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Neurobiochemical Roles of Low Molecular Weight Antioxidants on Oxidative Stress Biomarkers and Severity of Ischemic Stroke in Wistar Rats

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Abstract

Purpose: To evaluate the neurobiochemical role(s) of low molecular weight (LMWA) antioxidants in the treatment of surgically-induced ischemic stroke (IS) in wistar rats.

Methods: Ischemic stroke was induced in wistar rats using middle cerebral artery occlusion (MCAO). Neurological assessments were carried out using stair case, cylinder test and modified neurological severity score (mnss) methods. Low molecular weight antioxidants (vitamins C, E, α-lipoic acid, dimethyl sulfoxide and mannitol) were orally administered to the rats for two weeks in three different doses (22.5, 45 and 67.5 mg/kg). Serum and brain tissue homogenate of the animals were then assessed for antioxidants and oxidative stress biomarkers (SOD, CAT, GPX activities and MDA concentrations) respectively.

Results: Significant increase (P<0.05) in the antioxidant enzymes activities and significant (P<0.05) reduction of MDA concentration in the groups treated with low molecular weight antioxidants (LMWA) in a dose dependent manner.

Conclusion: It can be concluded from this study that LMWA reduces oxidative stress in surgically-induced ischemic stroke rats and underscores relevance of the role of LMWA in the treatment of IS rats.

Keywords: Neurochemical; Ischemic stroke; Middle cerebral artery occlusion; Low molecular weight antioxidant; Oxidative stress

List of Abbreviations: IS: Ischemic stroke; MCAO: Middle cerebral artery occlusion; mNSS: Modified neurological severity score; LMWA: Low molecular weight antioxidants; ROS: Reactive oxygen species; SOD: Super oxide dismutase; CAT: Catalase; GPX: Glutathione peroxidase; MDA: Malondialdehyde; PBS: Phosphate buffer saline; BW: Body weight; SINT: Stroke induced but not treated; NSINT: Non-stroke induced and non-treated

Introduction

The most common cause of disability according to the World Health Organization is stroke, each year 15 million individuals suffer from stroke in the world. Of these, 5 million surrender and an additional 5 million are permanently disabled [1]. Ischemic stroke is the most common type of stroke accounting for about 88% of all neurological cases; which is as a result of brain being denied blood supply denying the brain oxygen and glucose, thereby instigating the ischemic cascade [2]. Excessive generation of free radicals like reactive oxygen species (ROS) and the impairment of endogenous antioxidants defense mechanisms such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) begin immediately after the onset of IS. These result in secondary events like tissue hypoxia and cellular dysfunction, consequently, resulting in neuronal malfunction and death [3].

The mechanism and pathogenesis of ischemic injury include; excitotoxicity, oxidative stress (OS), inflammation and apoptosis [4-7]. In addition, OS plays a key role in the mechanism and pathogenesis of ischemic/reperfusion injury [5]. During ischemic injury,

neuron and glial cells are destroyed due to the overproduction of free radicals; and this interacts with important biomolecules such as nucleic acids, lipids and proteins [6]. Because of high oxidative metabolic activity, extreme production of ROS, the neurons non replicating nature and also a high membrane-to-cytoplasm ratio, the brain is therefore vulnerable to oxidative stress [7,8]. Other sources of lipid peroxidation reactions in the brain are high levels of polyunsaturated fatty acids in the membrane lipids and transition metals such as iron, and these are capable of catalyzing the production of reactive radicals in the brain [9-10]. Lipids and Apart from proteins that are classical target of free radicals attack, gene transcriptional processes associated with pathologic events are also there target such as activator protein-1 and nuclear factor-kB, both of which are activated by free radicals [11-12]. The brain uses enzymatic metabolism as the major defense mechanism to combat reducing its severity [13]. Antioxidants reduce the severity of oxidative stress either by forming a less active radicals or by quenching the damaging free radical chain reactions on macromolecules such as proteins, lipids, carbohydrates or DNA by been oxidize itself [14-16].

The frequency of stroke hospitalization in Africa ranges from 0.9%-4.0% as documented, it accounted for 0.5%-45% of all cases of neurological admissions and have been reported to be the eight leading cause of death in Nigeria among other diseases [17]. It was discovered in Africa, that the liability of the extended hospitalization on the family of stroke patients, the consequences of incapacity from the IS injury, and the economic indication on survival of stroke patients as well as the overstretching of hospital facilities have been previously studied [18]. And it was discovered that; most of the people lives in rural areas, where underdevelopment due to poverty, socialism, unavailability and inaccessibility to good and qualitative health care have played major roles in tendency, management and prognosis of stroke [19].

Worldwide, it was found that stroke is the most common cause of incapacity and the second leading cause of death [20]. So many researches have been conducted and reported on the objective of understanding the pathophysiology of cerebral ischemia but therapeutic options still remain very limited [21]. Regrettably, little therapeutics has been known to treat IS. Currently, the only FDA approved drug for stroke patient's treatment is the tissue plasminogen activator (tPA). However, due to its slight therapeutic window of less than 4.5h and safety margins, only less than 5% of patients can benefit from this medication [22]. In view of this, more new approaches are needed in the treatment of stroke. Thus, the study was designed to study the neurochemical potentials of low molecular weight antioxidants in the treatment of Wistar strain rats surgically induced with ischemic stroke.

Materials and Methods

Animals and Treatment

One hundred (100) 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, Usmanu 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 conditions at room temperature of 35-37.5 °C and were subjected to a 12-hour light/12-hour dark cycle. They were fed with grower's mash of vital® feed *ad-libitum*, randomly divided into eight groups as stroke induced and treated with vitamin C, vitamin E, uric acid, alpha lipoic acid, dimethyl sulfoxide and mannitol (VC, VE, UA, ALA, DMSO, Man) respectively. The two other groups were stroke induced non-treated and non-stroke induced non-treated (SINT, NSINT).

Stroke Induction

The focal cerebral ischemic model was conducted using MCAO method as described by Sprat *et al.* [23]. IS was induced in this study by occluding the middle cerebral artery (MCA) in wistar rats. Ketamine and Xylazine at the dose of 80mg/kg and 5mg/kg body weight respectively were used to anesthize the rats. The anesthetic 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 scrubbed area to gain access to the common carotid artery (CCA). The artery was ligated proximally, a niche incision was created on the CCA distally using 25G needle, a silicon-coated suture with the diameter and length of 0.35mm and 5mm, 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 anesthetic 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 was used to examined the level of IS and progress in the treatment in various groups. This method was performed following the method of Chen *et al.* [24]. 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 Jover *et al.* [25]. A wooden beam (1m in length, 1.5cm in diameter) was placed 60cm above the floor between the animal's room and a starting point. Each rat was placed on the beam at the starting point and allowed to walk in the direction of the room; every successful rat would walk into its room in 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 20s were scored 5, those that crossed in 25s were scored 4, those that crossed in 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 Baird *et al.* [26] 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 feed on the topmost step. The staircase is comprised of five steps and each step is scored 1 point with maximum of 5points 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 to 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 Gharbawie *et al.* [27] 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 was put off so as to allow the rats to explore better, 1 point was scored for standing for 5seconds inside the cylinder and up to the maximum of 5points for standing for 5 minutes.

Blood Sample Collection

Following the two weeks of the antioxidants supplementation to the experimental rats, blood samples were collected from the rats through cardiac puncture. The rats were anesthetized using chloroform in a glass jar. After proper anesthesia, the rats were laid on their back, an index finger was placed at the level of the lowest ribs without pressure been applied, the heart was located around this area, just about 1cm above the last ribs approximately. A syringe at an angle of 45 degree was held, the needle was inserted between the intercostal muscles to gain access to the heart. Blood was drawn, immediately poured into plain tubes and spun using bench top centrifuge at 3000rpm for 5 minutes using bench top centrifuge, the supernatant was removed and stored at -20 ˚C until required for analyses for oxidative stress indices.

Brain Tissue Extraction and Homogenization

This was done according to the method of Rezanejad *et al.* [28]. Micro dissecting scissors was used to open the skin at the midline of the head of the rats, cutting from the roof of the skull to the mid-eye area, scissors was used to fold back the skin flaps, and the skull was then cut at the midline fissure without cutting into the brain tissue. The raised skull cap was removed with the curved forceps, applying slight pressure. The brain was then released from the skull cavity by running a micro spatula underneath and along the length of the brain from the olfactory lobes to the beginning of the spinal cord. After gently transferring the brain to a 60mm petri dish, the tissues were rinsed with phosphate buffer saline (PBS) to remove any red blood cells and clots.

The brain were then transferred to a second petri dish and cut into small pieces in ice chilled 10% PBS solution, slices were sonicated for 45 min in 100 cycles [29]. The extracts were separated from cells debris by centrifugation at 1500rpm using bench top centrifuge for 5minutes, and then the supernatant was collected and used for the assay.

Biochemical Analyses

Estimation of Catalase (CAT) Activity

The commercial Cayman's Catalase Assay Kit was obtained from Cayman® Chemical Company, Ann Arbor, USA and was used to estimate activities of serum catalase according to the manufacturer's instructions following the method of Johansson and Borg [30]. Three wells were designated as sample, standard 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 H_2O_2 were added to each well to initiate the reaction. The plate was covered with 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 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 Marklund [31].

Estimation of Glutathione peroxidase (GPx) Activity

The commercial assay kits of Cayman obtained from Cayman® chemical company, Ann Arbor, USA was used to carry out the analysis of GPx in accordance with the manufacture'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 cumenehydroperoxide 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 [32].

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

$$\text{GPx activity} = \frac{Abs\,/\,\text{min}}{0.00373\mu/\text{M}} \times \frac{0.19\,ml}{0.02\,ml} = \text{nmol/mn/ml}$$

Estimation of Lipid Peroxidation (MDA) Concentration

Estimation of lipid peroxidation was performed using Cayman's assay kits obtained from Cayman® Chemical Company, Ann Arbor, USA, following the manufacture'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 minutse 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 10^5mol/l/cm) following the method highlighted by Niehans and Samuelson [33].

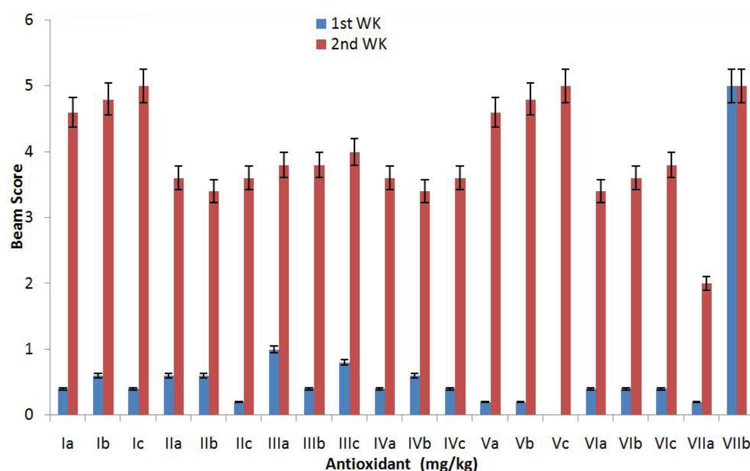
Data Analysis

Statistical programme for Social Sciences (SPSS22.0) was used to analyze the data. Results were expressed as means±SD. All Data were analyzed 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 after stroke induction (SI). 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 improvement from their first score.

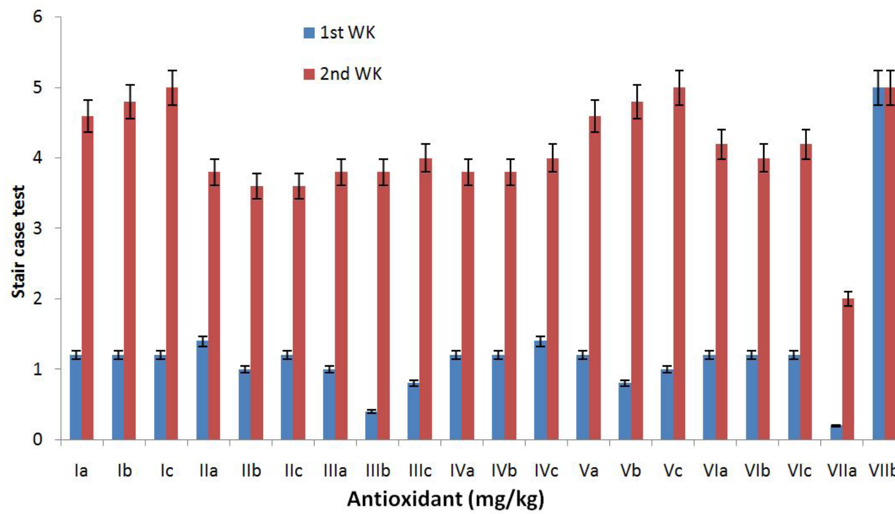


Ia- VC- Vitamin C 22.5mg/kg, Ib- VC- Vitamin C 45mg/kg, Ic-VC- Vitamin C 67.5mg/kg, IIa-VE-Vitamin E 22.5mg/kg, IIb- VE-Vitamin E 45mg/kg, IIc-VE-Vitamin E 67.5mg/kg, IIIa- UA-Uric Acid 22.5mg/kg, IIIb- UA-Uric Acid 45mg/kg, IIIc- UA-Uric Acid 67.5mg/kg, IVa- ALA-Alpha Lipoic Acid 22.5mg/kg, IVb- ALA-Alpha Lipoic Acid 45mg/kg, IVc- ALA-Alpha Lipoic Acid 67.5mg/kg, Va- DMSO- Dimethyl sulfoxide 22.5mg/kg, Vb-DMSO- Dimethyl sulfoxide 45mg/kg, Vc-DMSO- Dimethyl sulfoxide 67.5mg/kg, VIa- Mann- Mannitol 22.5mg/kg, VIb- Mann-Mannitol 45mg/kg, VIc- Mann- Mannitol 67.5mg/kg, VIIa- SINT- Stroke induced non-treated, VIIb- NSINT- Non stroke induced non-treated

Figure 1: The effect of antioxidant supplementation on Motor function of IS rats using BWT

Staircase Test (SCT)

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

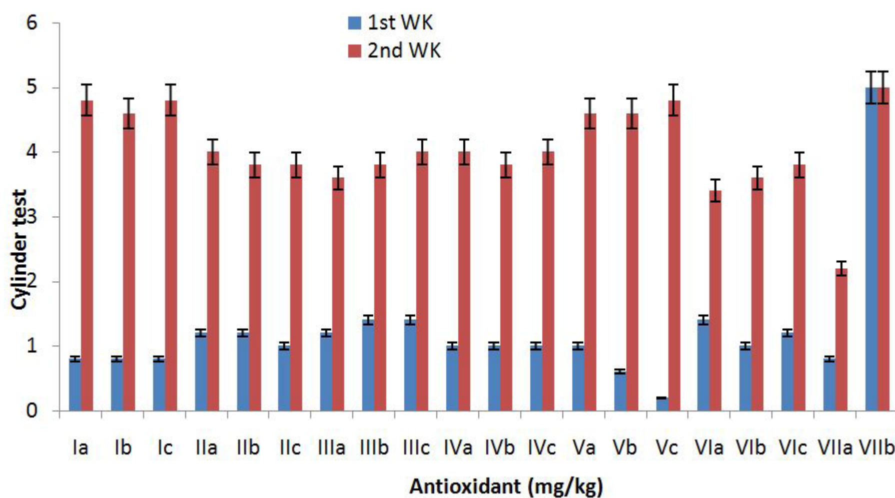


Ia- VC- Vitamin C 22.5mg/kg, Ib- VC- Vitamin C 45mg/kg, Ic-VC- Vitamin C 67.5mg/kg, IIa-VE-Vitamin E 22.5mg/kg, IIb- VE-Vitamin E 45mg/kg, IIc-VE-Vitamin E 67.5mg/kg, IIIa- UA-Uric Acid 22.5mg/kg, IIIb- UA-Uric Acid 45mg/kg, IIIc-UA-Uric Acid 67.5mg/kg, IVa- ALA-Alpha Lipoic Acid 22.5mg/kg, IVb- ALA-Alpha Lipoic Acid 45mg/kg, IVc- ALA-Alpha Lipoic Acid 67.5mg/kg, Va- DMSO- Dimethyl sulfoxide 22.5mg/kg, Vb-DMSO- Dimethyl sulfoxide 45mg/kg, Vc-DMSO-Dimethyl sulfoxide 67.5mg/kg, VIa- Mann- Mannitol 22.5mg/kg, VIb- Mann- Mannitol 45mg/kg, VIc- Mann- Mannitol 67.5mg/kg, VIIa- SINT- Stroke induced non-treated, VIIb- NSINT- Non stroke induced non-treated

Figure 2: The effect of antioxidant supplementation on Motor function of IS rats using SCT

Cylinder Test (CT)

Figure 3 shows the result of cylinder test performed to assess motor function of the experimental rats after SI. The scores indicated that rats from 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.

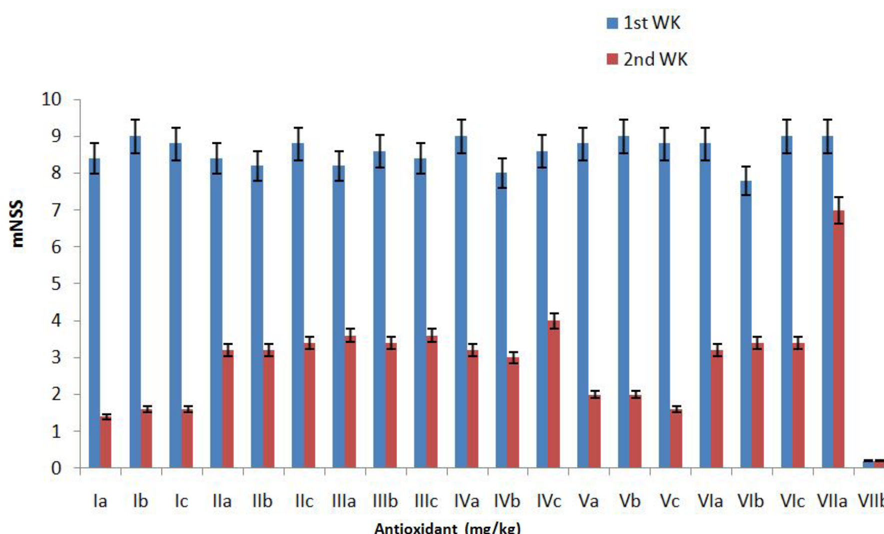


Ia- VC- Vitamin C 22.5mg/kg, Ib- VC- Vitamin C 45mg/kg, Ic-VC- Vitamin C 67.5mg/kg, IIa-VE-Vitamin E 22.5mg/kg, IIb- VE-Vitamin E 45mg/kg, IIc-VE-Vitamin E 67.5mg/kg, IIIa- UA-Uric Acid 22.5mg/kg, IIIb- UA-Uric Acid 45mg/kg, IIIc-UA-Uric Acid 67.5mg/kg, IVa- ALA-Alpha Lipoic Acid 22.5mg/kg, IVb- ALA-Alpha Lipoic Acid 45mg/kg, IVc- ALA-Alpha Lipoic Acid 67.5mg/kg, Va- DMSO- Dimethyl sulfoxide 22.5mg/kg, Vb-DMSO- Dimethyl sulfoxide 45mg/kg, Vc-DMSO-Dimethyl sulfoxide 67.5mg/kg, VIa- Mann- Mannitol 22.5mg/kg, VIb- Mann- Mannitol 45mg/kg, VIc- Mann- Mannitol 67.5mg/kg, VIIa- SINT- Stroke induced non-treated, VIIb- NSINT- Non stroke induced non-treated

Figure 3: The effect of antioxidant supplementation on Motor function of IS rats using CT

Modified Neurological Severity Score (mnss)

Figure 4 shows the outcome of the neurological assessment (modified neurological severity score) of all the experimental groups. The NSINT group of rats showed no neurological changes. There were considerable improvements in the neurological response in the rats treated with the antioxidants as indicated by their scores. The SINT group did not show significant improvement in their neurological score.



Ia- VC- Vitamin C 22.5mg/kg, Ib- VC- Vitamin C 45mg/kg, Ic-VC- Vitamin C 67.5mg/kg, IIa-VE-Vitamin E 22.5mg/kg, IIb- VE-Vitamin E 45mg/kg, IIc-VE-Vitamin E 67.5mg/kg, IIIa- UA-Uric Acid 22.5mg/kg, IIIb- UA-Uric Acid 45mg/kg, IIIc- UA-Uric Acid 67.5mg/kg, IVa- ALA-Alpha Lipoic Acid 22.5mg/kg, IVb- ALA-Alpha Lipoic Acid 45mg/kg, IVc- ALA-Alpha Lipoic Acid 67.5mg/kg, Va- DMSO- Dimethyl sulfoxide 22.5mg/kg, Vb-DMSO- Dimethyl sulfoxide 45mg/kg, Vc-DMSO- Dimethyl sulfoxide 67.5mg/kg, VIa- Mann- Mannitol 22.5mg/kg, VIb- Mann- Mannitol 45mg/kg, VIc- Mann- Mannitol 67.5mg/kg, VIIa- SINT- Stroke induced non-treated, VIIb- NSINT- Non stroke induced non-treated

Figure 4: The effect of antioxidant supplementation on Motor function of IS rats using mNSS

Oxidative Stress Analyses

Effect of Supplementation with LMWA on Serum and Brain Tissue Homogenates (BTH) SOD Activity of SI Rats: The effect of LMWA on the activity SOD on ischemic stroke of albino rats is presented in Table 1. The results indicated that SI caused significant ($P<0.05$) decrease in the activity of the enzyme SOD. Treatment with LMWA at 22.5mg/kg, 45mg/kg and 67.5mg/kg body weight (BW) resulted in significant ($P<0.05$) increase of SOD activity across the groups when compared with the SOD activity of rats in

	Serum SOD Activities				BTH SOD Activities			
Groups	0mg/kg	22.5mg/kg	45 mg/kg	67.5 mg/kg	0mg/kg	22.5mg/kg	45 mg/kg	67.5 mg/kg
VC		48.58±3.28^{a,x}	74.53±2.59^{a,z}	74.53±2.59^{a,z}		71.90±3.71^{ac,x}	93.09±3.87^{a,y}	104.48±6.38^{a,z}
VE		32.80±2.29^{b,x}	45.33±1.00^{b,y}	45.33±1.00^{b,y}		75.99±7.96^{ab,x}	86.41±3.36^{a,y}	89.05±4.67^{b,y}
UA		20.43±2.31^{cd,x}	31.35±0.50^{cd,y}	31.35±0.50^{cd,y}		65.66±4.89^{ac,x}	74.53±2.59b^{c,y}	75.71±5.41^{cf,y}
ALA		24.59±1.24^{df,x}	29.62±0.94^{df,z}	29.62±0.94^{df,z}		83.96±2.74^{b,x}	97.58±3.69^{a,y}	131.34±7.83^{d,z}
DMSO		65.66±4.89^{e,x}	100.88±4.52^{e,y}	100.88±4.52^{e,y}		73.69±3.77^{ab,x}	84.11±4.86^{ab,y}	131.34±7.83^{de,z}
Man		19.06±0.49^{cf,x}	27.49±1.48^{cf,z}	27.49±1.48^{cf,z}		61.96±1.51^{c,x}	65.49±3.01^{c,x}	70.95±2.30^{f,y}
SINT	5.84±1.60^{g,x}				42.60±1.37^{d,x}			
NSINT	108.38±8.14^{h,x}				150.65±9.79^{e,x}			

Means with different superscript letters are significantly different ($P<0.05$) across the rows and down the columns;

VC: Vitamin C; **VE:** Vitamin E; **UA:** Uric acid; **ALA:** Alpha lipoic acid; **DMSO:** Dimethyl sulfoxide Man Mannitol **SINT:** Stroke induced non-treated; **NSINT:** Non stroke induced non-treated: **SOD:** Superoxide dismutase, **BTH:** Brain tissue homogenates

Table 1: Effect of Antioxidants supplementation on Serum and BTH on SOD Activities of experimental rats

	Serum CAT Activities				BTH CAT Activities			
Groups	0mg/kg	22.5mg/kg	45 mg/kg	67.5 mg/kg	0mg/kg	22.5mg/kg	45 mg/kg	67.5 mg/kg
VC		33.96±1.33^{a,x}	46.42±0.99^{a,y}	62.69±0.55^{a,z}		67.50±0.39^{a,x}	80.25±6.00^{a,y}	90.59±0.87^{a,z}
VE		23.59±2.58^{be,x}	33.65±1.85^{b,y}	41.01±1.23^{b,z}		58.58±0.59^{b,x}	69.49±0.59^{be,y}	70.05±0.00^{b,y}
UA		15.51±1.00^{c,x}	24.17±1.05^{cd,y}	45.42±1.05^{bc,z}		55.46±0.39^{c,x}	58.58±0.59^{c,y}	61.41±0.59^{c,z}
ALA		24.29±1.47^{cd,x}	30.54±0.55^{bd,y}	48.28±0.86^{d,y}		69.77±0.39^{d,x}	86.20±1.27^{d,y}	94.42±0.39^{d,z}
DMSO		34.92±0.81^{a,x}	48.25±0.59^{a,y}	70.49±0.33^{de,z}		64.67±0.81^{e,x}	69.49±0.59^{e,y}	86.20±1.27^{e,z}
Man		17.64±1.12^{ce,x}	21.60±1.71^{ce,y}	26.56±1.85^{f,z}		53.19±0.92^{f,x}	55.60±0.39^{cf,y}	58.72±0.00^{f,z}
SINT	0.89±0.14^{f,x}				38.04±1.36^{g,x}			
NSINT	69.91±8.87^{g,x}				104.90±0.59^{h,x}			

Means with different superscript letters are significantly different ($P<0.05$) across the rows and down the columns;

VC: Vitamin C; **VE:** Vitamin E; **UA:** Uric acid; **ALA:** Alpha lipoic acid; **DMSO:** Dimethyl sulfoxide Man Mannitol **SINT:** Stroke induced non-treated; **NSINT:** Non stroke induced non-treated: **SOD:** Superoxide dismutase, **BTH:** Brain tissue homogenates

Table 2: Effect of Antioxidants supplementation on Serum and BTH on CAT Activities of experimental rats

SINT group in a concentration dependent manner. The effect on brain tissue homogenates SOD activities on the supplementation of antioxidants on ischemic stroke in wistar rats is presented in Table 2. The results indicated that SI caused significant ($P<0.05$) decrease in the activity of the enzyme SOD in SINT group. After supplementation of the antioxidants, SOD activity increased significantly ($P<0.005$) across the groups when compared with the SOD activity of rats in SINT group.

Effect of Supplementation with LMWA on Serum and Brain Tissue Homogenates (BTH) CAT Activity of SI Rats: Antioxidants supplementations effect on the serum activity of catalase is presented in Table 2. The results indicated that SI caused significant ($P<0.05$) decrease in the activity of the enzyme as shown by SINT group of the experimental rats. After the supplementation, the lowered catalase enzyme activity seen in SINT group was reverted in various antioxidants treatment groups when compared with the NSNT group. The effect of brain homogenates of CAT activity on the supplementation of antioxidants on ischemic stroke in wistar rats is presented in Table 2. The results indicated that SI caused significant ($P<0.05$) decrease in the activity of the enzyme CAT in SINT group. After supplementation of the antioxidants in all the groups, CAT activity increased significantly ($P<0.05$) across the groups when compared with the CAT of rats in SINT group.

Effect of Supplementation of LMWA on Serum and Brain Tissue Homogenates (BTH) GPx Activity of SI Rats: The effect of antioxidants supplementations on the serum activity of GPx is presented in Table 3. The results indicated that SI caused significant ($P<0.05$) decrease in the activity of GPx as shown by SINT group of the experimental rats. The effect of antioxidants supplementations on the brain tissue homogenates activity of GPx is presented in Table 3. The results indicated that SI caused significant ($P<0.05$) decrease in the activity of GPx as revealed by SINT group of the experimental rats. Following the supplementations, the GPx enzyme activity significantly ($P<0.05$) increased in various antioxidants treatment groups when compared with the NSNT and SINT groups.

Serum GPx Activities					BTH GPx Activities			
Groups	**0mg/kg**	**22.5mg/kg**	**45 mg/kg**	**67.5 mg/kg**	**0mg/kg**	**22.5mg/kg**	**45 mg/kg**	**67.5 mg/kg**
VC		$47.32\pm4.36^{a,x}$	$72.28\pm9.71^{a,y}$	$94.18\pm1.80^{a,z}$		$87.10\pm7.93^{a,x}$	$117.67\pm1.14^{a,y}$	$121.23\pm2.90^{a,z}$
VE		$31.07\pm2.79^{bd,x}$	$53.49\pm4.41^{bd,y}$	$57.56\pm2.90^{bdf,y}$		$53.49\pm4.41^{b,x}$	$72.28\pm9.71^{b,y}$	$83.03\pm2.90^{b,y}$
UA		$26.49\pm1.39^{bcf,x}$	$35.66\pm3.12^{cf,y}$	$57.56\pm7.34^{bc,z}$		$35.66\pm3.12^{c,x}$	$53.49\pm4.41^{ce,y}$	$58.58\pm9.00^{c,z}$
ALA		$25.47\pm0.00^{cd,x}$	$45.84\pm12.99^{cd,y}$	$66.73\pm2.13^{cd,z}$		$90.67\pm4.63^{a,x}$	$116.65\pm2.13^{a,y}$	$139.57\pm4.89^{d,z}$
DMSO		$75.89\pm1.14^{e,x}$	$89.14\pm5.97^{e,y}$	$106.46\pm2.13^{eh,z}$		$89.14\pm5.97^{ad,x}$	$96.78\pm3.12^{d,y}$	$111.55\pm1.14^{d,z}$
Man		$19.36\pm7.11^{df,x}$	$38.20\pm1.39^{ef,y}$	$62.14\pm6.13^{cdf,z}$		$47.37\pm6.64^{bd,x}$	$50.43\pm4.89^{e,x}$	$49.92\pm7.77^{cf,x}$
SINT	$3.57\pm1.39^{f,x}$				$30.05\pm2.13^{ce,x}$			
NSINT	$113.59\pm7.77^{g,x}$				$158.41\pm7.07^{f,x}$			

Means with different superscript letters are significantly different ($P<0.05$) across the rows and down the columns;
VC: Vitamin C; **VE:** Vitamin E; **UA:** Uric acid; **ALA:** Alpha lipoic acid; **DMSO:** Dimethyl sulfoxide Man Mannitol **SINT:** Stroke induced non-treated; **NSINT:** Non stroke induced non-treated: **SOD:** Superoxide dismutase, **BTH:** Brain tissue homogenates

Table 3: Effect of Antioxidants supplementation on Serum and BTH on GPx Activities of experimental rats

Effect of Supplementation of LMWA on the Serum and Brain Tissue Homogenates (BTH) MDA Concentration of SI Rats: The effect of antioxidant supplementations on the serum MDA concentration is presented in Table 4. The results indicated that SI caused significant ($P<0.05$) increase in the concentration of MDA in the SINT group. After being given various antioxidants at 22.5mg/kg, 45mg/kg and 67.5mg/kg BW, the concentration of the MDA significantly ($P<0.05$) decrease in all supplemented groups in a concentration dependent manner. The effect of antioxidant supplementations on the brain tissue homogenate of MDA concentration is presented in Table 4. The results indicated that SI caused significant ($P<0.05$) increase in the concentration of MDA in the SINT group. After given various antioxidants at 22.5mg/kg, 45mg/kg and 67.5mg/kg BW to the experimental rats, the concentration of MDA significantly ($P<0.05$) decrease in a concentration dependent manner across the treated groups.

Serum MDA Concentrations					BTH MDA Concentration			
Groups	**0mg/kg**	**22.5mg/kg**	**45 mg/kg**	**67.5 mg/kg**	**0mg/kg**	**22.5mg/kg**	**45 mg/kg**	**67.5 mg/kg**
VC		$0.67\pm0.36^{a,x}$	$0.53\pm0.01^{a,y}$	$0.31\pm0.02^{a,z}$		$0.95\pm0.13^{a,x}$	$1.00\pm0.00^{a,y}$	$0.87\pm0.00^{a,z}$
VE		$0.89\pm0.43^{a,x}$	$0.73\pm0.04^{b,y}$	$0.53\pm0.02^{b,z}$		$1.14\pm0.18^{ac,x}$	$1.12\pm0.02^{b,x}$	$1.08\pm0.00^{bc,X}$
UA		$1.67\pm0.17^{b,x}$	$0.84\pm0.02^{cf,y}$	$0.75\pm0.11^{c,z}$		$1.67\pm0.17^{b,x}$	$1.04\pm0.024^{ab,y}$	$0.99\pm0.00^{c,y}$
ALA		$1.28\pm0.14^{c,x}$	$0.74\pm0.08^{bd,y}$	$0.65\pm0.01^{d,y}$		$1.15\pm0.08^{ac,x}$	$0.97\pm0.00^{a,y}$	$0.87\pm0.00^{d,z}$
DMSO		$0.60\pm0.31^{a,x}$	$0.24\pm0.05^{e,y}$	$0.19\pm0.15^{eh,y}$		$1.28\pm0.14^{c,x}$	$1.00\pm0.00^{a,y}$	$0.79\pm0.01^{ad,z}$
Man		$2.05\pm0.31^{d,x}$	$0.85\pm0.04^{f,y}$	$0.85\pm0.00^{f,y}$		$2.77\pm0.12^{d,x}$	$1.85\pm0.04^{c,y}$	$1.78\pm0.01^{e,y}$
SINT	$2.75\pm0.08^{e,x}$				$4.77\pm0.10^{e,x}$			
NSINT	$0.13\pm0.05^{f,x}$				$0.77\pm0.07^{a,x}$			

Means with different superscript letters are significantly different ($P<0.05$) across the rows and down the columns; **VC:** Vitamin C; **VE:** Vitamin E; **UA:** Uric acid; **ALA:** Alpha lipoic acid; **DMSO:** Dimethyl sulfoxide Man Mannitol **SINT:** Stroke induced non-treated; **NSINT:** Non stroke induced non-treated: **SOD:** Superoxide dismutase, **BTH:** Brain tissue homogenates

Table 4: Effect of Antioxidants supplementation on Serum and BTH on MDA Concentrations of experimental rats

Discussion

A significant ($P<0.05$) decrease in the antioxidant enzymes (SOD, CAT, GPX) activities and a significant ($P<0.05$) increase in the concentration of MDA (Table 1-4) was observed in IS induced rats. This could be as a result of an increase in the production of reactive oxygen species (ROS) and free radicals produced after induction of the ischemic stroke, leading to the depletion of the enzymatic antioxidants such as SOD, CAT, GPX, thus leading to oxidative stress (OS) which is a major player in the pathophysiology of neurodegenerative diseases such as ischemic stroke, traumatic brain injury etc. This is in line with the report of Gilgun-Sherki *et al.* [34], it was stated in there study that free radicals can cause destruction to important cellular macromolecules such as carbohydrates, lipids, proteins and nucleic acids leading to consequent cell death by modes of apoptosis or necrosis.

A significant ($P<0.05$) increase in the activities of antioxidant enzymes was observed following treatment with LMWA at 22.5mg/kg, 45mg/kg and 67.5mg/kg body weight (BW) for all the groups supplemented, Antioxidant enzyme activities increased significantly ($P<0.05$) across the groups and OS biomarkers MDA concentration significantly ($P<0.05$) decreased when compared with the antioxidants enzyme activities and MDA concentration of rats in SINT group in a concentration dependent manner. The increase in the activities of antioxidants enzyme activities seen in vitamin C is ascribed to the report by some researchers [35], where they reported that vitamin C is an important antioxidant that has capacity to limit oxidative lipid damage in biological systems. In addition, it is also found to be a potent water affinity scavenger in biological fluids and tissues [35]. Vitamin C is a strong reducing agent and by donating electron(s), thus it neutralizes ROS directly, which always causes oxidative stress by stealing electrons. It was also reported by [35] that, ascorbic acid is a low molecular weight antioxidant that scavenges the ROS through electron transfer rapidly and prevents lipid peroxidation as reported by Flora and Tandon [36].

Furthermore, it has been demonstrated by several studies that vitamin C forms the first line of antioxidant defense and effectively protects the lipid plasma and lipoproteins against detectable per-oxidative damage under many different types of oxidizing conditions [37]. Our result is in agreement with Huang *et al.* [38] a research work that discovered that dehydroascorbic acid; a blood-brain barrier–transportable form of vitamin C, caused dose-dependent increase in post-reperfusion cerebral blood flow, with reductions in the infarct volume, neurological deficit, and mortality. Also, in the studies by [39-41] it was found that an increase in antioxidant vitamin C intake resulted in a decreased risk of stroke.

The positive outcome of the vitamin E (VE) treated group of this study compared to SINT group could be connected to the mode of action of vitamin E, VE is a chain breaking antioxidant and it can be transported into neurons and neuronal mitochondria which tolerates VE to take part directly in the regulation of the destruction which is as a result of oxidant stress according to the report by Yagamata *et al.* [42]. The main mechanism of action of VE is; adding hydrogen to peroxy radicals (ROO°). Meanwhile, VE can reacts with single oxygen ($^1O_2$) and hydroxyl radicals (HO°). Because of this, VE is therefore described as an antioxidant known as chain-breaking that blocks reactive oxygen metabolic cascades [43]. Our observation is similar to the report of Inci *et al.* [44], where vitamin E was observed to modify oxidative stress pathways and improves neurological results in many animal studies, so also, administration of vitamin E cause a neuro-protective effect by decreasing the rate of lipid peroxidation. Again, Carmen and Oyvind [45] reported that vitamin E is a lipid soluble antioxidant which prevents the formation of lipid peroxide. Because vitamin E is one of the potent antioxidant, it exert its action in many organs of the body, it can also cross the BBB and accumulates to therapeutic levels in CNS, which in turn may prevent spatial learning deficit as seen in animal model of aging [46], improve cognitive function, as seen in animal model of Alzheimer's with repetitive concussive brain injury and provide neuro-protection from any other types of insults [47].

The effectiveness seen in uric acid (UA) treatment could be attributed to the way uric acid functions as an antioxidant because its protection system includes the use of endogenous antioxidant enzymes (SOD and GPx), vitamin E which is free radical scavengers and β-carotenes in the cell lipid portion, ascorbic acid and UA in the aqueous phase [48]. UA is a strong free radical scavenger; it can also act as a chelator of metal ions, such as iron and copper, by transforming them to poorly volatile forms. One of the most essential antioxidants in human biological fluids is uric acid [49]. The findings of this study further pointed out the function of UA as an antioxidant and the study is in agreement with the report of Waring *et al.* [50] where they stated that administration of UA in healthy volunteers with low baseline serum concentrations increased the antioxidant ability [50]. Also, it was reported by Yu *et al.* [51] that uric acid suppresses the accumulation of ROS and lipid peroxidation following an exposure to glutamate or ischemic injury as it was shown by a confocal laser scanning microscopy [51]. Many studies have demonstrated the importance of UA in the management of stroke; in a study carried out by Amaro *et al.* [52] it was reported that increase UA levels were associated with an improved rate of excellent recovery independently of baseline variables. Moreover, increased serum levels of UA were significantly associated with smaller infarction volumes [52]. Wu *et al.* [53] conducted a study on 1351 ischemic and 380 cerebral hemorrhage patients and showed that reduced uric acid levels correlate with poor outcomes in acute ischemic stroke patients, but not in cerebral hemorrhagic patients. Wu *et al.* [53] stated that high serum of uric acid level is associated with good neurological outcome at the time of hospital discharge in patients with acute ischemic stroke.

ALA showed a promising result in our findings, the mechanism by which ALA improved the status of our ischemic stroke ALA treated group may be because, ALA is a strong thiol antioxidant with the following important mechanisms of action: ROS scavenging capacity, regeneration of endogenous antioxidants particularly endogenous antioxidants that are necessary for repair systems and has a metal chelating capacity as stated by Biewenga *et al.* [54]. This result is in line with the report of Connell and Saleh [55]

where they stated that ALA produced a dose-dependent neuro-protection against neuronal cell death as observed in a previously confirmed, novel model of ischemia–reperfusion injury. Also the finding is consistent with the research that demonstrated the beneficial effects of ALA in animals subjected to cerebral ischemia–reperfusion. Such benefits include a decrease in ischemic damage, a decrease in the level of neuronal ROS, and enhanced post-ischemic survival [56]. Our findings are in accordance with the research reported by Choi *et al.* [57] where it was argued that the possible mechanism by which ALA promotes favorable outcomes may be related to the activation of the insulin receptor; which is a well-known neuro-protectant. In addition, ALA has been reported to be safe and effective in the treatment of IS, from a previous study by Schreibelt *et al.* [58] ALA has a stabilizing effect on the BBB, making it an attractive therapeutic agent for the treatment of ischemic stroke. Destruction of BBB is a common event in ischemic stroke and is aggravated by reperfusion [59]. Therefore, one of the most important targets for the effective treatment of cerebral ischemia is the protection of the BBB against damage as reported by Choi *et al.* [60].

It is evident in this study that DMSO group of supplementation improved significantly much more than other treated groups. DMSO is a simple organic compound that has so many biological actions including antioxidant, anti-inflamatory, anti-nociceptive and radio-protective effects as reported by Shigetoshi *et al.* [61]. In addition, DMSO is a highly soluble substance that distributes throughout the entire body including brain very rapidly [61]. Furthermore, it solubilizes many lipophilic compounds and increases penetration of many drugs into the brain. Accordingly, it is frequently used as a vehicle in pharmacological studies in cerebral ischemia [62]. DMSO treatment resulted in an improved outcome after transient cerebral ischemia measured by MRI and by a functional neuro-score. This study is also in agreement with the work of Bartdutzky *et al.* [63] where they reported that DMSO has already proven its neuro-protective effects in rats in different doses when administered before and after permanent ischemia of 24 h and MRI observations showed a persistent diffusion/perfusion mismatch.

Treatment with mannitol significantly increased the activities of antioxidants enzymes and decreased significantly the concentration of oxidative stress marker; MDA when compared with the SINT control group. The observed effect of management could be as a result of anti-edema ability which could also have contributed to antioxidant effect Yilmaz *et al.* [64]. Reported that early administration of mannitol after head trauma is more effective in reducing oxidative damage and avoiding cellular damage than the antioxidant enzymes, however administration of mannitol and 7.5% hypertonic saline to reduce intracranial pressure by drawing water from interstitial and intracellular areas to an intravascular area also increases the levels of catalase and GSH-Px enzymes. It has been suggested that these enzymes would reduce the production of MDA, which is a harmful substance for cells and would thereby reduce cellular damage.

Conclusion

In conclusion, our study on the treatment of ischemic stroke using LMWA for two weeks reveals that there was reduction of activities of antioxidant enzymes and increase in the concentration of oxidative stress biomarker suggesting the presence oxidative stress in the stroke induced non treated (SINT) group. All the antioxidants used in this study increases the antioxidant enzyme activities and reduced the concentration of oxidative stress biomarker following two weeks of treatment. These findings might provide new insights into the understanding of the pathogenesis of ischemic stroke as well as open new therapeutic possibilities.

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