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RESEARCH ARTICLE

EFFECT OF SOME LOW MOLECULAR MASS ANTIOXIDANTS IN THE MANAGEMENT OF TRAUMATIC BRAIN INJURY IN ALBINO RATS

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ABSTRACT

Excessive generation of reactive oxygen species (ROS) and impairment of endogenous antioxidant defense mechanism begins immediately after traumatic brain injury (TBI), resulting in secondary events leading to neuronal dysfunction and death. This study reports the role of some low molecular mass antioxidantsin the management of TBI. Winstar rats subjected to closed head injury using an accelerated impact device were administered 22.5mg/kg and 45mg/kg body weight of some of the low molecular mass antioxidants (LMMA) for two weeks. Modified Glasgow coma scale (MGCS) was performed to ascertain the level of consciousness. Blood and brain tissues were collected and analyzed for oxidative stress biomarkers and histological appearances respectively. The results indicated that TBI caused significant decrease ($P<0.05$) superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, and significantly increase ($P<0.05$) malondialdehyde (MDA) concentration. Supplementation with some LMMA however reverted the trend and decreased the healing time and mortality. Histology also showed interparanchymal hemorrhage in traumatized-non-treated rats group, and healing/normal brain sections in the TBI antioxidants supplemented groups. Supplementation of antioxidants to TBI induced rats reduced the impact of oxidative stress and may be responsible for the observed reduction in mortality rate and healing time in the traumatised rats.

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INTRODUCTION

Traumatic brain injury (TBI) is a worldwide problem and its management can pose enormous challenge to health care delivery as well as significantly stretching the hospital resources including manpower and facilities (Eghwrudjakpor and Allison, 2010). In Africa, road traffic accident (RTA) is responsible for the majority of head and spinal cord injuries causing 80% of all injuries in Nigeria alone. Nigeria's death from RTAs is higher than those of both the industralised and other developing countries (Awojebi, 1987), probably due to poor road network and recklessness in driving.

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Two different mechanisms usually determine the damage after TBI. The primary insult occurring immediately after the injury, followed by the secondary pathological processes such as oxidative stress (Ferguson *et al*., 2010). Oxidative stress (OS) has been implicated as potential contributor to the pathogenesis of acute central nervous system (CNS) injury, it has a significant role in secondary damage, and it is responsible for mortality following TBI(Cook *et al.*, 2010). The brain is principally susceptible to OS because of its high rate of oxidative metabolic activity, intense or extreme production of reactive oxygen metabolites (Ames *et al.*, 1993), non-replicating nature of neurons, and high membrane-to-cytoplasm ratio (Evans 1993). Antioxidants known to be free-radical scavengers can, therefore, protect against per oxidative tissue

damage. It reduces the severity of oxidative stress either by forming a less active radical or by quenching the damaging free radical chain reaction on substrates such as proteins, lipids, carbohydrates or DNA (Dekkers *et al*., 1996). Although TBI is a problem of major medical and socioeconomic significance, its pathogenesis is not well understood (Blumberg's *et al*., 1994) and is often difficult to manage leading to primary and secondary lesions of varying severity (Graham *et al*., 1989). The immune system response to damage done by the closed head injury as a result of free radicals produced neutrophils to remove the damaged tissue (Ishaq *et al*., 2013). The cells of the immune system such as T cells, B cells, and macrophages have membranes that are particularly rich in long chain unsaturated fatty acids, thus making them more vulnerable to free radicals oxidation initiated by the closed head injury than other cells (Ishaq *et al*., 2013). The brain itself is rich in unsaturated fatty acids and consumes large amounts of oxygen that may generate excess free radicals which could overwhelm the antioxidant defense systems that can exacerbate the brain injury (Ishaq *et al*., 2013).

Antioxidant system can be classified into two major groups: enzymes and low molecular weight antioxidants (LMWA) also called non enzymatic antioxidant. These enzymes include superoxide dismutase (SOD), catalase, and glutathione peroxidase. The levels of the enzymes vary in different brain regions and among various species (Watson, 1993). LMWA in the brain include a concerted system of water- and lipid-soluble molecules like GSH, ascorbic acid, histidine-related compounds (carnosine, homocarnosine, and anserine), melatonin, uric acid, lipoic acid, and tocopherols (vitamin E) (Watson, 1993). These are extremely important in minimizing OS. However, the cell can synthesize only a limited number of these molecules (*i.e.* GSH, carnosine). The majority of LMWA are derived from dietary sources. Notably, the ascorbate concentration is unusually high in the brain (Halliwell, 1996). Decreased levels of antioxidants or inhibition of the antioxidant enzymes cause OS that may damage cells (Chaudière and FerrariIliou, 1999). This study was therefore designed to investigate the effect of low molecular weight antioxidants in the management of TBI in experimental rats.

MATERIALS AND METHODS

Chemicals

Superoxide dismutase assay kit (Item No. 706002), catalase assay kit (Item No. 707002), glutathione peroxidase assay kit (Item No. 703102) and lipid hydroperoxide (LPO) assay kit (Items No. 705002 and 705003) were all obtained from Cayman® Chemical Company, Ann Arbor, USA. Ketamine hydrochloride was obtained from Rotexmedica®, Trittau, Germany.

Antioxidants

Ascorbic acid, (vitamin C), and ∝-tocopherol (vitamin E), Zinc, Manganese, Copper, and Mannitol were obtained from PAL®Pharmaceutical Industrial LTD Kano, Nigeria. Glutathione was obtained from Sigma® Chemicals Limited, Paderborn, Germany, while dimethyl sulfoxide (DMSO) was obtained from Cayman®Chemicals Company, Ann Arbor, USA.

Head trauma model and management

The experimental protocol was approved by the Ethical and Animal Care and Usage Committee of the Usmanu Danfodiyo University, Sokoto, Nigeria. Ninety (90), apparentlyhealthy albino rats of Winstar strain weighing about 200g were randomly divided into eighteen groups of five rats each as follows.

Group	Antioxidants
I	TBI treated with Vitamin C 22.5mg/kg
II	TBI treated with Vitamin C 45mg/kg
III	TBI treated with Vitamin E 22.5mg/kg
IV	TBI treated with Vitamin E 45mg/kg
V	TBI treated with DMSO 22.5mg/kg
VI	TBI treated with DMSO 45mg/kg
VII	TBI treated with GSH 22.5mg/kg
VIII	TBI treated with GSH 45mg/kg
IX	TBI treated with Zinc 22.5mg/kg
X	TBI treated with Zinc 45mg/kg
XI	TBI treated with Copper 22.5mg/kg
XII	TBI treated with Copper 45mg/kg
XIII	TBI treated with Manganese 22.5mg/kg
XIV	TBI treated with Manganese 45mg/kg
XV	TBI treated with Mannitol 22.5mg/kg
XVI	TBI treated with Mannitol 45mg/kg
XVII	TBI-untreated
XVIII	Non-TBI-untreated

Heath and Vink (1995) model was used to induce TBI in the rats. The experimental rats were properly restrained and anaesthesized using a dissociative anaesthetic agent, Ketamine at a dose of 80mg/kg body weight intramuscularly, intubated and were ventilated on a Harvard Rodent ventilator. The body temperature of the animals was maintained througho ut with a thermostat- ically-controlled heating pad set at 37ºC. The skull was exposed by midline incision and a stainless steel disc, measuring 10mm in diameter and 3mm in depth was cemented centrally along the control suture, between the lambda and the bregma with a polyacrylamide adhesive. The experimental animals were secured in a prone position on a 10 cm deep foam bed. Injury was induced by dropping an eighty gram (80g) brass weight from a distance of 1m. The stainless steel disc was immediately removed from the skull and sutured; the animals were weaned off the ventilator and allowed to recover in the cages.

Modified Glasgow coma scale (MGCS) was carried out on each experimental rat using the model described by Platt *et al.* (2001). MGCS was done to record indirectly the motor activity, brain stem reflexes and level of conciousness of all the traumatised rats. This was done to ascertain that trauma was induced. MGCS was commenced immediately the rats began to show signs of recovery from anaesthesia, which was about 5-6 hours post-trauma. Supplementation with various antioxidants began twelve hours after TBI induction. After two weeks of supplementation with appropriate amount of Vitamin C, Vitamin E, GSH, Zinc, Manganese, Copper, Mannitol and DMSO,the rats were anaesthesized using chloroform in a transparent glass jar and blood sample was collected from each rat by cardiac puncture. Each blood sample centrifuged at 3000rpm for 5 minutes using bench top centrifuge and the

supernatant was removed and stored at -20°C until required for analyses for oxidative stress indices.

Biochemical Analysis

Superoxide dismutase was assayed using Cayman's Superoxide Dismutase Assay Kit (Marklund, 1980). Catalase was assayed using Cayman's Catalase Assay Kit (Johansson and Borg, 1988). Glutathione peroxidase was assayed using Cayman's Glutathione Peroxidase Assay Kit (Paglia and Valentine, 1967). Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was measured by the method of Niehans and Samuelson (1968).

Histological assessment

The experimental rats were anesthesized with chloroform in a transparent glass jar. Brain tissues were extracted from the anesthesized rats and the tissues immersed in 10% formaldehyde. Coronary brain section (40µm thick) was cut with serial sections collected at 1mm intervals. The paraffin embedded brain tissues were stained at a thickness of 4µm using Leica RM 2125RT microtome. Sections were stained with Haematoxylin and Eosin (H&E) for morphological assessment of injury and detection of inflammatory cells, including polymorphonuclear cells (neutrophils, monocytes and macrophages-like cells) defined by large irregular cytoplasm. The images of stained specimens were captured by digital photo camera.

Statistical analysis

Results were presented as means ± standard deviation. Data were analysed by one-way analysis of variance (ANOVA). Duncans New Multiple Range Test was used for multiple comparisons. Statistical Package for Social Sciences (SPSS) Version 16 was used for the analysis.

RESULTS

After the induction of the brain injury, there were clear signs of trauma because all the traumatised rats manifested changes in behavior, feeding, motor activity, brain stem reflexes, and level of consciousness. They exhibited changes like continuous closure of the eyes, refuse to eat, non-responsive to pinching, auricular and auditory reflexes. Figure1 showed the number of survivors and casualty in each treatment group. Rats with MGCS category between 9-14 and 15-18 survived as indicated in the tables below and were used for the experiment. Experimental rats that had MGCS scores between 3-8 died during the experiment. The rats in the non-traumatised non-treated (NTNT) and those treated/supplemented with Vitamin C 45mg/kg and DMSO 22.5mg/kg and 45mg/kg-treated (Groups II, V and VI respectively) survived without any casualty. Figure 2 showed TBI caused significant ($P<0.05$) decrease in the activity of SOD as indicated by the TNT group, while antioxidant supplementations significantly ($P<0.05$) increased SOD activity in a concentration dependent manner. The result indicated that Supplementation of the antioxidants at 22.5mg/kg body weight (BW) changed the SOD activity above the non-traumatised rat status except in groups supplemented with 22.5mg/kg BW zinc and mannitol. At 45mg/kg BW, the activity of SOD doubled compared to the activity of SOD in non-traumatised rats. DMSO, Vitamin C, vitamin E and Glutathione appeared to perform better than the other antioxidants in terms of SOD activity.

The effect of supplementation of antioxidants on the activity of CAT is presented in Figure 3. The results indicated that TBI caused significant ($P<0.05$) decrease in the activity of the enzyme as shown by TNT group. Supplementation at 22.5mg/kg body weight, reverted the activity to that of non-traumatised status, except in groups supplemented with Mn and GSH. At 45mg/kg body weight, the activity of CAT almost doubled compared to the activity of CAT in NTNT group. Antioxidants; Vitamin E, Copper, DMSO, Vitamin C, and Zinc at higher dose of 45mg/kg showed higher activity in the enzyme CAT than all other group in the experimental rats. The serum GPX activity is presented in figure 4. TNT group indicated that TBI caused significant ($P<0.05$) decrease in the activity of the enzyme GPX. Various antioxidants treatments significantly ($P<0.05$) increased the activity as compared with the NTNT group status, except in groups supplemented with Mn, GSH and Zinc at 22.5mg/kg body weight. At 45mg/kg body weight, the activity of GPX significantly ($P<0.05$) increased to doubled compared to the activity of GPX in NTNT rats group. Vit C, Vit E, Cu and DMSO presented higher activity of the enzyme. Figure 5 showed that there was significant ($P<0.05$) increased in the concentration of MDA in the TNT group of the experiment. After various antioxidants supplementation at 22.5mg/kg body weight, the concentration of MDA significantly ($P<0.05$) decreased in the treated groups. The concentration of MDA decreased more at 45mg/kg body weight in Vit C and DMSO.

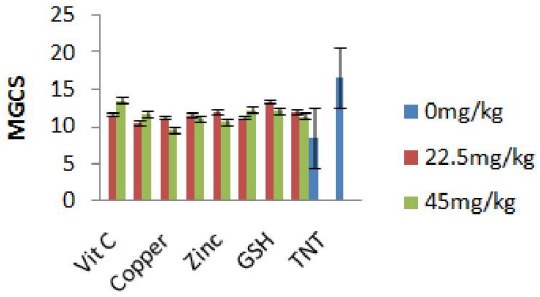

Fig. 1. MGCS of TBI Rats

Histology

Histological assessment is presented in Plates 1 to 10. Plate 1 indicated that the brain section of the TNT showed intraparanchymal hemorrhage with reactive gliosis. Plate 2 showed that the group NTNT showed brain section with normal cerebellum. Plate 3 shows vitamin C supplemented group with normal cerebellum, Plate 4, the GSH supplemented group indicated brain section of normal ventricular lining. Plate 5 is the DMSO supplemented group showing normal histological brain tissue. Plate 6 of the histological slide shows normal brain tissue section after TBI induction and treated with zinc. Mild cerebral edema was seen in the histilogical slide of the group treated with mannitol as evident plate 7. Plate 8 indicated a moderate cerebral edema after the treatment with Cu. Manganese treated group showed mild inflamtion and

neutrophilic infiltration in plate 9. Normal brain section is seen in plate 10 following the treatment with vitamin E.

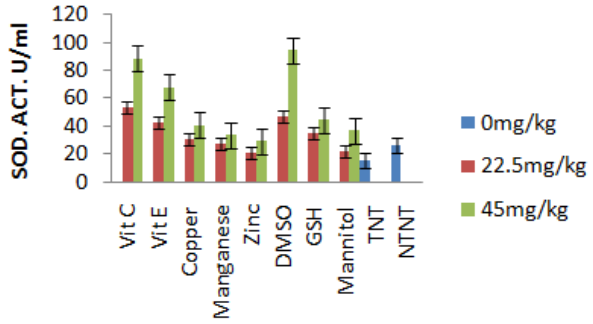

Fig.2. Effects of Antioxidants on SOD Activity of TBI Rats

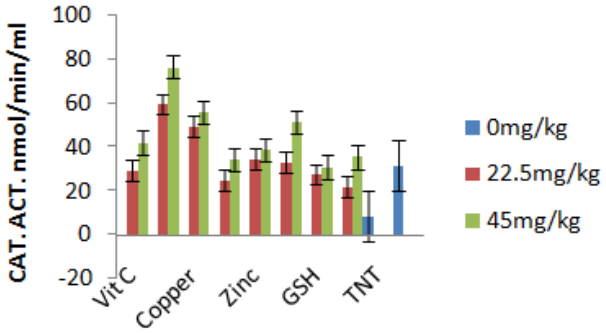

Fig.3. Effects of Antioxidants on CAT Activity of TBI Rats

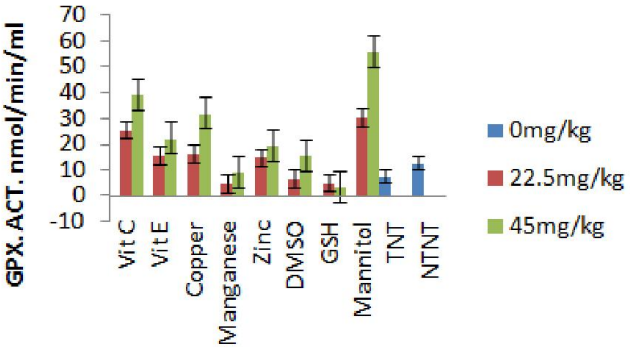

Fig.4. Effects of Antioxidants on GPX Activity of TBI Rats

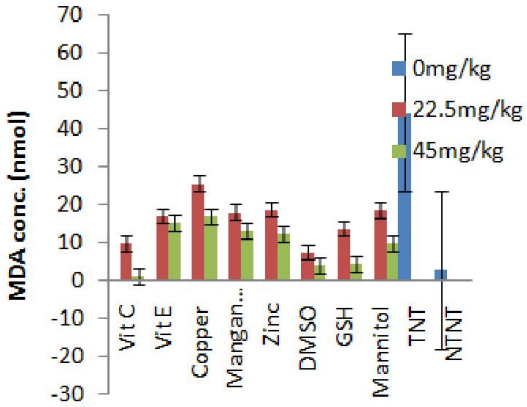

Fig.5. Effects of Antioxidants on MDA concentration of TBI Rats

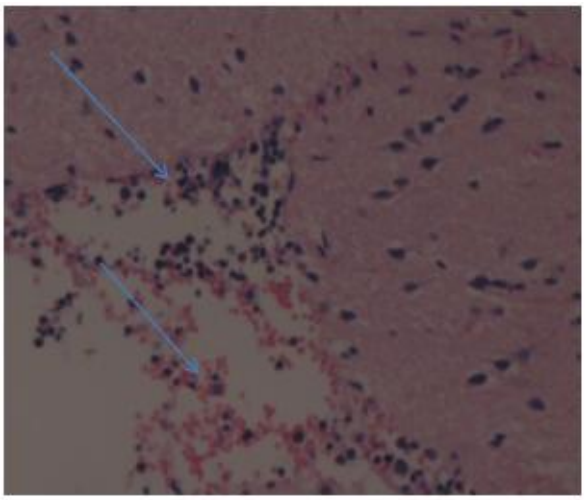

Plate 1. TNT: Section of the brain showing intraparanchymal hemorrhage and reactive gliosis in rats traumatized without treatment

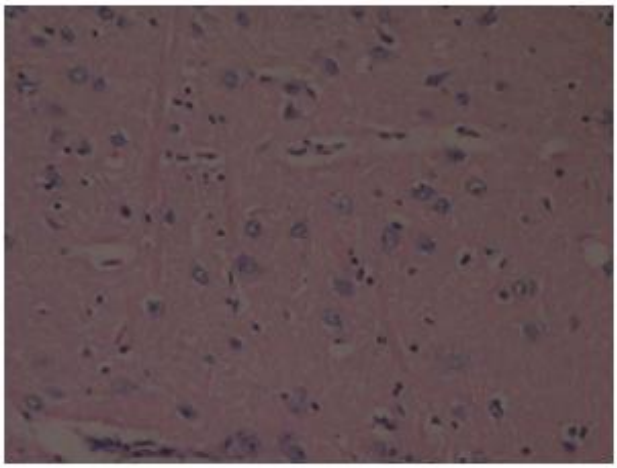

Plate 2. NTNT: Section of the brain showing normal brain tissue

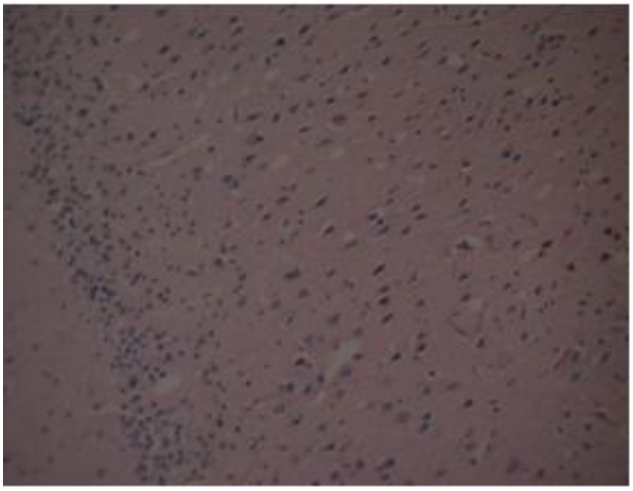

Plate 3. Vitamin C: Section of the brain showing normal cerebellum

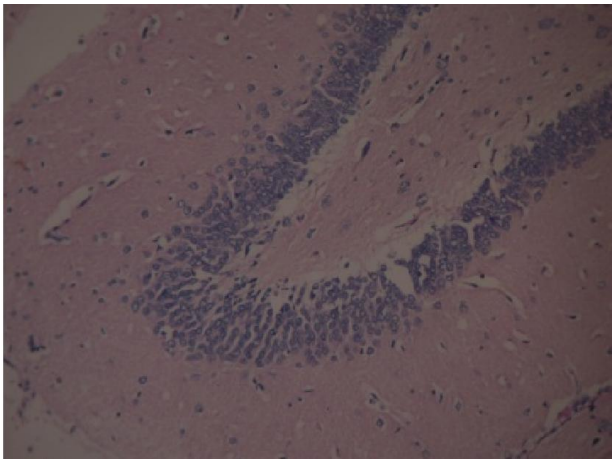

Plate 4. GSH: Section of the brain showing normal ventricular lining

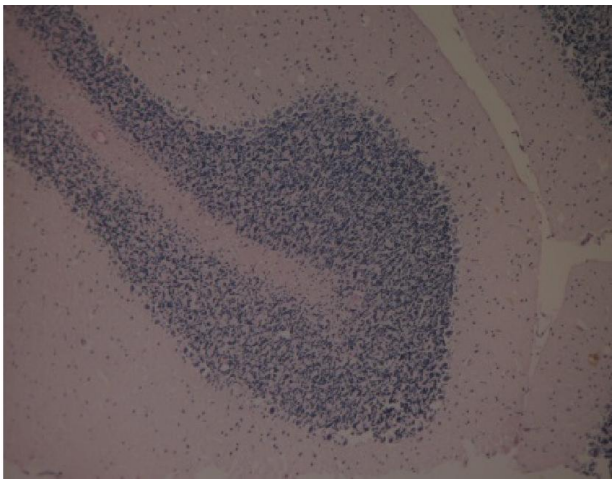

Plate 5. DMSO: Section of the brain showing normal brain tissues

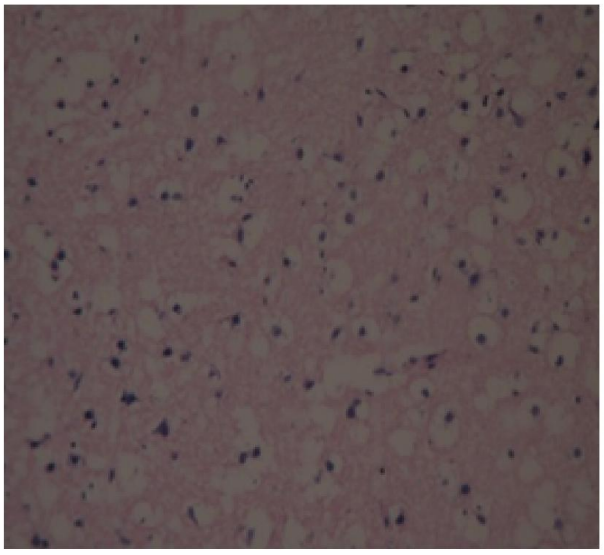

Plate 6. Zn:Section of the Brain showing normal brain tissue after treatment with Zn

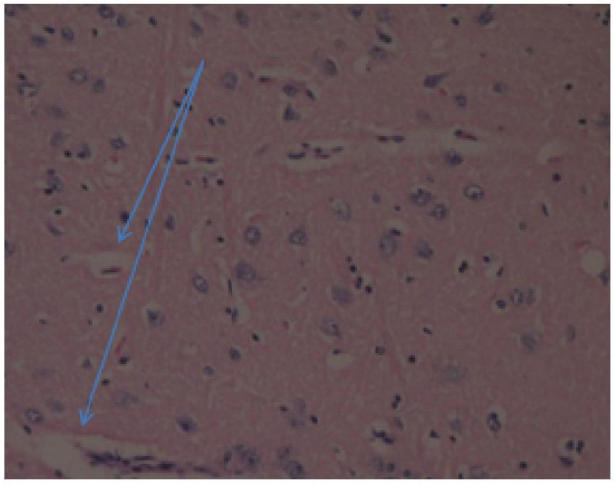

Plate 7. Mannitol: Section of the brain showing mild cerebral edema

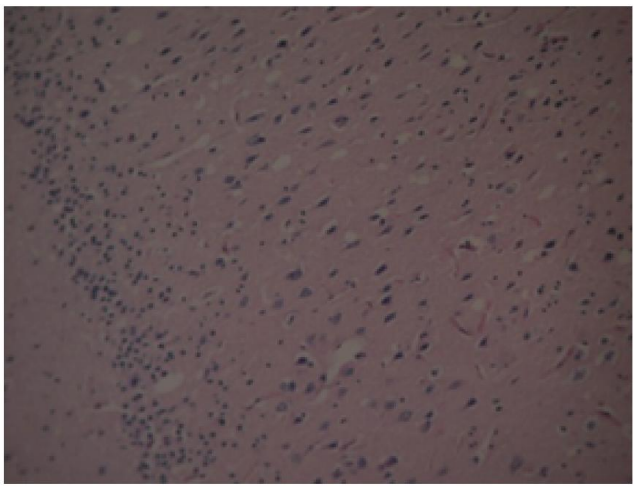

Plate 8. Cu: Section of the brain showing moderate cerebral edema

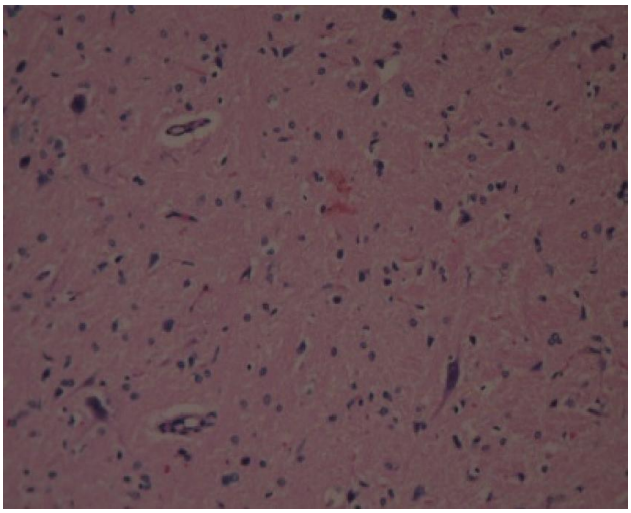

Plate 9. Manganese: Section of the brain showing mild inflammation and neutrophilic infiltration

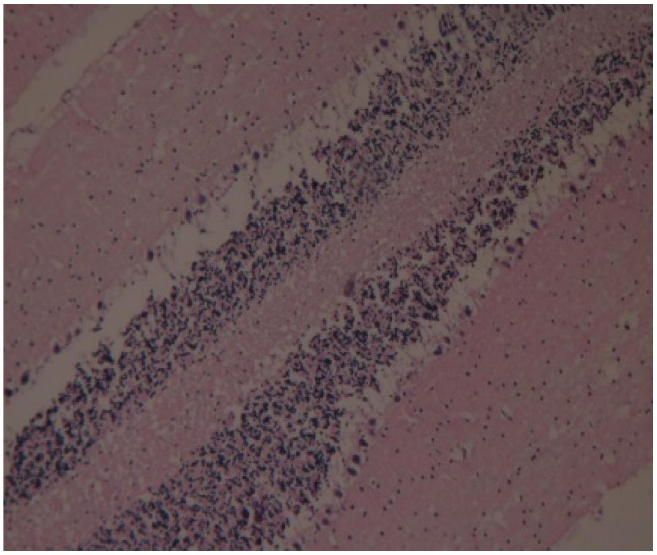

Plate 10. Vit. E: Section of the brain howing normal brain section

DISCUSSION

Oxidative stress (OS) has been implicated as a possible contributor to the pathogenesis of acute central nervous system (CNS) injury and it is found to be responsible for mortality following TBI (Cook *et al*., 2010).TBI triggers a pathway of neuronal death involving loss of cellular calcium homeostasis, tissue acidosis, and OS (Kochanek *et al*., 2000). The observed significant ($P<0.05$) decrease in the activities of serum antioxidant enzymes; SOD, CAT, GPX activities and increased plasma MDA concentration in all the TBI-induced-treated groups compared to non TBI-induced- non treated and TBI-induced non treated group, suggested a role of oxidative stress in TBI. Treatment of the TBI-induced rats with varying doses of vitamin C caused significant increase in TBI antioxidant status with decrease in MDA concentration; this might be due to the fact that vitamin C is found to be more abundant in tissues, where ROS production is more important. Also, it might be that it is because of the ability of vitamin C to regenerate vitamin E and GSH, which are very potent against ROS. Findings of this work are in agreement with the result of Rabec and Pierce (1994), who reported that ascorbate is highly efficient in trapping free radicals, and preventing them from forming lipid hydroperoxide that can be generated during TBI. Eghwrudjakpor and Allison (2010) also reported that ascorbic acid is a very efficient free-radical scavenger because it neutralizes or removes the impurities formed by the free radicals, produced during head insult.

Treatment of TBI-induced rats with 22.5mg/kg and 45mg/kg of vitamin E caused the activity of the enzymes (SOD, CAT, and GPX) to significantly increase, while the concentration of plasma MDA significantly decreased in this study. Inci *et al.* (1998) reported that vitamin E resulted in the increase of the activities of SOD, CAT and GPX, while the concentration of MDA significantly decreased. This might be due to the characteristics of vitamin E as the most relevant chain-breaking antioxidant and abundance in cells and mitochondria membrane. It is also known that vitamin E acts directly on ROS, and it can also react with various antioxidants such as vitamin C, GSH, β-carotene to bring about synergistic activity. All these antioxidants mentioned have the ability to regenerate vitamin E.Inci *et al.* (1998) also reported that vitamin E is promising in modifying OS pathways and improving neurological outcome in many animal studies. The administration of vitamin E also causes a neuroprotective effect by decreasing the rate of lipid peroxidation. Carmen and Oyvind (2001) reported that vitamin E is a lipid-soluble antioxidant which prevents the formation of lipid peroxide.

It was observed in this study that treatment with DMSO significantly increased the activities of SOD, CAT and it decreased the concentration of MDA in the treated groups, when compared with the control groups. DMSO is widely used as a solvent for various drugs and an effective neuroprotectant. It also averts glutamate-induced neuronal cell death (Lu and Mattson, 2001). DMSO is reported to offer an option as a preventive measure in patients undergoing procedures with an increased risk of developing perinterventional brain ischaemia, such as carotid coronary artery bypass surgery (Karaca *et al*., 2002). Treatment with glutathione significantly increased the activities of SOD, CAT, GPX, and it decreased the concentration of MDA in the treated-TBI-induced group, when compared with TNT and NTNT though the activity of GPX was less compared to TNT. Raffa *et al*. (2011) reported thatglutathione is the brain major or dominant antioxidant, implicated in the pathophysiology of brain diseases.Glutathione participates in the reduction of oxyradical and its level in the brain is high, especially during early development. Glutathione a dominant co-enzyme in the GPX molecule, which converts peroxides and hydroxyl radical into nontoxicor inert forms, often with the concomitantoxidation of reduced glutathione (GSHr) into the oxidized form glutathione disulfide (GSSG) and glutathione reductase recycles GSSG to GSH (Drevet, 2006).

Treatment with manganese (Mn) significantly increased the activities of SOD, CAT and decreased the concentration of MDA in the treated groups when compared with the control group (TNT). The molecular mechanisms underlying the protective effect of Mn-SOD have previously been explored by other studies (Bruno-Barcena*et al*., 2004), in general, H_2O_2 itself is not a strong oxidant. In the presence of ferrous iron ($Fe2^{+}$), however, H_2O_2 produces toxic hydroxyl radical ($OH\cdot$) through the Fenton reaction (Bruno-Barcena*et al*., 2004). Hydroxyl radical oxidizes thiol (–SH) groups of the mitochondrial permeability transition pore complex, thus inducing pore opening. The opening of nonspecific channels in the mitochondrial inner membrane may cause a depolarization of the change in membrane potentials. Thus, inhibition of $OH\cdot$ production may help maintain the membrane potentials and inhibit cell death induced by H_2O_2. Because endogenous production of O_2^- is necessary for the oxidation of labile iron–sulfur clusters and the regeneration of Fe^{2+} from ferric iron (Fe^{3+}), Mn-SOD, which can eliminate O_2^- and block the supply ofFe^{2+}, will efficiently decrease the generation of $OH^{\cdot}$.

Zinc supplementation significantly increased the activity of SOD, CAT, and GPX but decreased the concentration of the MDA in the treated group when compared with the control groups. Zinc is reported to be an essential trace element for all

forms of life and that zinc supplementation is a therapeutic tool in managing a long list of illness (Bhowmik *et al*., 2010).Zinc is reported to help enhance memory and improve mental ability, especially for foetal brain development. It is found in the vesicles of mossy fiber system of the brain hipopocampus. These fibers play a role in enhancing memory and thinking skills. This might dictate the decrease in the activity of the antioxidant enzymes (SOD, CAT and GPX) and the increase in the concentration of MDA in our TBI-inducednon-treated group. Zinc is an essential trace mineral, it is required for the metabolic activity of 300 of the body's enzymes, and is considered essential for cell division and the synthesis of DNA and protein, which are potential target of ROS. It is also a coenzyme for Cu/Zn SOD (Peluffo *et al*., 2005). Zinc is a cofactor for many enzymes which might be the reason why there was increase in the activities of oxidative stress enzyme, thereby producing a synergistic effect, and therefore ameliorating the oxidative stress effect.

It was observed that treatment with varying concentrations of mannitol significantly increased the activity of SOD, CAT, GPX and decreased the concentration of MDA in the treated TBI group when compared with the traumatised non treated/control group. This is in concordance with the study of Yilmaz *et al*. (2007), he reported that 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 the enzymes catalase and GSH-Px, these enzymes would ultimately reduce the production of MDA which is a harmful substance for cells and would thereby reduce cellular damage
The morphological assessment of injury and detection of inflammatory cells which includes the polymorphonucleosites (neutrophils, monocytes and macrophages like cells) defined by large irregular cytoplasm in the TNT group indicated that there was injury inflicted which might be the reason why casualty is much in the group. Normal section of the histological slides in the various treatment groups indicated that supplementation was effective and this could be the reason why the mortality rate decreased in the various groups supplemented with antioxidants.

Conclusion

This study provide evidence of the oxidative stress in TBI as shown in decrease of oxidative stress markers after the induction of the injury. Supplementation of TBI rats with various low molecular mass antioxidants however ameliorate the impact of oxidative stress in TBI rats and this could be responsible for the observed reduction in mortality and healing time.

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