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Combination of Citicoline and L-NAME Restores Neurological Functions, Reverts Biochemical Alterations and Reduces Neuronal Damage in Transient Focal Cerebral Ischemic Rats

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Abstract

Objectives: To investigate the neuroprotective effect of combination of Citicoline (membrane stabilizer) and L-NAME (NOS inhibitor) in middle cerebral artery occluded/reperfused rats (MCAO/R). Methods: Citicoline (250 mg/kg b.w.) followed by L-NAME (3 mg/kg b.w.) was administered 30 minutes before (Pre) performing middle cerebral artery occlusion/reperfusion (MCAO/R) in Sprague Dawley rats. Parameters such as Glutamate, Aspartate, Nitrite/Nitrate, Sodium potassium ATPase (Na^+K^+ATPase) content, expression of TNF- α and IL-10, and histopathology was studied. Neurological scoring was also performed in the MCAO/R rats. Findings: Pre ischemic administration of citicoline and L-NAME has improved neurological deficit ($p<0.001$). Combination treatment has also ameliorated the glutamate level in MCAO/R rats ($p<0.05$). Combination of these drugs has also reduced the nitrate/nitrite content ($p<0.001$) and also sodium potassium ATPase (Na^+K^+ATPase) ($p<0.001$) significantly. Citicoline and L-NAME combination treatment has also down regulated the expression of TNF-α and up regulated the expression of IL-10 in middle cerebral artery occlusion/reperfusion rats. Citicoline and L-NAME combination was also observed to reduce the neuronal damage which is evident from the histopathology (H and E) of brain sections of MCAO/R rats. Novelty of the Study: Though Citicoline and L-NAME has been evaluated and proven for their neuroprotection separately, the effect of these two drugs when administered together as a combination has not been studied. This study is first of its kind to determine the neuroprotective effect of combination of citicoline and L-NAME. Conclusion/Application: Combination of citicoline and L-NAME has profoundly improved neuroprotection and ameliorated excitotoxicity when administered 30 minutes before ischemia.

Keywords: Cerebral Ischemia, Citicoline, L-NAME, Middle Cerebral Artery Occlusion/Reperfusion, Transient Focal Cerebral Ischemia

1. Introduction

Stroke is the third major devastating disorder worldwide causing substantial neuronal loss ultimately affecting the quality of life[1]. Till date there are no highly effective and safe treatments available for acute ischemic stroke other than thrombolytics and endovascular surgical procedures. However, the scope of such treatment therapies is limited especially in developing countries[2]. Therefore, there is a serious demand for safe and efficacious neuroprotective drugs to ameliorate neuronal damage and to extend the therapeutic time window[3]. Stroke is a debilitating disorder involving multiple complex pathophysiological cascades such as glutamate accumulation, failure of ionic pumps, abnormal calcium influx, free radical formation,

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lipid peroxidation, oxidative stress, accumulation of nitric oxide, nitrosative stress, mitochondrial membrane damage, damage of blood brain barrier and necrotic/apoptotic neuronal death. Although wide variety of candidates have shown promising therapeutic outcome and has been identified as potential molecules, their actions were only submissive due to the multicomplex pathophysiology of ischemic stroke[4]. It is apparent from this, that a multi target approach should be pursued for better neuroprotection in ischemic stroke. For this reason, the application of manifold drugs, wherein two or more physiological event is targeted was identified to be the rational therapeutic internvention in ischemic stroke[5]. Subsequently various authors evaluated the efficacy of combined therapeutic approaches[6,7]. Hence in the current investigation combination of citicoline (membrane stabilizer) and L-NAME (non selective NOS inhibitor) has been study for neuroprotection in transient focal cerebral ischemic rats. Another crucial factor to be considered in developing the effective therapeutic intervention for ischemic stroke, is the appropriate therapeutic time window. Our previous results show that citicoline and L-NAME confer better neuroprotection when administered before ischemia. Hence, the combination of these drugs was administered pre ischemically in the present study. Neuroprotective effect was determined in terms of neurological functioning, glutamate, aspartate, Nitrite/Nitrate, Sodium potassium ATPase (Na^+K^+ATPase) content, expression of TNF- α and IL-10, and histopathology.

2. Materials and Methods

2.1 Chemicals

L-NAME was procured from Sigma, India. Citilin (i.p.) and 4-0 Nylon monofilament TM Ethicon was purchased from the Pharmacy of Sri Ramachandra University, India. Primary antibody for TNF-α (#sc-52746) and IL-10 (#sc-365858), and HRPO-conjugated goat anti-mouse secondary antibody (#sc-365858) were purchased from Santa Cruz Biotechnology (USA). All other chemicals were purchased from Himedia laboratories, India and were of analytical grade.

2.2 Animals and Ethics Approval

Number of animals required for the study was approved by the Institutional Animal Ethics Committee of Sri Ramachandra University (IAEC/XXXIII/SRU/250/2013). Male Sprague Dawley rats of 290-340 g bodyweight were used in the study. Rats were housed individually in cages. The air exchange ratio and air cycle per hour in the animal room was maintained at 55:45 and 12-15 cycles. Rats were maintained under 22 ± 3°C and 30-70% temperature and relative humidity respectively. An artificial photoperiod of 12 h light and 12 h dark was provided and the rats were supplied with feed and water ad libitum.

2.3 Middle Cerebral Artery Occlusion and Reperfusion (MCAO/R)

Middle cerebral artery occlusion and reperfusion method was used to develop transient focal cerebral ischemia in the rats following modified method of Longa et al8. Rats were injected with 350 mg/kg chloral hydrate intraperitoneally to anesthetize. An cut was made on the upper thoracic region of the rat to expose the bifurcation of right common carotid artery. 4-0 nylon filament was coated with 0.01% poly-l-lysine and inserted into the external carotid artery after making a nick in the external carotid artery. The filament was advanced into the internal carotid artery until a slight resistance is felt. Filament was detained with the external carotid artery by tying it with non absorbable surgical suture. After 2 hours of ischemia, the suture is removed and the filament is slowly pulled out to allow reperfusion. Then the animal is sutured and maintained in a cage under a lamp to maintain the body temperature.

2.4 Animal Grouping and Experimental Design

Animals were segregated into three groups i.e., sham, Ischemic/Reperfusion (IR) and pre ischemic/reperfusion. Each group consisted of 12 Sprague Dawley rats. Sham operated group did not undergo the middle cerebral artery occlusion/reperfusion, only external incision was made and the animals were sutured. IR group underwent MCAO/R surgery. Both sham and IR group received 0.9 ml of saline. Citicoline and L-NAME was administered to the other group at a dose of 250 mg/kg .w. and 3 mg/kg b.w. respectively, 30 minutes before ischemia. An hour after the onset of ischemia, the filament was removed and reperfusion allowed. Ischemia and reperfusion time was fixed based on the earlier research[9]. 72 hours after reperfusion behavioural assessment such as anxiety, cognition and assessment of motor activity was performed in all the groups and then

sacrificed. Brains of the sacrificed animals were immediately removed and fixed in formalin (10%) for histopathology.

2.5 Assessment of Neurological Functioning

2.5.1 Neurological Scoring

Neurological deficit was scored following the modified method of Bederson scoring[10]. The score is allotted as followed;

Rats showing no visible signs of neurological deficit - Score 0.

Contralateral forelimb flexion - Score 1.

Decreased reaction to lateral push - Score 2.
Movement in all directions - Score 3.
Circling - Score 4.

2.6 Biochemical Parameters

2.6.1 Glutamate

Glutamate content was estimated following Babu method[11]. Brain tissue was homogenized in 0.1 N HCL and 80% ethanol. 200 µl was used for about 10 mg of tissue. The homogenate was centrifuged at 4500 rpm for 20 minutes at 25°C. The supernatant was collected and glutamate were estimated in HPTLC (CAMAG - version 1.3.4, CSA). Silica gel GF254 was used as the stationary phase. Mobile phase is prepared with 65:15:25 (v/v) ratio of n-butanol, glacial acetic acid and water. (0.2% ninhydrin - detection agent, Wavelength - 486 nm). L-Glutamic acid was used as standard at a concentration of 20-200 ng to plot calibration curve.

2.6.2 Nitrite/Nitrate

Nitrite/Nitrate was estimated following Green et al. method[12]. To the homogenate (0.2 ml) 0.4 ml of sulphosalicylic acid (35%) and 1.8 ml of saline was added to precipitate the protein. The mixture was centrifuged at 4000 rpm for 10 minutes to remove the precipitate. To 1 ml the supernatant, 2 ml Griess reagent was added and mixed well. The mixture was then allowed to stand for 20 minutes in dark. The color intensity was read at 412 nm using an UV/Visible spectrophotometer (Perkin Elmer, Lambda 25, USA). The content of nitrite was calculated using standard graph of sodium nitrite.

The results were expressed in micromoles of nitrite/nitrate per gram tissue.

2.6.3 Na^+K^+ATPase

Na^+K^+ATPase was estimated following the method of Sovoboda and Mossinger[13]. 50 µl of 600 mM Nacl, 50 µl of 50 mM Kcl, 50 µl of 80 mM ATP and 50 µl of 1 mM sodium EDTA was added to 250 µl of 184mM Tris HCl and pre incubated for 10 minutes at 37oC and then the mixture was added to 25 µl of 10% homogenate and again incubated for an hour at 37°C. The reaction was arrested by adding 10% TCA and the precipitate was centrifuged at 3500 rpm for 10 minutes and released inorganic phosphorous was determined using Spectrophotometer (Perkin Elmer) at 640 nm.

2.6.4 Western Blot Analysis (TNF-α and IL-10)

Expression of TNF-α and IL-10 was determined by western blotting following the method of Lee et al[14] with β-actin as marker[14]. Samples were homogenised in 0.1 M ice cold tris HCL and centrifuged at 3500 rpm for 10 minutes. Protein content of the supernatant was determined using Bradford method. Aliquots containing 50µg of protein was loaded along with β-mercaptoethanol and bromophenol blue in 12% SDS PAGE. Seggregated proteins were transferred to PVDF membranes and blocked overnight with 3% BSA in tris buffered saline. Membrane was then washed in tris buffered saline and probed with primary antibodies and then washed again with tris buffered saline thrice for five minutes and then probed with secondary antibodies. Finally, membrane was incubated with BCIP/NBT substrate for 10 minutes. Bands were visualized under a scanner and quantified using Bio ID software (Vilber Lourmat, Marne-la-Vallee, France).

2.6.5 Histopathological Examination

Brain sections of about 4-5 µm thickness was prepared and stained with Haematoxylin and Eosin (H and E) after processing. Changes in the cortex, hippocampus (CA1, CA2 and CA3), striatum and hypothalamus was examined and lesion scored as follows;

0-10%: No morphological changes and few dark stained cells - Score 1.

11-30%: Mild oedema or dark neurons or pyknotic cells - Score 2.

31-50%: Moderate number of dark neurons - Score 3.

51-70%: Moderate edema, necrosis and severe morphological changes - Score 4.

71-100%: Severe oedema, necrosis and infarction - Score 5.

The sum of histological scores of cortex, hippocampus (CA1, CA2 and CA3), striatum and hypothalamus was calculated and expressed as percentage damage.

3. Statistical Analysis

Behavioural and percentage neuronal damage was expressed as mean ± SEM. One way ANOVA followed by Tukey's multiple comparison test as post hoc was performed to find the statistical differences between the groups. For neurological deficits, the values are represented as median and expressed in interquartile range. Comparisons of the neurological scores were analysed with Mann-Whitney U test. GraphPad Prism 5 software (version 5.03) was used for all statistical calculations. Statistical significance was set at $p<0.05$.

4. Results

One animal was dead at 24 hours after reperfusion in the middle cerebral artery occlusion/reperfusion (IR) group.

4.1 Effect of Combination of Citicoline and L-NAME on Neurological Scoring

Rats in the IR group exhibited significant neurological deficit than sham operated (median = 4.0, $p<0.001$). IR rats exhibited signs such as decreased reaction to lateral push, irregular movements in all directions and all circling. Pre-ischemic administration of combination of Citicoline and L-NAME has improved the neurological functioning, only one animal out of 12 exhibited contralateral fore limb flexion. Therefore, combination treatment has significantly ameliorated neurological deficits (median = 0.0 $p<0.001$) (Figure 1).

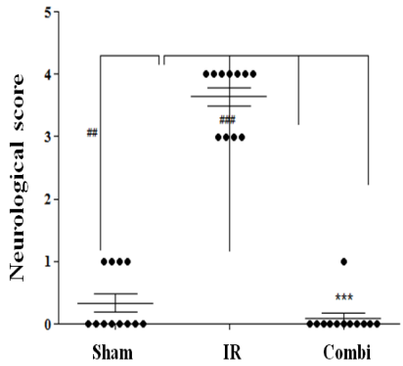

Figure 1. Effect of combination of citicoline and L-NAME on neurological functioning.

Effect of combination of citicoline and L-NAME on neurological deficit. Data expressed as Mean ± SEM, # indicates comparison between sham and IR (# - $p<0.5$, ## - $p<0.01$ and ## - $p<0.01$), * indicates comparison between IR and treatment groups (* - $p<0.5$, ** - $p<0.01$ and ** - $p<0.001$).

4.2 Effect of Combination of Citicoline and L-NAME on Biochemical Markers

4.2.1 Glutamate

A significant increase in ipsilateral glutamate content was observed in the IR rats than sham operated rats [$F(2, 32) = 7.173$, $p<0.01$]. Glutamate content was found to be significantly reduced in the combination treatment ($p<0.01$). Combination treatment of Citicoline and L-NAME has significantly reduced the glutamate content (Figure 2). Effect of combination of citicoline and L-NAME on glutamate. Data expressed as Mean ± SEM, # indicates comparison between sham and IR (# - $p<0.5$, ## - $p<0.01$, ### - $p<0.01$), * indicates comparison between IR and treatment groups (* - $p<0.5$, ** - $p<0.01$ and *** - $p<0.001$).

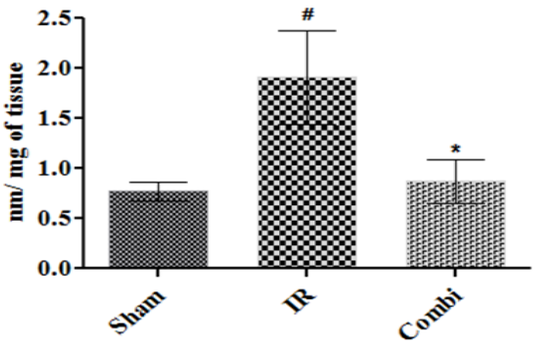

Figure 2. Effect of combination of citicoline and L-NAME on glutamate.

4.2.2 Nitrite/Nitrite

Nitrate/nitrite was significantly increased in the MCAO/R group than sham operated [F (2, 32) = 39.30, p<0.001]. A significant reduction in the nitrate/nitrite content was observed in the combination group (p<0.001). Therefore combination treatment has greatly reduced the nitrate/nitrite content in the ischemic reperfusion rats (Figure 3).

Effect of combination of citicoline and L-NAME on Nitrite/Nitrate and Na^+K^+ATPase. Data expressed as Mean ± SEM, # indicates comparison between sham and IR (# - p<0.5, ## - p<0.01 and ### - p<0.001), * indicates comparison between IR and treatment groups (* - p<0.5, ** - p<0.01 and *** - p<0.001). Percentage increase and decrease in the enzyme level were indicated within the bar.

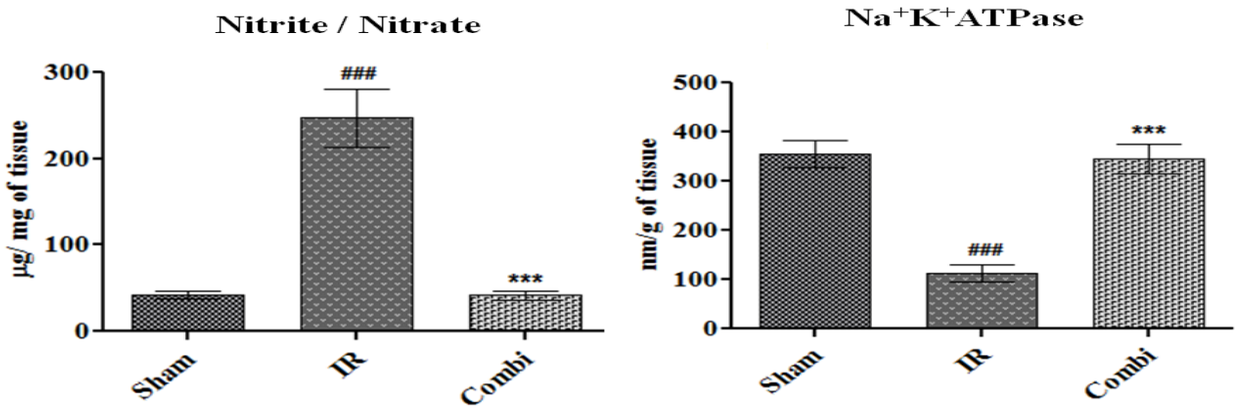

Figure 3. Effect of combination of citicoline and L-NAME on nitrite/nitrate and Na^+K^+ ATPase.

4.2.3 Na^+K^+ATPase

Na^+K^+ATPase has significantly decreased [F (2, 32) = 26.70, p<0.001] in the ipsilateral striatum of the IR brain when compared to the sham operated. Citicoline when administered along with L-NAME has significantly increased Na^+K^+ATPase (p<0.001). Thus, combination treatment has effectively improved Na^+K^+ATPase activity (Figure 3).

4.3 Effect of Combination of Citicoline and L-NAME on Expression of TNF-α and IL-10

TNF-α was upregulated in the MCAO/R group than the sham operated [F (2, 32) = 5.199, p<0.05]. Pre ischemic administration of citicoline with L-NAME down regulated the expression of TNF-α (p<0.05) (Figure 4). With regard to IL-10, it is down regulated in the MCAO/R group than the sham operated [F (2, 32) = 4.257, p<0.05]. Expression of IL-10 was also observed to be significantly up regulated in the combination rats (p<0.05) than the ischemic group (Figure 4).

4.4 Effect of Combination of Citicoline and L-NAME on Neuronal Damage

Occlusion of middle cerebral artery in rats induced severe neuronal damage in the ischemic reperfusion rats (IR) than sham operated (1.09%). IR group exhibited about 73.5 % neuronal damage characterized by necrotic focus in the hippocampus and cortex, and infarction in the hypothalamus with pyknotic cells in the penumbra. The striatum of the IR group also appeared severely vacuolated. Apparent reduction in neuronal damage was revealed in the combination group. The striatum of the combination group revealed only few dark stained neurons and moderate vacuolation. Combination treatment also exhibited cortical necrosis. Preoptic area and the hypothalamus appeared normal. Neuronal damage of was reduced to about 20 % in the group treated with Citicoline and L-NAME combination (Figure 5 and Figure 6).

Histological changes in the pre optic area of sham, ischemic and combiantion treatment. Inset: Subgross view of ipsilateral brain hemisphere (1.25 x) (Bar = 50 µm).

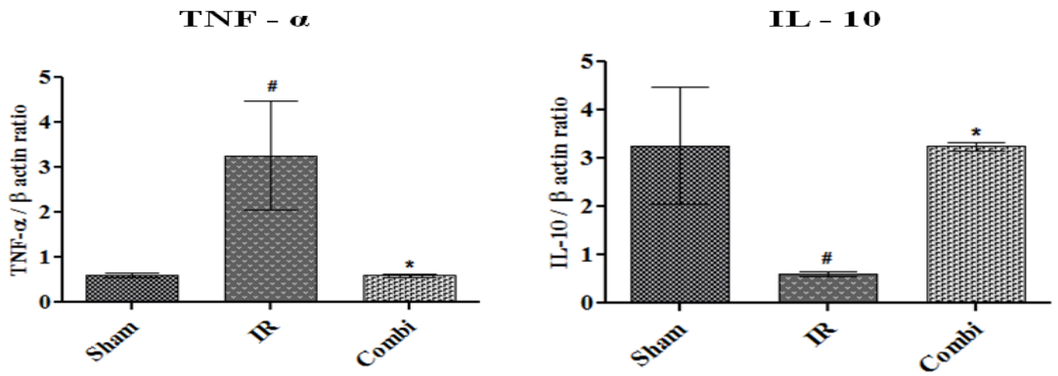

Figure 4. Effect of combination of citicoline and L-NAME on protein expression.

Effect of combination of citicoline and L-NAME on TNF-α and IL-10. Data expressed as Mean ± SEM, # indicates comparison between sham and IR (# - p<0.5, ## - p<0.01 and ### - p<0.001), * indicates comparison between IR and treatment groups (*- p<0.5, ** - p<0.01 and *** - p<0.001). Percentage increase and decrease in the enzyme level were indicated within the bar.

5. Discussion

Numerous literatures reveal Citicoline, as a potential neuroprotective candidate in cerebral ischemia due to its membrane stabilizing effects[15,16].Literatures also reveal L-NAME a nonselective NOS inhibitor as a prospective clinically potent neuroprotectant[17–19].

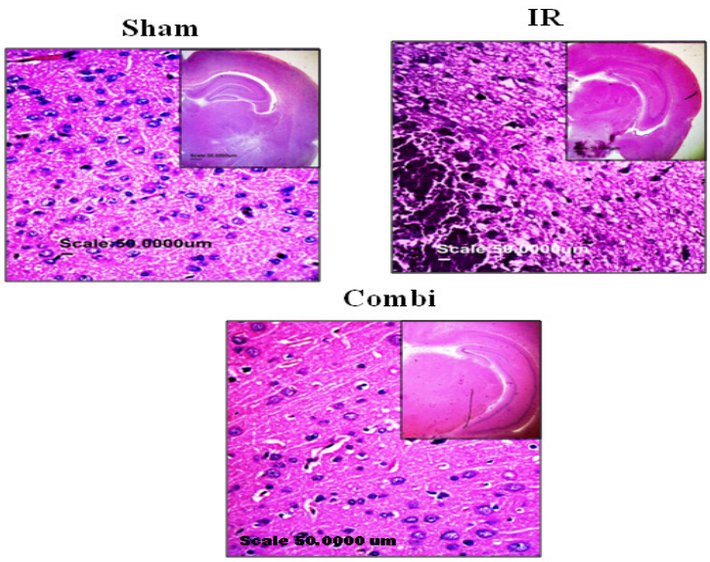

Figure 5. Effect of combination of citicoline and L-NAME on histopathology (pre optic area).

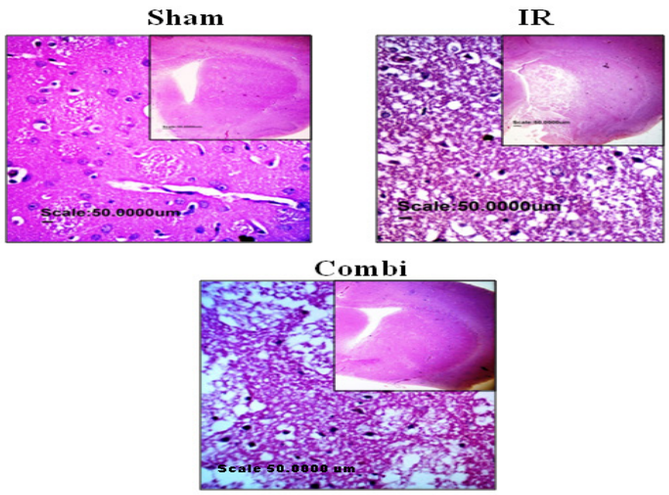

Figure 6. Effect of combination of citicoline and L-NAME on histopathology (striatum).

Histological changes in the striatum of sham, ischemic and combination treatment. Inset: Subgross view of ipsilateral brain hemisphere (1.25 x) (Bar = 50 μm).

Therefore in the current study, the combination effect of Citicoline with L-NAME on middle cerebral artery occluded/reperfused rats have been investigated. Neuroprotection conferred by Citicoline and L-NAME has been substantiated based on the findings from neurological scoring, perpetuation of biochemical markers, TNF-α and IL-10 expression, and histopathology of brain. Rats treated with combination of Citicoline and L-NAME has shown improved neurological function than the ischemic reperfusion rats. It has been reported in various studies that Citicoline and L-NAME improves neurological functioning[20–23]. Citicoline is reported to protect the neuronal membrane and preserve neuronal plasticity, which may be the reason for recuperation of neurological functioning[24]. L-NAME restrains the production of overt nitric oxide and thereby inhibits the generation of nitrosative stress and maintains mitochondrial function, thereby preserves neuronal plasticity and improves neurological functioning[25]. Administration Citicoline and L-NAME combination has reduced the glutamate levels in the IR rats. It is reported by several investigators that citicoline prevents fatty acid release, stimulates phosphotidyl choline synthesis, preserves cardiolipin and sphingomyelin, increases glutathione synthesis and glutathione reductase activity, and restores Na^+/K^+-ATPase[26]. It was revealed that Citicoline enhances the uptake of glutamate in cultured rat astrocytes and it is also reported that citicoline plays dual role in glutamate excitotoxicity, wherein it increases both neuronal ATP and astrocyte clearance of extracellular glutamate[27]. Since, citicoline preserves the membrane structures and reduces glutamate level, the generation of nitric oxide is greatly reduced. L-NAME administered in combination with citicoline may be involved in the scavenging of residual nitric oxide generated due to ischemia and there by further ameliorates glutamate excitotoxicity[28]. The decrease in the nitrite/nitrate levels may be corroborated to the inhibition of Nitric oxide synthase by L-NAME[29]. Increased activity of sodium potassium ATPase may be related to its membrane protection effect i.e. inhibition of fatty acid release, triggering phosphotidyl choline synthesis and preservation of cardiolipin and sphingomyelin[30]. Expression of TNF-α is decreased in the combination treatment of citicoline and L-NAME. This finding is in corroboration with the finding that L-NAME decreases the expression of TNF-α[31]. As citicoline protects the neuronal membrane and L-NAME prevents the nitric oxide surge after ischemic event due to its ability to inhibit NOS, the expression of the inflammatory cytokine TNF-α is reduced in the combination group. In addition the better neuroprotective effect of combination of L-NAME and citicoline may also be related to the fact that TNF-α exerts neurotoxicity only in the presence of elevated Inducible Nitric Oxide Synthase (iNOS)31. Since, L-NAME is a non selective NOS inhibitor, subtle inhibition of iNOS may be corroborated to the above finding. With regard to histopathology, the reduction in the neuronal damage in combination group may be related to membrane stabilizing property of citicoline and also due to the reduction of nitric oxide generation. Inhibition of NOS by L-NAME reduces the generation of nitric oxide, prevents nitrosative stress and sustains mitochondrial integrity and thereby greatly decreases the neuronal damage[32].

6. Conclusion

It is interpreted from the current investigation that combination of citicoline (membrane stabilizer) along with L-NAME (non selective NOS inhibitor) improves neurological functioning, restores biochemical changes, decreases the expression of inflammatory marker TNF-α, increases the expression of the anti inflammatory cytokine, IL-10 and thereby reduces neuronal damage. Overall combination of citicoline and L-NAME exerts better neuroprotection in middle cerebral artery occluded/reperfused rats.

7. Conflict of Interest

None declared by the authors.

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