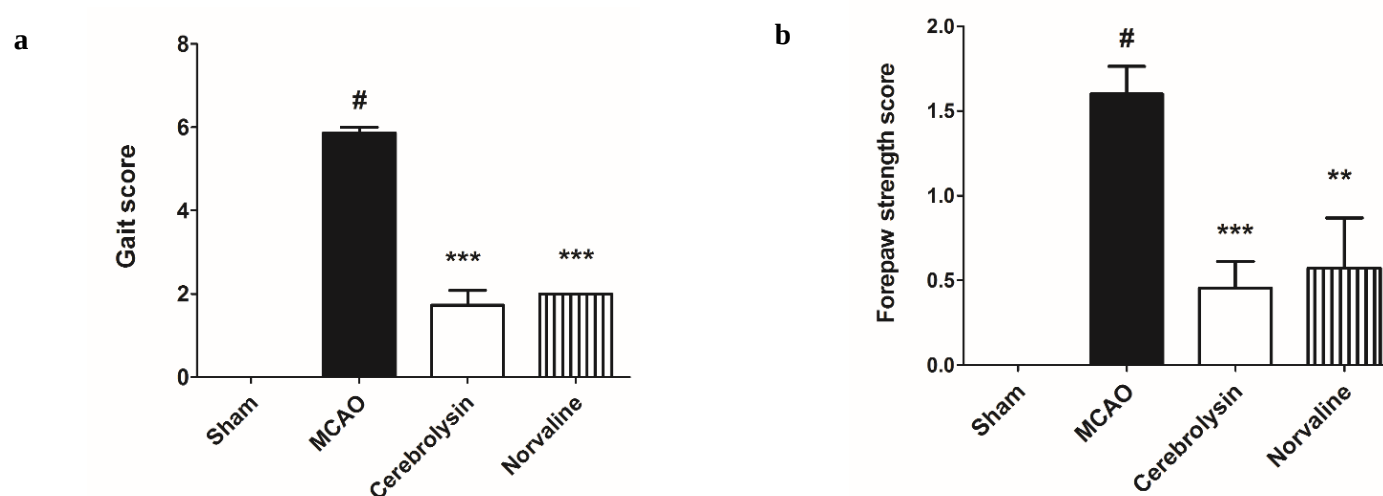
Figure 1: Effect of MCAO and treatment on: a) infarct volume & b) serum AGEs level. Data are expressed as mean $\pm$ SEM. # significantly different from sham group, **, *** significantly different from MCAO group at $p < 0.01$ or $P < 0.001$ respectively using one way ANOVA followed by Tukey's post hoc test.



On the behaviour level, gait scores were significantly increased in MACO rats compared to the sham group (5.8 vs 0). While, gait scores were significantly reduced by 65 and 70 % after treatment with L-norvaline and cerebrolysin respectively compared to MCAO group (Figure 2a).

Also, sham-operated rats used both forepaws to grip items, but MCAO rats were unable to use their right forepaws scoring 1.6 for MCAO compared to 0.25 for sham group. Treatment with L-norvaline and cerebrolysin showed significantly reduced forepaw strength by 64 and 71 % respectively compared to MCAO group (Figure 2b).

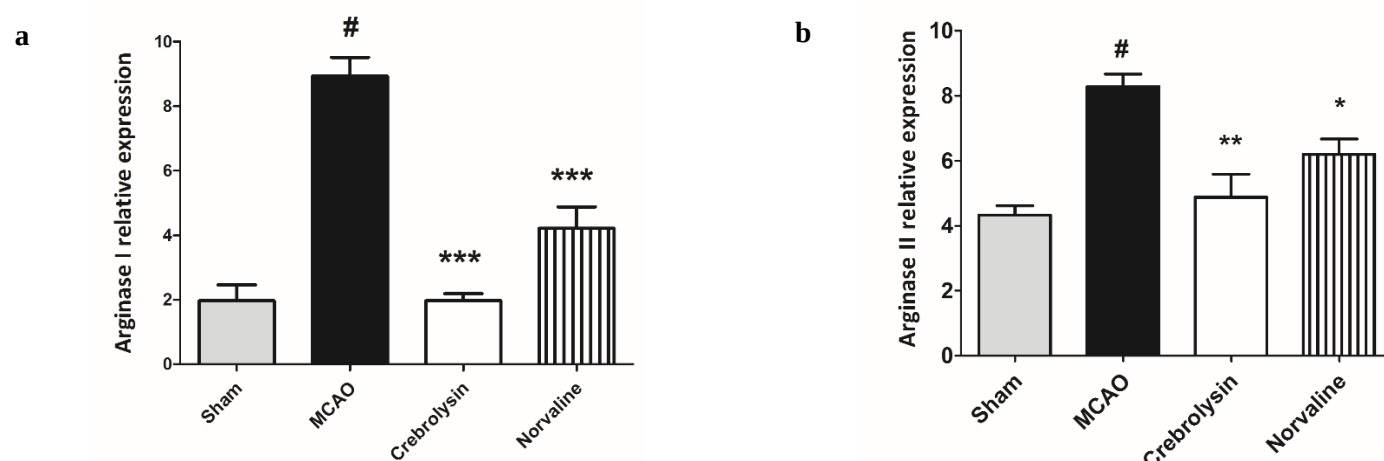
Figure 2: Effect of MCAO and treatment on: a) gait scores & b) forepaw strength score. Data are expressed as mean $\pm$ SEM. # significantly different from sham group, **, *** significantly different from MCAO group at $p < 0.01$ or $P < 0.001$ respectively using one way ANOVA followed by Tukey's post hoc test.



After MCAO, the expression of Arginase I was significantly increased compared with sham group (9.50 vs 1.96). Treatment with L-norvaline and cerebrolysin, induced a significant decrease in Arginase I expression compared with MCAO group by 52 and 78 % respectively (Figure 3a).

Also, Arginase II expression was increased following MCAO compared to sham group (8.28 vs 4.32) and treatment with L-norvaline and cerebrolysin showed a significant decrease in Arginase II expression compared with MCAO group by 25 and 41% respectively (Figure 3b).

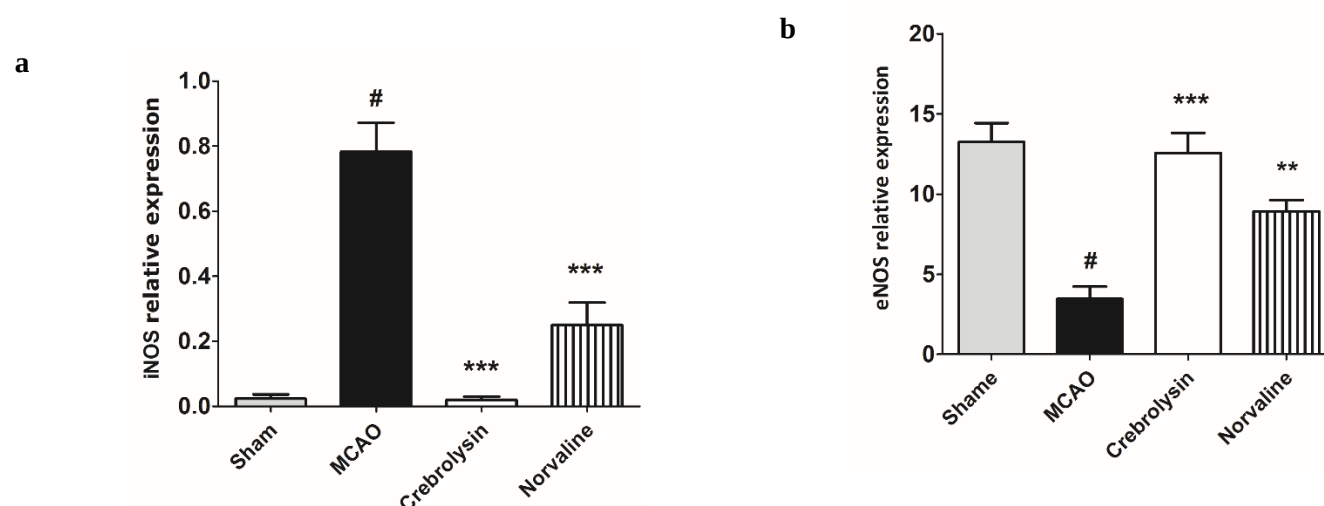
Figure 3: Effects of MCAO and treatment on: a) Arginase I & b) Arginase II relative expression. Data are expressed as mean $\pm$ SEM. # significantly different from sham group, *, **, *** significantly different from MCAO group at $P < 0.05$, $P < 0.01$ or $P < 0.001$ respectively using one way ANOVA followed by Tukey's post hoc test.



Our results have shown that, MCAO caused a significant increase in iNOS expression compared to sham-operated group (0.78 vs. 0.02). iNOS expression was decreased in brain tissue of rats treated with L-norvaline and cerebrolysin by 68 and 97% respectively compared with MCAO group (Figure 4a).

The expression eNOS in MCAO group was significantly decreased compared to sham-operated group (3.97 vs 13.90). While, treatment with L-norvaline and cerebrolysin induced a significant rise in eNOS expression by 124 and 209 % respectively compared with MCAO group (Figure 4b).

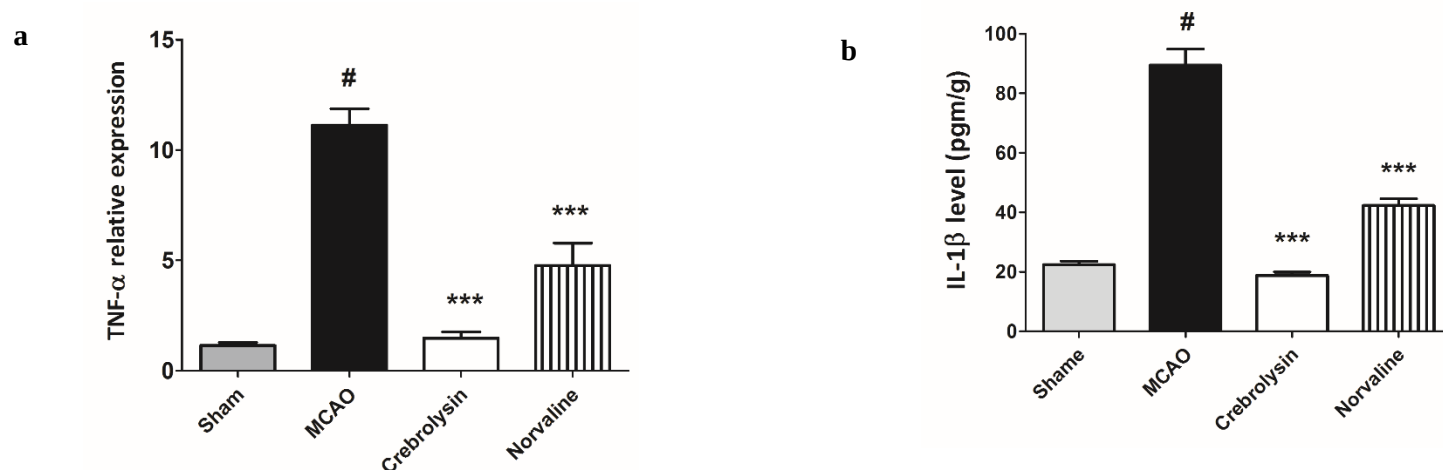
Figure 4: Effects of MCAO and treatment on: a) iNOS & b) eNOS expression. Data are expressed as mean $\pm$ SEM. # significantly different from sham group, **, *** significantly different from MCAO group at $p < 0.01$ or $P < 0.001$ respectively using one way ANOVA followed by Tukey's post hoc test.



Moreover, MCAO group showed significantly increased TNF- α expression compared to sham-operated group (11.13 vs 1.14). On the other hand, treatment with L-norvaline and cerebrolysin caused a significant reduction in TNF- α expression by 63 and 84 % respectively compared with MCAO group (Figure 5a).

Similarly, brain IL-1 β level in MCAO group was significantly increased compared to sham-operated group (11.13 vs 1.14 pg/g). The level of IL-1 β was significantly reduced by treatment with L-norvaline and cerebrolysin by 52 and 78 % respectively compared with MCAO group (Figure 5b).

Figure 5: Effects of MCAO and treatment on: a) TNF- α expression and b) IL-1 β level. Data are expressed as mean $\pm$ SEM. # significantly different from sham group, *** significantly different from MCAO group at $P < 0.001$ using one way ANOVA followed by Tukey's post hoc test.



Discussion

Stroke is a complex and devastating neurological condition [45, 46]. Currently, thrombolysis with tissue Plasminogen Activator (t-PA) is the only effective therapy, but due to its narrow therapeutic window and safety concern; fewer than 5% of stroke patients receive this treatment [47]. It is crucial to expand the narrow therapeutic opportunities for this devastating condition.

Cerebral ischemia evokes a strong inflammatory response characterized by release of cytokines, chemokines, adhesion molecules and proteolytic enzymes that exacerbate tissue damage [48, 49]. Therefore, in this work, we focused on the inflammatory response to brain ischemia and the potential role of L-norvaline as arginase inhibitor against ischemic brain injury induced in rats.

In the present work, no infarction was observed in the sham-operated group, whereas extensive lesions were developed in the MCAO group. Similar changes induced by MCAO have been previously reported [39, 40]. In addition, serum AGEs were increased in MCAO rats as previously reported [50].

No neurological deficits were observed in the sham-operated group, while the ischemic group exhibited significant increase in scores of gait and forepaw grasp ability. These behavioural tests indicate a severe deficit in the neurological function of the ischemic rats which could be attributed to the brain damage evidenced by the observed infarction.

The results of the current study showed an increase in Arginase I and II expression in MCAO compared to sham-operated rats as previously reported [51, 52].

Our results have shown an elevation in iNOS expression as previously reported [42]. While, eNOS expression was decreased as shown previously mentioned [6, 52].

The current study has shown a state of brain inflammation as evidenced by the elevation in TNF- α and IL- β . It is well known that stroke activates gene expression of several pro-inflammatory mediators, including TNF- α and IL-1 β [42] that stimulate the expression of cellular adhesion molecules on endothelial cells, and promote leukocyte adherence and migration into brain parenchyma [53-57] and may participate in disruption of the Blood Brain Barrier (BBB) [58].

These results support the involvement of inflammation in ischemic brain injury and point out the possible role of arginase in this process.

The present study examined the effect of L-norvaline on ischemic stroke. Animals that were treated with L-norvaline showed significant reduction in infarct volume. Although experimental data from studies using arginase inhibitors in stroke are lacking, a previous study [59] demonstrated that systemic arginase inhibition reduced myocardial infarct size by 51% in rats. In addition, L-norvaline caused a significant reduction in serum AGEs which may be due its ability to reduce ROS production as previously described [10].

In addition, arginase inhibition by L-norvaline caused a significant improvement in scores of gait and forepaw use ability that might be attributed to the reduction in brain damage and infarct volume.

The reduction in infarct volume after treatment with L-norvaline may be attributed to its ability to decrease AGEs production [10] and increase the availability of L-arginine to be consumed by eNOS to produce NO [6, 60]. NO confers neuroprotective effects during cerebral ischemia by upregulating brain-derived neurotrophic factor (BDNF) expression that exerts protective effects in ischemia [61]. NO also augments cerebral blood flow following ischemia [62, 63], inhibits platelet aggregation and leukocyte activation [64]. On the other hand, NO itself can dampen inflammatory reactions by causing nitrosylation of the NF- κ B subunits p65 and p50 [65-67].

In addition, previous reports have shown that arginase inhibitors suppressed arginase I and II expression [68] and increased eNOS expression [68] in aortic endothelial cells of mice.

This reduction in arginase expression may be due to increased eNOS expression and increased circulating NO levels [68, 69] and/or decreased TNF- α [52] through NO that can dampen inflammatory reactions as mentioned previously [65-67]. Moreover, the reduction in ROS and AGEs [10] mediated by arginase inhibitors reduces inflammation and inflammatory mediators [70, 71].

A recent study [72] has shown that inhibition of arginase significantly attenuated TNF- α expression and increased eNOS expression in diabetic nephropathy in mice. Inhibition of

production of TNF- α leads to decreased ROS production, that plays a crucial role in the pathogenesis of diabetes and neurodegenerative disorders [70, 73].

In addition, elevated TNF- α level increases iNOS expression [74], worsens cerebral injury and edema after transient focal cerebral ischemia in rats [75]. Therefore, inhibition of TNF- α expression reduces iNOS expression. Also, inhibition of TNF- α may reduce the migration of neutrophils and macrophages into the brain as they do in other organ systems [57, 76].

Treatment of rats with arginase inhibitor; L-norvaline reduced the level of IL-1 β in brain tissue due to its ability to conserve L-arginine [77] that has many benefits like reducing lipid peroxidation, plasma levels of soluble adhesion molecules, myocardial stunning and arrhythmias [78]. Similar to TNF- α , IL-1 β has also been shown to promote neutrophil tissue infiltration and the induction of endothelial cell adhesion molecule expression [57, 79].

Conclusion

The arginase inhibitor, L-norvaline showed improvement of ischemic rats. Inhibition of arginase reduced the expression of arginase enzyme itself, iNOS, TNF- α and IL-1 β as well as increased the expression of eNOS. Motor activity, walking in normal pattern and use of forepaw was significantly improved by the treatment with L-norvaline. Also, AGEs and infarct volume were significantly reduced in treated groups compared to the ischemic group. This may indicate the possible beneficial effect of treatment of ischemic patients using arginase inhibitors as L-norvaline.

Conflicts of Interest

The authors declare no conflicts of interest.

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