Neurobehavioral and Neurobiochemical Improvement of Induced Ischemic Stroke Rats Treated With Alpha Lipoic and Uric Acids

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Abstract: Excessive generation of reactive oxygen species (ROS) and impairment of endogenous antioxidant defense mechanisms have been associated with ischaemic stroke (IS), resulting in secondary events leading to neuronal dysfunction and cell death. This study reports the neurobehavioral and neurobiochemical improvement of alpha lipoic acid and uric acid in Wistar rats induced with IS. Thirty rats of Wistar strain were subdivided into six groups of five rats each. Ischaemic stroke was induced in the rats using Middle Cerebral Artery Occlusion (MCAO). The IS rats were administered 45 and 67.5mg/kg body weight of alpha lipoic acid and uric acid orally for two weeks. Various neurological assessment methods (stair case, cylinder test and modified neurological severity score (mins)) were carried used to assess the behaviour of the experimental rats before induction of stroke, during and after treatment. Ischemic stroke caused a significant decrease (P<0.05) in the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxide (GPX) activities. There was also a significant (P<0.05) increase in the serum concentration of oxidative stress biomarker, thiobarbituric acid reactive species (TBARS) of the stroke induced not treated group of rats. Administration of alpha lipoic and uric acids cause a significant increase (P<0.05) in the activities of CAT, SOD and GPX activities of the rats compared to their activities in stroke non treated rats. The serum level of TBARS decreased significantly (P<0.05) in rats treated with alpha lipoic acid and uric acids compared to those of the two control groups (stroke induced non-treated and non-stroke induced non-treated) rats. There was also a significant improvement in the neurobehavioral characteristics of the rats as a result of treatment with uric and alpha lipoic acids. This study observed that treatment with alpha lipoic and uric acids boost the antioxidant status of the IS rats and may be responsible for the observed neurobehavioral improvement in the IS and treated rats.

Keywords: ischaemic stroke, Alpha lipoic acid, Uric acid, Oxidative stress.

INTRODUCTION

Globally ischemic stroke (IS) is the major cause of disability and death, but therapeutic choices are still limited. IS as a result of middle cerebral artery occlusion (MCAO) lead to loss of blood supply to part of the brain initiating ischemic cascade [1], accounts for up to 80% of all stroke cases (Ginsberg, 2008). Reduction in cerebral blood flow beyond its normal threshold results into hypoxia, hypoglycemia, the failure of ATP-dependent pumps and a disruption of ionic equilibrium, resulting to the generation of reactive oxygen species (ROS) and free radicals [2]. Excessive generation of reactive oxygen species (ROS) and impairment of endogenous antioxidant defense mechanism begins immediately after the onset of a neurodegenerative disorder such as ischemic stroke (IS) and traumatic brain injury resulting in secondary events leading to neuronal dysfunction and cell death [3]. Research into antioxidant and anti-inflammatory therapy to prevent and/or scavenge ROS and free radical generation has provided hope that the damage due to reperfusion injury may be mitigated, and that the window of opportunity for therapeutic treatment, such as tissue-plasminogen activator (TPA), may be extended beyond the currently accepted time frame of 3 to 4 hours following the onset of stroke [4]. The major
player in the pathophysiology of ischemic stroke is oxidative stress and inflammation, based on the importance of this relationship [5] so many researches have focused on the importance of antioxidant to manage neurodegenerative diseases such as ischemic stroke and traumatic brain injury. Alpha lipoic acid (ALA) is an endogenous antioxidant produced majorly from the mitochondria of the heart, liver and kidney. ALA is a compound that has two sulphydryl (thiol) groups and a free hydroxyl group that act as an electron donor thereby scavenging free radical. Although, Dihydrolipoic acid, a reduced form of ALA has created interest in maintaining endothelial and vascular function [3].

In addition, ALA exhibits metal-chelating activity and is an essential cofactor for enzymes, it is involved in the Krebs cycle and the electron transport chain [7, 8]. ALA also regenerates glutathione (GSH), thereby maintaining optimal levels of other antioxidants, such as vitamins C and E, superoxide (SOD), and catalase (CAT) [9]. Accordingly, among the many health-promoting activities of ALA are its antioxidant, anti-inflammatory, anti-apoptotic, anticancer, cardio-protective, and neuroprotective functions [10]. Moreover, because of its amphiphilic nature, ALA readily penetrates all cells and tissues of the body, including the blood brain barrier (BBB). ALA also effectively increases the production of acetylcholine (neurotransmitter) and thereby maintains the integrity of the brain [11]. These findings strongly suggest a role for ALA in the treatment of various neurological disorders. However, only a few experimental studies, involving different animal and clinical models following traumatic head injury, cerebral ischemia, and seizures, have examined the neuroprotective nature of ALA [6, 12, 13, 14]. Uric acid (UA), the end product of purine metabolism, is reported to exert pro-oxidant or antioxidant properties depending on its altered concentrations in various diseases. Elevated serum UA levels can be responsible for gout arthritis due to its pro-oxidant activity [15]. On the opposite, UA exerts a strong anti-oxidant activity on the serum, where its levels can be easily measured and have been utilized as a marker of the oxidative balance with its clinical correlates. Indeed, reduced serum UA levels have been associated with worse disease outcomes in Parkinson's disease and multiple sclerosis [16, 17]. In addition, several studies reported that increased serum UA levels were associated with a wide range of cardiovascular disease, including coronary heart disease, stroke and hypertension [18]. Serum uric acid (SUA) is a major natural antioxidant and increased levels have been associated to a slower progression of several neurodegenerative disorders but also to an improvement in neurological and immune functions [19]. It has been reported that administration of uric acid (UA) was associated with a reduced infarct growth and improved outcome in stroke patients with hyperglycemia during acute stroke [20]. Uric acid is one of the most important antioxidants in plasma, and its concentration is almost 10-fold higher than other antioxidants. Urate (the soluble form of uric acid in blood) scavenges superoxide, hydroxyl radical, oxygen, and can chelate transition metals. Uric acid blocks the reaction of superoxide anion with nitric oxide that can injure cells by nitrosylating the tyrosine residues of proteins, and it also prevents the degradation of extracellular superoxide dismutase, an enzyme critical in maintaining endothelial and vascular function [3].

MATERIALS AND METHODS
Animals and Treatment
Twenty apparently healthy rats of Wistar strain weighing between 180 and 200g were obtained from the Animal House of the Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine, Usman Danfodiyo University, Sokoto, Nigeria. The Ethics Committee of the Department of Veterinary Physiology and Biochemistry approved the animal experiment with ethical clearance number VPB/EC/17/16. The rats were housed under a standard condition at room temperature of 35-37.5°C and were subjected to a 12-hour light/12-hour dark cycle. The rats were fed with a grower mash of vital feed ad-libitum. They were randomly divided into twenty groups of five rats each as shown in table 1 below.

Stroke induction
The focal cerebral ischemic model was conducted using MCAO method as described by [21]. IS was induced in this study by occluding the middle cerebral artery (MCA) in winstar rats. Ketamine and Xylazine at the dose of 80mg/kg and 5mg/kg body weight respectively were used to anaesthize the rats. The anaesthetic condition was maintained until the end of the occluding period. The neck regions of the rats were shaved and scrubbed with savlon®, incision was done at the scrubed area to gain access to the common carotid artery (CCA). The artery was ligated proximally, a nitch incision was created on the CCA distally using a 25G needle, and absorbable suture material chromic catgut was inserted through the nitch incision into the artery until resistance was felt. A silicon-coated suture with the diameter and length of 0.35mm and 5 mm, respectively was manipulated through the external and internal carotid arteries to block the MCA. The incisions made were closed using a non-absorbable suture material (nylon) and the rats were allowed to recover from the anaesthetic condition in the cages. During the induction, the heart rate and rectal temperatures were monitored.

Neurological Assessments
Modified Neurological Severity Score (mNSS)
Before and after treatment of the rats, mNSS were used to examine the level of IS and progress in the treatment in various groups. This method was performed following the method of [22]. The total score of 18 points consisting of five components:
Consciousness and respiration, cranial nerve function, motor function, sensory function and coordination. Eighteen different tasks were used to evaluate these functions. One point was given for failure to perform a task and zero for successful performance of the task. Scores ranged from zero in healthy uninjured rats to a maximum of 18 indicating severe neurological dysfunction with failure in all tasks. The mNSS immediately after stroke reflects the initial severity of injury. Immediately after the initial evaluation of mNSS the rats were assigned to different treatment groups.

**Beam walking test (BWT)**

Beam test was carried out using the method reported by [23]. A wooden beam (1 m in length, 1.5 cm in diameter) was placed 60 cm above the floor between the animal’s room and a starting point. A rat was placed on the beam at the starting point and allowed to walk in the direction of the room. A successful animal would walk into its room in the 20s. Failure to reach the destination or falling (tumbling) from the beam is recorded as motor–coordination deficit. Experimental rats were graded as follows; those that crossed in the 20s were scored 5, which is the maximum score; those that crossed in 25s were scored 4, those that crossed in the 30s were scored 3, while those that fell down or fail to move got 0 and 1 respectively.

**Staircase test (SCT)**

This test was conducted by modifying the method of [24] in order to assess the independent use of the forelimbs of the rats. It was performed by placing the experimental rat on a wooden staircase and placing food on the topmost step. The staircase is comprised of five steps and each step scores 1 point with maximum of 5 points for any rats that was able to get to the top. Food deprivation and daily pre-training was done for four weeks prior to the experiment so as to make the experimental rats get familiar with the test.

**Cylinder test (CT)**

Cylinder test was carried out on the experimental rats to investigate the neural basis of spatial and motor behavior. The method reported by [25] was modified by constructing a locally made glass cylinder and used to carry out the test. The rats were placed in the cylinder and were observed; the light is put off so as to allow the rats to explore better, 1 point was scored for standing for 5 seconds inside the cylinder and up to the maximum of 5 points for standing for 5 minutes.

**Blood sample collection**

Following the two weeks of the antioxidant supplementation to the experimental rats, blood samples were collected from the rats through cardiac puncture. The rats were anaesthetized using chloroform in a glass jar. After proper anaesthesia the rats were laid on dorsal recumbency and then the needle was inserted between the intercostal muscles to gain access to the heart. Blood was collected and poured into plain tubes and spun with centrifuge at 3000rpm for 5 minutes using a bench top centrifuge, the supernatant was removed and stored at -20°C until required for analyses for oxidative stress indices.

**Biochemical Analyses**

**Estimation of Catalase (CAT) Activity**

The commercial Cayman’s Catalase Assay Kit was obtained from the Cayman® chemical company, Ann Arbor, USA and was used to estimate activities of serum catalase in accordance to the manufacturer’s instructions according to the method of (61). Three wells were designated as a sample, standardization and control. To each well, 100µl of assay buffer and 30µl of methanol were added. To standard well, 20µl of prepared standard (Formaldehyde Standards) were added and to sample well 20µl of serum were added. 20µl of H₂O₂ were added to each well to initiate the reaction. The plate was covered with a lid and incubated on a shaker for 20 minutes at room temperature. To each well, 30µl of potassium hydroxide were added to terminate the reaction and 30µl of purpald were then added. The plate was covered once again and incubated for 10 minutes at room temperature on a shaker. Once again, to each well, 10µl of potassium periodate were added, covered and incubated for 5 minutes on a shaker. The absorbance was read at 540nm using Rayto RT 2100C plate reader produced by Shanghai International Holding Corporate, Humberg, Germany.

**Estimation of Superoxide dismutase (SOD) Activity**

Cayman’s Superoxide Dismutase Assay Kit from Cayman® chemical company, Ann Arbor, USA was used according to the manufacturer’s instructions to quantify the serum activities of superoxide dismutase. Two wells were designated as standard and sample. To each well 200µl of the diluted radical detector, 10µl each of the prepared standard to the serum were added to the standard well and sample well respectively. Twenty microliter (20µl) of diluted xanthine oxidase was added to both standard and sample wells to initiate the reaction. The plate was shaken for a few seconds and covered with cover plate. The plate was then incubated on a shaker at room temperature for 20 minutes and absorbance was read at 450nm using Rayto RT 2100C plate reader produced by Shanghai International Holding Corporate, Humberg, Germany. With the method described by (62).

**Estimation of Glutathione peroxidase (GPx) Activity**

The commercial assay kits of Cayman obtained from the Cayman® chemical company, Ann Arbor, USA was used to carry out the analysis of GPx in accordance with the manufacturer’s guide. In brief, three wells were designated as sample, non-enzymatic and positive control. To sample well, 100µl of assay buffer, 50µl of co-substrate mixture and 20µl of serum were added. To non-enzymatic well, 120µl of assay buffer and 50µl of co-substrate mixture were added and...
to positive control well 100µl of assay buffer, 50µl of co-substrate mixture and 20µl of diluted GPx were added. The reaction was initiated by adding 20µl of cumene hydroperoxide to each well and the plate was carefully shaken for a few seconds. The absorbance was read at 340nm using Rayto RT 2100C plate reader produced by Shanghai International Holding Corporate, Humberg, Germany, once every 3 minutes according to the method described by (63).

$$\Delta \text{Abs/min} = \frac{\text{Abs (time 2)} - \text{Abs (time 1)}}{\text{Time (2 min)} - \text{Time (1 min)}}$$

GPx activity = \frac{\text{Abs / min}}{0.00373 \ \mu\text{M} (0.02 \ \text{ml})} = \text{nmol/mm/ml}

Estimation of Lipid Peroxidation (MDA) Concentration

Estimation of lipid peroxidation was performed using Cayman’s assay kits obtained from the Cayman® chemical company, Ann Arbor, USA, following the manufacturer’s instruction. Into a test tube, 0.1ml of serum was added and treated with 2 ml of TBA-TCA-HCl (1:1:1 ratio) reagent (Thiobarbituric acid 0.37%, 0.25N HCl and 15% TCA). The tube was placed in water-bath for 15 minutes, cooled and centrifuged at room temperature for 10 minutes at 1000 RPM. The absorbance of clear supernatant was measured against reference blank at 535 nm using Rayto RT 2100C plate reader produced by Shanghai International Holding Corporate, Humberg, Germany. The concentration of TBARS was calculated using the molar extinction coefficient of malondialdehyde (1.5 x 10^5 Mol/l/cm) following the method highlighted by (64).

DATA ANALYSIS

Statistical programme in Social Sciences (SPSS 22.0) was used to analyse the data. Results were expressed as means ± SD. The data were analysed by one-way analysis of variance (ANOVA); Turkey Alpha Post-hoc Test was used for multiple comparisons between groups and concentrations.

RESULTS

Beam walking test (BWT). Figure 1 shows the results of the beam walking test performed to assess motor function of the experimental rats before, after stroke induction (SI) and during treatment. The scores indicated that rats in the treated groups improved on their first BWT score from 0-1 to 3-5 while the SINT group rats did not show much improvement from their first score.

Staircase test (SCT). Figure 2 presents the results of staircase climbing test performed to assess motor function of the experimental rats before, after SI and during treatment. The scores indicated that rats from the treated groups improved on their first score from 0-1 to 1.2 to 3-5 while the SINT group rats did not show much improvement from their first score.

Cylinder test (CT). Figure 3 shows the result of the cylinder test performed to assess motor function of the experimental rats before, after SI and during treatment. The scores indicate that rats from the treated groups improved on their first score from 0-1 to 3-5 while the SINT group rats did not show much improvement from their first score. The SNINT group showed the same results for the two tests.

Oxidative stress analyses

Effect of treatment of IS rats with ALA and UA on SOD activities: The effect of LMWA on the activity SOD on ischemic stroke of albino rats is presented in figure 5. The results indicated that SI caused significantly (P<0.05) decrease in the activity of the enzyme SOD in the group of rats that was not treated. Treatment with ALA and UA at 45mg/kg and 67.5mg/kg body weight (BW) resulted in significant (P<0.05) increase of SOD activities.

Effect of treatment of IS rats with ALA and UA on CAT activities: Antioxidants supplementation effect on the serum activity of catalase is presented in figure 6. The results indicated that SI caused significantly (P<0.05) decrease in the activity of the enzyme as shown by stroke-induced and not treated group of the experimental rats. After the treatment with ALA and UA, the lowered catalase enzyme activity seen in stroke-induced non-treated group was reverted in the treatment groups when compared with the non-stroke induced non-treated group.

Effect of treatment of IS rats with ALA and UA on GPx activities: The effect of ALA and UA supplementation on the serum activity of GPx is presented in figure 7. The results indicated that SI caused significantly (P<0.05) decrease in the activity of GPx as shown by stroke-induced non-treated group of the experimental rats. Following the supplementation, the GPx enzyme activities significantly (P<0.05) increased in the treatment groups when compared with the non-stroke induced non-treated and stroke induced non-treated groups.

Effect of treatment of IS rats with ALA and UA on MDA activities: The effect of antioxidant supplementation on the serum MDA concentration is presented in figure 8. The results indicated that SI caused significant (P<0.05) increase in the concentration of MDA in the stroke-induced non-treated group. After treatment with ALA and UA at 45mg/kg and 67.5mg/kg BW doses, the concentration of the MDA significantly (P<0.05) decrease in all the treated groups in a concentration dependent manner.
DISCUSSION

Ischemic stroke is caused by an interruption of cerebral blood flow, usually the middle cerebral artery (MCA), ultimately leading to hypoperfusion of tissue and neuronal cell death [26]. Oxidative stress is one of the main mechanisms implicated in the pathophysiology of ischemic stroke because the ischemic brain is particularly susceptible to oxidative stress compared with other organs due to its high consumption of oxygen and it relatively has low antioxidant capacity. Immediately after the onset of brain ischemia, a rapid increase of oxidative stress mediators like ROS or reactive nitrogen species (RNS) occurs. During reperfusion, oxidative stress reaches higher peaks that may overwhelm the scavenging capacity of endogenous antioxidant (SOD, CAT, GPx, uric acid) systems [27]. Thereby decreasing the endogenous antioxidant capacity and consequently, this decrease is associated with poor clinical, radiological and neurological outcomes [28].
The significant (P<0.05) decrease in antioxidant enzymes (SOD, CAT and GPX) activities status and a significant (P<0.05) increase of oxidative stress biomarker (TBARS) in stroke induced non treated (SINT) group indicated that induction of ischemic stroke in albino rats caused an increase in the production of reactive oxygen species, which resulted into oxidative stress and thereby overwhelming the endogenous antioxidant activity, ultimately, the antioxidant become inadequate, consumed and inactivated following ischemia and couldn’t defend the body system from damage. Treatment with different doses of alpha ALA reverted the antioxidant enzyme activities significantly in a concentration dependent manner. It is important to know that ALA can exist in both R- and S-enantiomeric forms, naturally found ALA is an R-isomer that can bind to the ε-amino moiety of lysine residues by an amide linkage, thus making this isomer essential as a co-factor in the mitochondrial energy metabolism. Chemically synthesized LA, in contrast, is a racemate (a mixture of equal amount of R- and S-LA), and is believed to exert additional beneficial bioactivities when administered exogenously [29] because it can augment the endogenous antioxidant in the body to fight against reactive oxygen species created as a result of IS. Alpha-lipoic acid is an antioxidant commonly used for treatment of many neurological disorders such as diabetic polyneuropathy, multiple sclerosis and neurological disorders such as IS and traumatic brain injury. ALA is not toxic at therapeutic doses and it is absorbed from the diet, crosses the blood–brain barrier [30, 31] and still maintain its structure and function. This strong antioxidant influences a number of cellular processes, including direct radical scavenging, recycling, metal chelation, regeneration of endogenous antioxidants, and modulation of transcription factor activity. It has been shown to improve endothelial function and blood flow, and accelerate glutathione synthesis, which plays a crucial role in regulating the expression of several antioxidant and anti-inflammatory genes [30]. One of the ultimate goal of therapy following ischemic stroke is to record improvement in the functional outcome of the neurological ability of the experimental rats; this was assessed using cylinder test, stair case test and beam walking test. The neurological tests significantly improved following the administration of ALA when compared to control group of the rats, this is agreement with the report of [31] where it was reported that functional outcome of an ischemic stroke treated rats was assessed by various neurobehavioral tests and was significantly improved in the aLA group when compared with the control group over a relatively long period of time (56 days).

Treatment with different doses of Uric acid (UA) changed the status of the antioxidant enzymes (SOD, CAT and GPX) activities significantly in a concentration dependent manner. It also increased the oxidative stress biomarker (TBARS) when compared with the control group. The reason for the result could be because UA have a cellular protective antiradical mechanism, it is also reported that uric acid is said to be a neuroprotectant due to its antioxidants action as advocated by Heo and Lee 2010 [32]. In addition, the result of this research work is similar to those reported by Rajeshwar et al. [33] were he reported that the levels of serum UA were significantly elevated in ischemic stroke patients within 24 hours after symptom onset and the results did not change even after adjusting the potential confounders.

CONCLUSION

Alpha lipoic acid and Uric acid has widely proven there neuroprotective properties in experimental models of acute cerebral ischemia. In this study, the exogenous administration of UA and ALA as an antioxidant have shown measurable beneficial biological effects. Furthermore, there is increasing evidence arising from recent research that the two antioxidants could be a therapeutic option in the treatment of ischemic stroke. This beneficial effect is more apparent in the management of patient vulnerable to oxidative stress and reperfusion injury.

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