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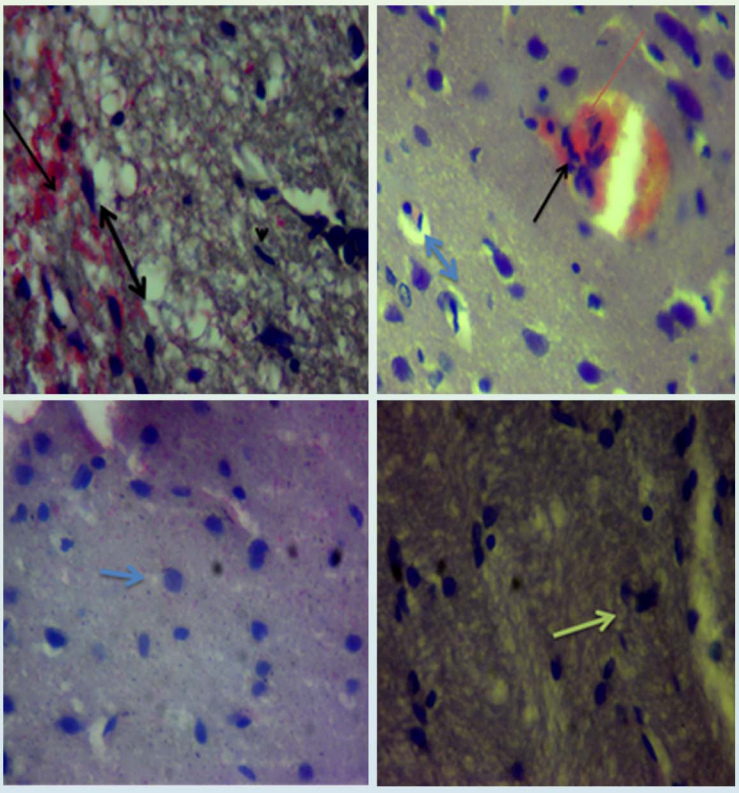

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AIM AND SCOPES
Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

READERSHIP

Biophysics	Biochemistry
Biology	Biomedical Engineering
Pharmacology	PhysiologyGenetics
Cardiology	Neurology
Oncology	Psychiatry
Neuroscience	Neuropharmacology

Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide, ageing, antioxidants, neuropathy, traumatic brain injury, pain, spinal cord injury, Alzheimer's Disease, Parkinson's Disease.

RESEARCH ARTICLE

J Cell Neurosci Oxid Stress 2020;12(1): 922-936.

Ascorbic acid treatment modulated traumatic brain injury-induced oxidative stress and neuropathic pain in rats

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List of Abbreviations;

ARE, *Antioxidant response element;* ***AA,*** *Ascorbic acid;* ***CAT,*** *Catalase;* ***GPx,*** *Glutathione peroxidase;* ***MDA,*** *Malondialdehyde;* ***mNSS,*** *Modified Neurological Severity Score;* ***NOS,*** *Nitric oxide synthase;* ***Nrf2,*** *Nuclear factor erythroid related factor;* ***NF-KB,*** *Nuclear factor kappa;* ***ROS,*** *Reactive oxygen species;* ***GSH,*** *Reduced glutathione;* ***SOD,*** *Superoxide dismutase;* ***TNF-α,*** *Tumor necrosis factor-alpha;* ***WHO,*** *World Health Organization;* ***XDH,*** *Xanthine dehydrogenase;* ***XO,*** *Xanthine oxidase*

Abstract

Generation of reactive oxygen species (ROS) during traumatic brain injury (TBI) has been identified as an important factor that is responsible for disease progression and cell death, particularly in secondary injury process. Ascorbic acid (AA) is an exogenous antioxidant that can be used to quench ROS in neurodegeneration. Its antioxidant properties have been reported in some neurodegenerative conditions in rats. In the present study, we examined the neurotherapeutic effects of AA in TBI-induced rats. Three groups of seven rats each were used for this study. Group I was induced with TBI and treated with AA (67,5 mg/kg orally). Group II was traumatized but not treated (TNT), while group III (control) was neither traumatized nor treated (NTNT). Treatment started 30 min after TBI and lasted for 21 days. Morris water maze (MWM), elevated plus maze, and open field test were carried out in the rats. Antioxidant enzymes [(superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)] and their gene expressions were analyzed. Malondialdehyde level, S100B concentration, and histological studies were also

conducted. The treatment with AA improved learning and memory, locomotor function, and decreased anxiety in the treated groups compared to group II. S100B was significantly ($p<0.05$) lowered in the treated group as compared to the group II rats. Treatment with AA also decreases malondialdehyde level when compared to group II. There were increased activities of SOD, CAT, and GPx activities in the treated group when compared to the control group. These were in agreement with their gene expressions that are highly expressed in the same groups. In conclusion, present data suggest that AA induced neuroprotective effects via down-regulation of lipid peroxidation and up-regulation of antioxidant redox system in the TBI-induced rats.

Keywords: Antioxidant; Ascorbic acid; Neurotherapeutic pain; Oxidative stress.

Introduction

Traumatic brain injury, also known as intracranial injury, occurs when an external force traumatically injures the brain (Saatman et al. 2008).TBI can cause direct mechanical damage to the brain, and induces complex cellular and sub-cellular changes that lead to neurodegeneration and delayed neural cell loss (Angeloni et al. 2015). The direct mechanical impact causes immediate disruption of brain tissue at the time of exposure to the external force and results in contusion, damage to blood vessels (hemorrhage) (Dadas et al. 2018) and axonal shearing (Donate et al. 2017). While the delayed or secondary complex cellular injury is the result of cascades of metabolic, cellular and molecular events including: glutamate excitotoxicity, perturbation of cellular calcium homeostasis, increased free radical generation and lipid peroxidation (Dos Santos et al. 2018).

Generation of reactive oxygen species (ROS) following TBI is one of the most confirmed aspects of secondary injury to brain tissues. After brain injury, the overproduction of ROS leads to oxidative stress and tissue damage via several different cellular molecular pathways (Koza and Linesman, 2019). Radicals can cause damage to lipids, proteins, and nucleic acids (e.g. DNA), leading to subsequent cell death (Engwa, 2018). These ROS are scavenged and neutralize by antioxidant system such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) which react with superoxide anion and hydrogen peroxide and form non-reactive product.

DuringTBI, the capacity of these antioxidants sytem to neutralize the adverse effects of free radicals is compromised. Therefore, enhancing these endogenous antioxidants defense mechanisms may be neuroprotective during injury (Hole et al. 2011).

Ascorbic acid (AA) is one of the most important biomolecule, which acts as antioxidant and radical scavenger (Fetoui et al. 2018). In addition, AA serves as a cofactor in enzymatic synthesis of collagen, carnitine, coagulation factor V, and neurotransmitters by Cu and Fe hydroxylase, and monooxygenase enzymes. Neurons have been shown to maintain high concentration of intracellular AA (May, 2012). Numerous studies have reported that AA level is linked to a decreased risk of neurodegenerative conditions such as stroke (Suleiman et al. 2018). or glutamate induced neurodegeneration (Shah et al. 2015). Low level/intake of AA has been associated with increase reactive oxygen species release and oxidative stress (Kocot et al. 2017). In vivo and in vitro investigations have proven that some of the effects linked to AA are at transcriptional level (Moutinho et al.2019). The aim of this research work is to evaluate the effect of AA in the management of TBI.

Materials and Methods

Chemicals / reagents and assay kits

Ascorbic acid (vitamin C), was obtained from Sigma –Aldrich, Louk, Germany. Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSH) and MDA assay kits were obtained from Cayman® chemical company, Ann Arbor, USA. Ketamine hydrochloride was obtained from Rotexmedica®, Trittau, Germany.

Experimental design

Twenty-one male albino rats (4 weeks old, Wister strain) weighing 180g – 200g were acclimatized in the laboratory for 2 weeks before the experiment. The rats were randomly divided into one treatment group and 2 control groups (positive and negative controls). The treatment group (Group I) was induced with traumatic brain injury and treated with AA for 21 days. The positive control group (Group II) was traumatized but not treated (TNT) while the negative control (Group III) was neither traumatized nor treated (NTNT). The rats were exposed to

a 12 hours light/12-hour dark schedule in room temperature. They were maintained on Rats Pellet (Gold coin® feed), and clean water ad-libitum. The work was approved by the institution animal care and utilization committee University Putra Malaysia on 20th 10 2017 with no UPM/ACUC/AUP-RO75/2017.

Induction of TBI in the rats

Head injury was induced in the entire experimental animals except in the negative control group by weight drop method using an acceleration impact devise of Foda and Marmarou (1994). The experimental rats were properly restrained and anaesthetized using a dissociative anaesthetic agent Ketamine at a dose rate of 80mg/kg body weight. 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 the prone position on a 10cm deep foam bed. Injury was induced by dropping an eighty-gram brass weight from a distance of 1m. The stainless-steel disc was immediately removed from the skull and the animal was allowed to recover in the cage.

Assessment of locomotor activity and anxiety with Open Field Test

The open field apparatus was constructed of white plywood and measured 100 x 100cm with 30 cm walls. One of the walls was clear Plexiglas, so rat could be visible in the 2 apparatus. Blue lines were drawn on the floor with a marker and were visible through the clear Plexiglas floor. The lines divided the floor into sixteen 18 x 18 cm squares. A central square (33 cm in diameter) was drawn in the middle of the open field. Activity in the open-field was recorded by a computer-operated Digital camera system using ANY-maze software. Total distance (locomotor activity) center distance (the distance traveled in the center of the arena) were recorded. The center distance was divided by the total distance to obtain a center distance–total distance ratio. The center distance–total distance ratio can be used as an index of anxiety-related responses. The total distance was used as an index of locomotor activity

Learning and memory functions evaluation (Morris Water Maze Test)

Learning and memory were tested in the Morris water maze on days 15-20 after TBI using the method of Morris (1984). For each trial, the rat was placed in a circular tank with a hidden submerged platform. Each rat was tested on 4 trials a day with each trial beginning in a new quadrant of the tank. The order in which the rat was placed in the 4 quadrants was randomized each day for each rat. The rat was allowed to swim to the platform and was left on the platform for 20 seconds before being removed. If the platform was not found after 120 seconds, the rat was placed on the platform for 30 seconds before being removed.

A computerized video tracking system (Logitech digital camera attached to a computer with ANY-maze software) was used to record latency (time in seconds to find the platform), cumulative distance, and the average swim speed. After the first learning and acquisition trial period, each animal was given a probe trial, during which the platform was removed and each animal was allowed 60 s to search the pool. The amount of time that each animal spent in each quadrant was recorded (quadrant search time) (Standford, 2007).

Brain extraction and homogenization

This was done according to Rezanejad et al. (2014) using micro dissecting scissors, the skin was opened at the midline of the head, cutting from the roof of the skull to the mid-eye area, after folding back the skin flaps with the scissors, the skull was 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 60 mm petri dish, the tissues were rinsed with a phosphate buffered saline (PBS) to remove any red blood cells and clots. Then the brains were 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 cycle. Extract was separated from tissue by centrifugation at 1500 rpm for 5min, then supernatant was collected and use for the assay.

Estimation of calcium binding protein S100

The assay was done using ELISA kit from Fine Biotech based on the principle of sandwich ELISA

technology. The assay was done based on the quantification of antigen between two layers of antibodies: Capture and detection antibodies. These antibodies must bind to the two non-overlapping epitopes on the antigen. Anti-S100 B antibody was pre-coated onto 96 –well plates as the capture antibody. The biotin conjugated anti- S100 B antibody was used as detection antibodies. Horseradish peroxidase (HRP)-Streptavidin was used as label and tetramethyl blue (TMB) as substrate to visualize the reaction. The blue colour product form from HRP – TMB reaction changes to yellow when acidic stop solution is added. The intensity of the yellow colour measured at the absorbance of 450 nm is proportional to the S100 B concentration **(**Ingebrigtsen et al. 1995). The assay was done using ELISA kit from Fine Biotech**.** Standard, test sample and control wells were set on the 96 wells pre-coated plates. Aliquot of 0.1 ml of the six different concentrations of the standard solutions were added to the standard wells. 0.1 ml of standard dilution buffer was added to the control wells. Properly diluted samples of the brain tissue homogenates were added (0.1 ml) into the test sample wells. The plate was sealed with cover and incubated at 37 ºC for 90 minutes. The cover was removed and the plate was washed twice with wash buffer. Then 0.1 ml of diluted biotin- labeled antibody was added into all the wells. The plate was sealed and incubated at 37 ºC for 60 minutes after which the plate was uncovered and wash 3 times with wash buffer. Diluted HRP-Streptavidin (0.1ml) solution is added into each well in the plates covered and incubated at 37 ºC for 30 minutes. The plate was wash 5 times with wash buffer and 90 ul of TMB was added into each well, sealed and incubated at 37 ºC for 30 minutes.

At this stage colour change to blue was observed and immediately 50 ul of stop solution was added into each well and mixed thoroughly. The colour then changed to yellow immediately and absorbance was read with Micro plate reader at 450 nm. Concentration of S100 B was calculated using Myassay software (Myassay limited, Brighton, United Kingdom).

Estimation of SOD

These were assayed using Cayman's Assay Kit's, following the manufacturer's instructions. This assay utilizes a tetrazolium salt for the detection of superoxide radicals generated by the reaction between xanthine oxidase (XO) and hypoxanthine (Marklun 1980). Two wells were designated as standard and sample. To each well 200µl of the diluted radical detector (tetrazolium salt) was added then 10µl of standard to the standard well and 10µl of serum/ brain tissue homogenate to the sample wells. 20µl of diluted XO was added to both standard and sample wells to initiate the reaction. The plate was shaken for a few seconds to mix and covered with cover plate. The plate was then incubated on a shaker at room temperature for 20 minutes and absorbance was read at 460nm using Rayto (RT 2100C) plate reader.

Estimation of catalase activity (CAT)

This was assayed using Cayman's Catalase Assay Kit's manufacturer's instruction. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic hetrocycle with aldehydes, which upon oxidation changes from colourless to a purple coloured complex that measured at 540 nm (Johansson and Borg 1988).

Estimation of glutathione peroxidase activity (GPx)

GPx was assayed using Cayman's Glutathione Peroxidase Assay Kit and according to the manufacturer's instructions. This assay measures glutathione peroxidase activity indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced upon reduction of hydroperoxide by glutathione peroxidase, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (Ursini et al. 1988).

Estimation of lipid peroxidation

Lipid peroxidation as evidenced by the formation of Thiobarbituric acid (TBA) reactive substances (TBARS) was measured using Cayman's Assay Kit's. The assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid, forming an MDA-TBA_2 adduct that absorbs strongly at 535nm (Goulart et al. 2005). Into two test tubes labeled sample and standard, 100µl of serum/ brain tissue homogenate and standard were added respectively and treated with 100µl of TCA (trichloroacetic acid) reagent. Then 800 µl of color reagent (106 mg TBA, 10 ml TBA-acetic acid solution and 10 ml NaOH) was added to each test tube and vortex.

Tubes were heated in boiling water for one hour and cooled on ice to stop reaction and incubated for ten minutes on ice. After ten minutes, tubes were centrifuge for another ten minutes at 4000 rpm and stabilized at room temperature for 30minutes. After transferring 200 µl of the supernatant to the plate absorbance was read at 540 nm using the plate reader.

Gene expression study

Reverse Transcriptase-Polymerase Chain Reaction (RTPCR).

RNA extraction

The brain homogenate from all the experimental animals were used for RNA extraction, cDNA synthesis, and PCR analysis. Briefly, 250 μl of the brain homogenate was dispensed in 1.5ml sterile Eppendorf tubes and 750 μl of Trizol LS was added in 1:3 ratio. The mixture was resuspended by several up and down pi-petting and allowed to stand for 15 minutes at room temperature. Chloroform (200 μl) was added to the mixture, shaken vigorously for 15 seconds, and then allowed to stand at room temperature for 5 minutes before centrifugation at 12000 ×g for 15minutes at 4◦C.The upper clear aqueous phase containing RNA was gently removed from the two organic and DNA phases into new labeled 1.5ml tube and was used for RNA precipitation. Five hundred microlitres of 100% isopropanol was added to each tube and allowed to stand at room temperature for 10 minutes before centrifugation at12000 ×g for 10 minutes and the isopropanol was discarded while the RNA was washed with 1000 μl of 75% alcohol and centrifuged for 5 minutes at 7500 ×g. The alcohol was discarded and RNA pellet partially dried inside level 2 biosafety cabinets for 5 to 10minutes at the end of which 35 μl of sterile RNAse free water was added to re suspend the RNA for determination of concentration, purity, and subsequent use for downstream application. The RNA purity and concentration were determined using NanoDrop machine (Nanodrop 1000 Thermoscientific) (Chomczynski and Mackey, 1985; Lawal et al. 2017).

cDNA Synthesis

The extracted RNA was used to synthesize cDNA using Tetro cDNA synthesis kit (Bioline Pty limited, Australia) with the following reagent mixtures and conditions: RNA template (5 μl), RNase free water (7.0 μl), and random oligomers (1.0 μl). The mixture was briefly centrifuged and incubated at 65◦C for 2 minutes and rapidly chilled on ice for 5 minutes after which 4.0 μl of RT buffer, 1.0 μl of dNTPs, 1.0 μl of RiboSafe RNase inhibitor, and 1.0μl of reverse transcriptase were added to bring the total reaction volume to 20 μl. The mixture was gently mixed, briefly centrifuged, and incubated at 37◦C for 60minutes after which the temperature was raised to 85◦C for 5 minutes (Lawal et al. 2017).

PCR Amplification

The amplification was done with qpcr machine Realplex from Eppendoff. The synthesized cDNA was used as template for PCR amplification using PCR kit from PCR Biosystem with the following reagents volume and concentrations as recommended by the manufacturer: Two times qpcrBio SYGreen Blue mix (10 μl),10μM Forward primer (0.8 μl), 10μM Reverse primer (0.8 μl), Template DNA (1.0 μl) and PCR grade deionized water (up to 20 μl). The same procedure was adopted to amplify all the three genes of interest and Beta actin as reference gene using the following primers as shown in the table below (Mullis et al. 1986).

Expression of the genes

The threshold cycle (C_t) values were measured to detect the threshold of each of three genes of interest and Beta actin gene in all samples. Each sample was measured in triplicate and normalized to the reference Beta actin gene expression. The C_t value of each well was determined and the average of the three wells of each sample was calculated. Delta C_t (ΔC_t) for test gene of each sample was calculated using the equation:

$\Delta C_t = C_{t\ test}$ gene $- C_t$ Beta actin

Delta delta C_t ($\Delta\Delta C_t$) was calculated using the following equation:

$\Delta\Delta C_t = \Delta C_t\ t_{est}$ average - ΔC_t control group

The fold change in the test gene expression was finally calculated from the formula: Fold change = $2^{-\{\Delta\Delta Ct\}}$ (Livak and Schmittgen, 2001).

Histopathological Examination

Samples of the brain tissue extracted from all the experimental animals were fixed in 10% buffered formalin for 48hrs.The fixed tissues were dehydrated in graded concentration of alcohol (70%, 80%, 90% and

Gene	Forward primer	Reverse primer	Product size bp
GPX	5`GGACATCAGGAGAATGGCAAG-3`	3`TCGATGTCGATGGTGCGAAA-5`	323
CAT	5`-GGTCTGGGACTTCTGGAGT-3`	3`GATGGGTAATTGCCACTGG-5`	285
SOD	5`ACTTCGAGCAGAAGGCAAGC-3`	3`TGAGGTCCTGCAGTGGTACA-5`	133
Beta Actin	5`- ACAACCTTCTTGCAGCTCCT-3`	3`CCCATACCCACCATCACACC-5`	200

The mixture was briefly centrifuged and incubated in a PCR cycling conditions as shown in the Table 2.

***Table 1**: Primers for each gene with their product size.*

Cycles	Temperature(°C)	Time(S)	Steps
1	95	120	Initial denaturation
40	95	5	Denaturation
40	60	20	Annealing
40	60	20	Extension

***Table 2**; PCR cycling conditions.*

100%) using automatic tissue processor. The tissues were cleared using Xylene embedded with molten paraffin wax, blocked and labeled appropriately. Tissue sections of 5µm thick were made from the embedded tissues using a microtome knife attached to a microtome. The sectioned tissues were mounted on a grees-free, clean glass slide, dried at room temperature and stained with Heamatoxylin and eosin (H and E), Cresyl violet stain and Belchowsky stain. The slides were viewed under microscope (Olympus BX51TRF-CCD) at different magnifications (× 10, ×20, ×40)

Statistical Analysis

Results were analyzed using the statistical package - SPSS version 22. Results were expressed as means ± SD. Data were analyzed by one-way analysis of variance (ANOVA). If the F values were significant, the Tukey post-hoc test was used to compare groups. Gene expression fold changes by RT PCR considered significant at two-fold cut-off (P<0.05).

Results

Effect of AA on learning ability in the TBI-induced rats

Figure 1 shows the result of the MWM test carried out on days 15 to 20 of the experiment to test the ability of the rats to learn the position of the escape route located in one of the quadrant in the MWM tank. Rats in the TNT group spent significantly (P < 0.05) longer time (increased escape latency) to find the escape route than the AA group which exhibited a decreasing trend of escape latency from day 15 to 20.

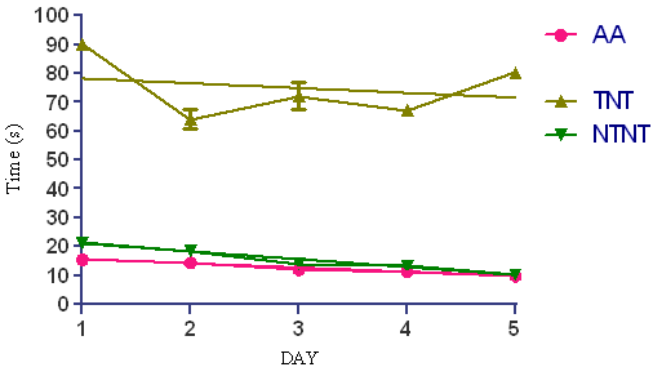

***Figure 1.** Effect of AA on learning in TBI induced rats. AA- Ascorbic acid, TNT- Traumatized Nontreated, NTNT – Non traumatized Non treated. (*p<0.05 versus control). (Mean±SD and n=7).*

Memory function in TBI rat treated with AA

In the Figure 2 (a and b), the result of the probe trial test in MWM showed that AA treated rats spent significantly (p < 0.05) longer duration in the target quadrant than the TNT rats (a). Similarly, the treated

group also had increased number of entries to the target quadrant compared to the TNT group (b).

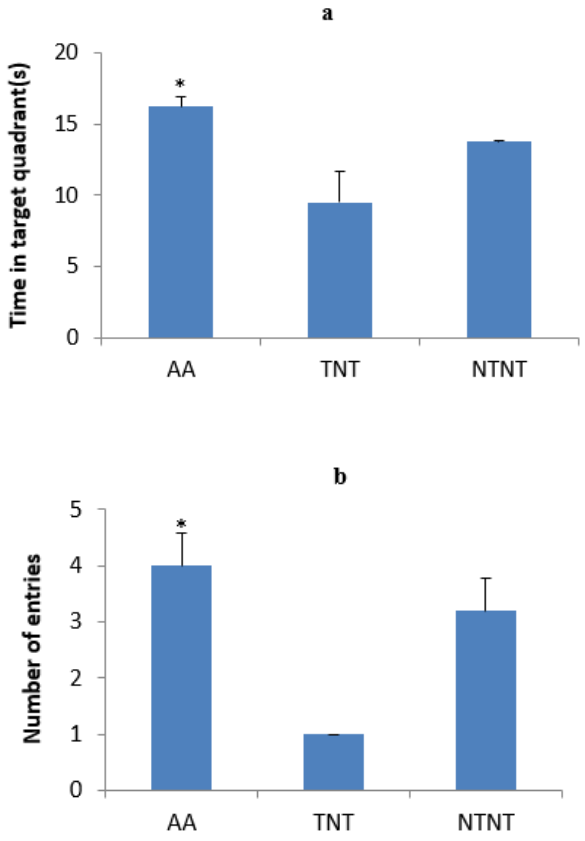

Figure 2. *Effect of AA on memory in rats induced with TBI; AA- Ascorbic acid, TNT- Traumatized Nontreated, NTNT – Non traumatized Nontreated. (*p<0.05 versus control). (Mean±SD and n=7).*

Locomotor activity in TBI rat Treated with AA

Locomotor activity was evaluated with open field test to compare distance travelled by rats in the open field box as presented in Figure 3. TNT group significantly ($p < 0.05$) travelled shorter distance compared to the NTNT group. However, in the AA treated group, the distance travelled is not significantly ($p < 0.05$) different from the NTNT group.

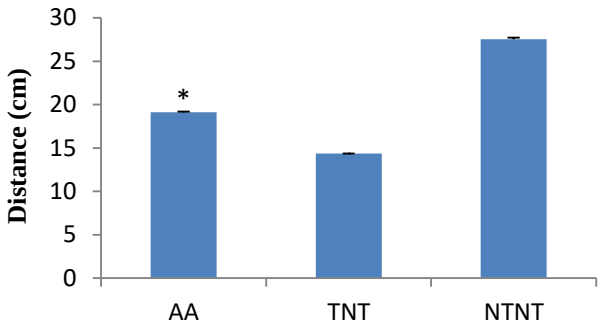

Figure 3. *Effects of AA on the locomotor activity in the rats with TBI; AA- Ascorbic acid, Traumatized Nontreated, NTNT – Non traumatized Nontreated (*p<0.05 versus control). (Mean±SD and n=7).*

Assessment of anxiety in TBI rat treated with AA

The Open Field Test was use to evaluate anxiety by comparing the center distance–total distance ratio between the experimental groups. The result presented in Figure 4 showed that the treated group had significantly ($p < 0.05$) higher ratio than the TNT group.

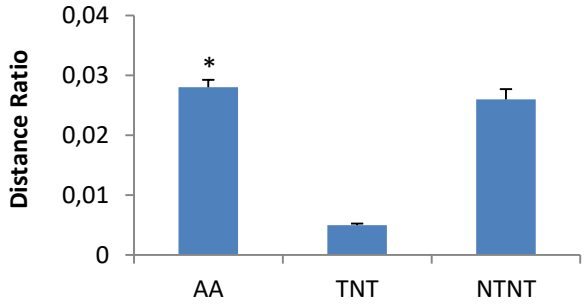

Figure 4. *Effects of AA on anxiety in TBI-induced rats. AA- Ascorbic acid, TNT- Traumatized Nontreated, NTNT – Non traumatized Nontreated. (*p<0.05 versus control). (Mean±SD and n=7).*

Estimation of TBI biomarker (S100B)

Estimation of TBI biomarker calcium binding protein (S100B) was carried out using ELISA technique in the cortex of the experimental rats as shown in Figure 5 The concentration of S100B in the TNT group was observed to be significantly ($p < 0.05$) higher compared to the NTNT group. The results in the treated group showed significantly ($p < 0.05$) lower concentration compared to the TNT group.

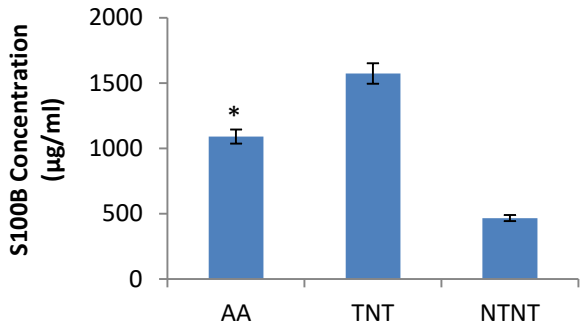

Figure 5. *Effect of AA on the S100B concentration in the brain cortex of TBI-induced rats. AA- Ascorbic acid, TNT- Traumatized Nontreated, NTNT – Non traumatized Non treated. (*p<0.05 versus control). (Mean±SD and n=7).*

Effect of AA on SOD activity in Serum and Brain cortex of TBI induced rats

The results presented in Figure 6 shows the activity of SOD in serum and brain cortex of TBI rats obtained at the end of the experiment. Both the serum and cortex activities of the enzyme were significant ($p < 0.05$) decreased in the TNT group compared to the NTNT group while the enzyme activities in the AA group were significantly ($p < 0.05$) increased compared to the TNT group. There was no significance ($p > 0.05$) in the level of the enzyme activities between the AA group and the NTNT group.

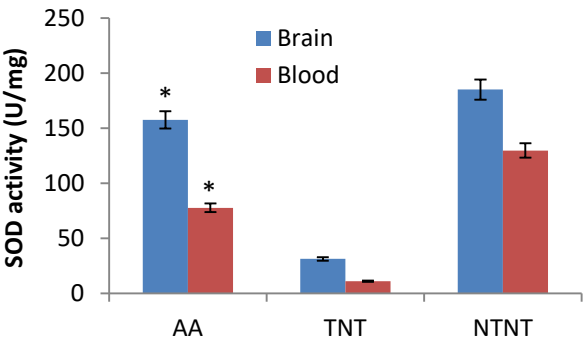

Figure 6. *Effects of AA on the SOD activity in the serum and brain cortex of TBI-induced rat. AA- Ascorbic acid, TNT- Traumatized Nontreated, NTNT – Non traumatized Non treated. (*p<0.05 versus control). (Mean±SD and n=7).*

Effect of AA on GPx activity in the serum and brain cortex of TBI-induced rats

Figure 7 shows the outcome of AA administration on the activity of GPx in the TBI-induced rats. A significant ($p<0.05$) decrease in the activities of the enzyme in the serum and cortex was observed in the TNT rats compared to both the NTNT rats and the AA treated rats. Comparing the result in the AA group to the NTNT group, there was no statistical difference ($p>0.05$).

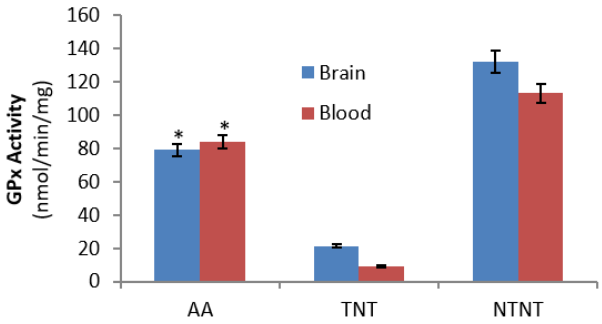

Figure 7. *Effects of AA on the GPx activity in the serum and brain cortex of TBI-induced rat; AA- Ascorbic acid, TNT- Traumatized Nontreated, NTNT – Non traumatized Nontreated * (p<0.05 versus control). (Mean±SD and n=7).*

Effect of AA on CAT activity in Serum and Brain cortex of TBI induced rats

There was a significant ($p<0.05$) decrease in the activities of the enzyme in the TNT group compared to the NTNT and the AA treated groups (Figure 8). However, no significant ($p>0.05$) difference was observed between the AA group and the NTNT group.

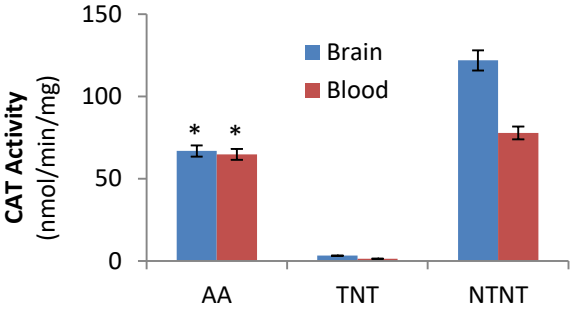

Figure 8. *Effects of AA on the catalase activity in the serum and brain cortex of TBI-induced rat. AA- Ascorbic acid, TNT- Traumatized Nontreated, NTNT – Non traumatized Nontreated * (p<0.05 versus control). (Mean±SD and n=7).*

Effect of AA on MDA level in serum and brain cortex of TBI-induced rats

The results in Figure 9 indicated significant ($P<0.05$) increase in levels of MDA in the serum and brain cortex of TNT rat compared to the NTNT rats. In the AA treated rats, the levels of MDA decreased significantly ($P<0.05$) compared to the TNT group. There

was no statistical difference in the MDA levels between the AA group and NTNT group.

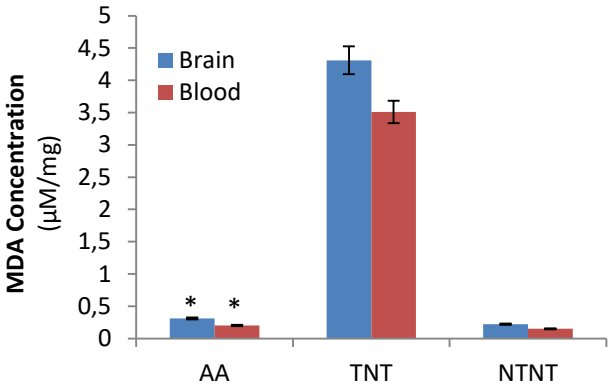

Figure 9. *Effects of AA on the MDA levels in the serum and brain cortex of TBI-induced rat. AA- Ascorbic acid, TNT-Traumatized Nontreated, NTNT – Non traumatized Nontreated * (p<0.05 versus control). (Mean±SD and n=7).*

Expression of SOD gene in the brain of Induced rats treated with AA.

Result showing the expression levels of SOD gene in Figure 10 below indicated up- regulation of SOD gene in AA treated rats. However, the fold change in the TNT group indicated down-regulation of SOD gene.

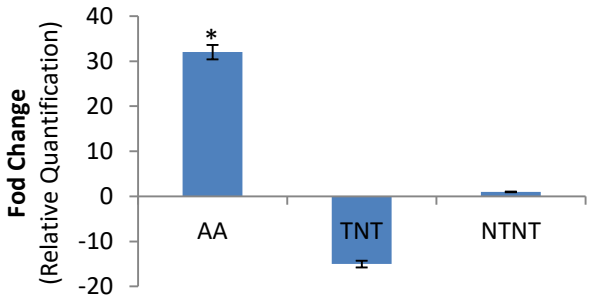

Figure 10. *Effects of AA on the SOD gene expression levels in the brain cortex of TBI-induced rat. AA- Ascorbic acid, TNT-Traumatized Nontreated, NTNT – Non traumatized Nontreated * (p<0.05 versus control). (Mean±SD and n=7).*

Expression of GPx gene in TBI rats treated with AA

Expression of GPx gene in the experimental rats is shown in Figure 11. In the AA treated group up-regulation of the gene was observed while in the TNT group the gene is down-regulated.

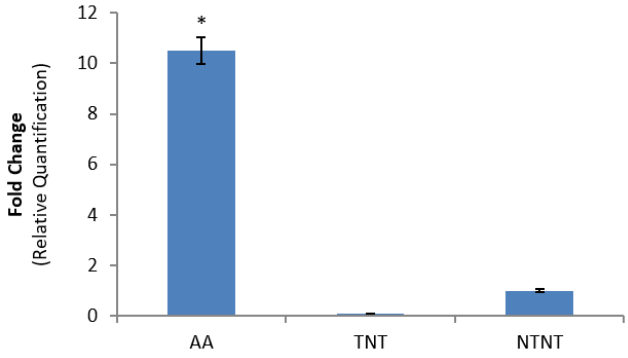

Figure 11. *Effects of AA on the GPx gene expression levels in the brain cortex of TBI-induced rat. AA- Ascorbic acid, TNT-Traumatized Nontreated, NTNT – Non traumatized Nontreated * (p<0.05 versus control). (Mean±SD and n=7).*

Expression of CAT gene in TBI rats treated with AA

Expression of the CAT gene shown below (Figure 12) indicated up-regulation of the gene in AA treated rats and down-regulation in the TNT rats

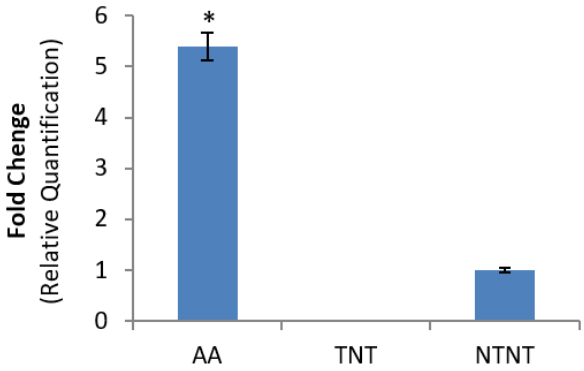

Figure 12. *Effects of AA on the CAT gene expression levels in the brain cortex of TBI-induced rat. AA- Ascorbic acid, TNT-Traumatized Nontreated, NTNT – Non traumatized Nontreated * (p<0.05 versus control). (Mean±SD and n=7).*

Histology

Plate 1 show that the photomicrographs taken from the histology slides (H and E) of brain tissues of all the groups. From the result of the NTNT group, normal neuronal cell appearance, layers and brain architecture were seen indicating absence of injury or abnormality. The tissue slides of the TNT group showed inflammation, necrosis, congestion and hemorrhage. These lesions were however not seen in the group treated with ascorbic acid.

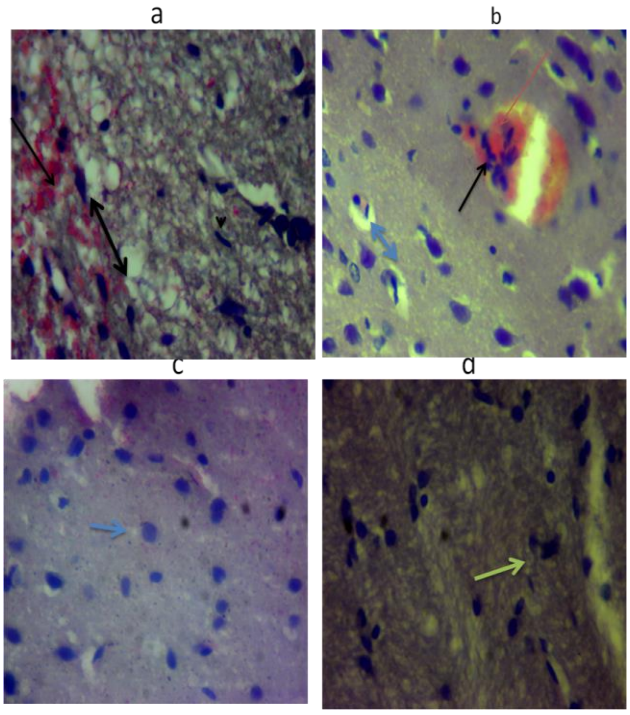

Plate 1(a – d). *Photomicrograph of the brain (cerebrum) of (a)TNT group showing spongiosis (double head arrows), haemorrhages in the neuropil (long arrows) and necrosis of the neurons (arrow heads), (b) TNT group showing vacoulation (double head arrow), congestion (red arrow) and inflammation (black arrow) (c) Normal rats showing normal architecture of the cerebrum, (d) AA treated rats showing normalized cells H and E, X40 TNT-Traumatized non treated, AA-Ascorbic acid*

Plate 2 indicated the photomicrograph of the cerebellum of rats stained with cresyl violet (Nissl stain). The purkinje cell layer of the cerebellum is distorted and cell lost were observed in the TNT rats. These lesions were reversed in the ascorbic acid treated rats as undisturbed cell layers were observed.

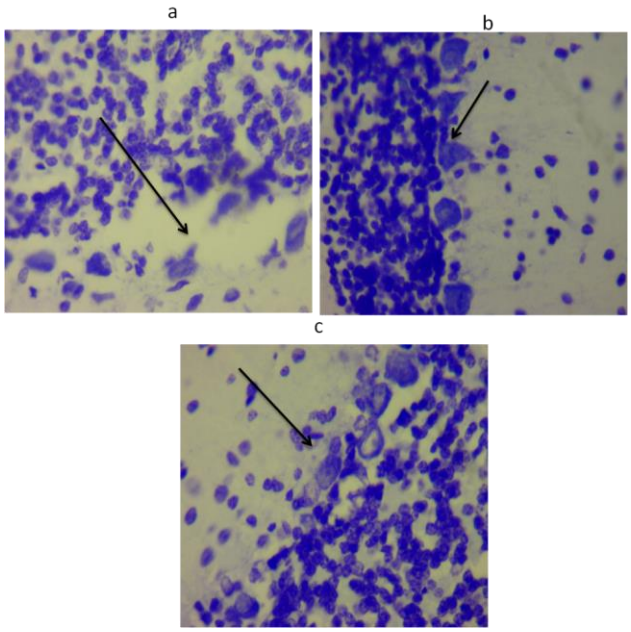

Plate 2. *Photomicrograph of the cerebellum of TBI and Normal rats; (a) TNT group showing loss of Purkinje cells (arrows) (b) Normal rats showing normal architecture of the cerebellum (c) AA treated rats showing undisturbed Purkinje cells layer TNT- Traumatized non treated, AA-Ascorbic acid, NTNT-non traumatized non treated Nissl stain, X40*

The photomicrograph from the silver nitrate stained (Bielchowsky stain) (plates 3) histological slides indicates the presence of axonal swelling (bulb) and axonal breaks in the TNT group.

Histology of the hippocampus was studied using Nissl stain (Plate 4).The photomicrograph taken from the slides indicated that the pyramidal cell layers of the hippocampus were distorted and cell lost were observed in the TNT rats. These lesions were reversed in the ascorbic acid treated rats as undisturbed cell layers were observed.

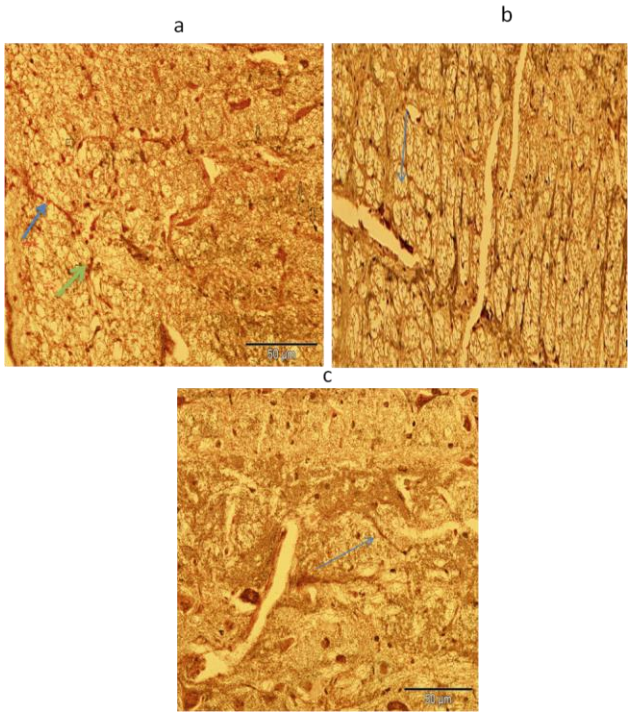

Plate 3 *Photomicrograph of the cerebrum of TBI and normal rats stained with Bielchowsky stain (a) TNT rats showing axonal bulb (Thick arrow) and axonal break (Thin arrow), (b) NTNT showing normal axon (arrow), (c) AA group showing axonal bulb (blue arrow) TNT- Traumatized non treated, AA- Ascorbic acid, NTNT-non traumatized non treated (Bielchowsky stain X40).*

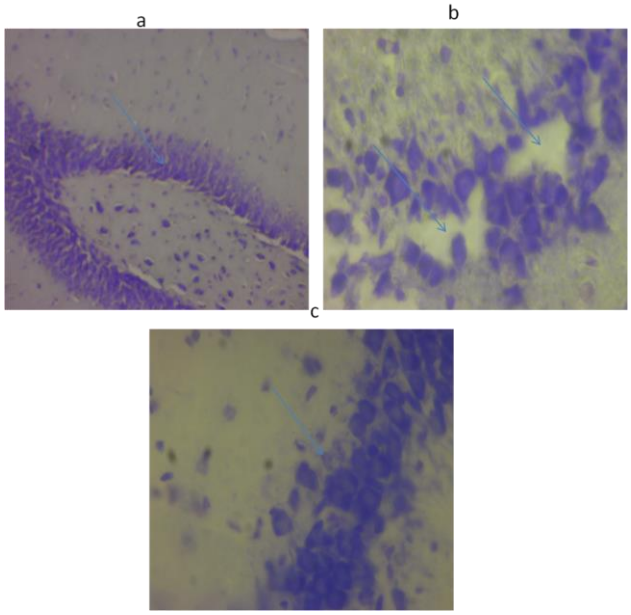

Plate 4 (a-d) *showing the photomicrograph of the hippocampus of the normal rats showing with intact cell layers(a), TNT rats showing distorted layers with cell lost (b), and AA treated rats showing restored cell layer(c). TNT – traumatized – non treated, AA– Ascorbic acid, Nissl stain (×40)*

Discussion

The findings of this study suggest that TBI by impact acceleration method in the rat induces a pronounced memory deficit which is attenuated by AA. This shows that AA has a promoting effect on memory task and increased learning ability in comparison with the TNT group. Other studies have reported similar results; one of our studies on the oral supplementation of AA indicated that it could attenuate memory deficit in TBI induced rats (Bulama et al. 2017). Two different studies also indicated that AA could be useful in retention of memory in rat model of Alzheimer's disease (Olajide *et al*. 2017) and memory impairments in Colchicine treated rats (Sil, *et al.* 2016).

The result of this study and those cited support the neurophysiologic role of AA. Prevention of the spatial memory deficits in rodents accompanying TBI induction has been targeted at inhibiting the secondary injury cascade to restore the lost memory (Walker and Tesco 2013)**.** AA is involved in protecting mitochondria from damage and preserved its function (Shahidi *et al*. 2008). This could be one of the possible mechanisms of AA on promoting learning and memory processes. AA scavenges ROS and prevent lipid per oxidation occurring in the brain. Oxidative damage of the hippocampus which has an important role in learning and memory processes can occur during the secondary injury. Therefore, AA might have protected the hippocampus and preserved its function. AA can modulate neurotransmitters such as cholinergic system which has an essential role in learning and memory processes.

The result of the open field test (Figure 3) indicated that rats in the TNT group travel short distance in the open field. This decreased distance travel indicates motor deficit due to TBI. TBI causes loss of motor function due to impact of secondary injury on motor control areas such frontal cortex and deregulation of neurotransmitters as reported by Dasuni et al. (2013). However, all the treated rats have increased total distance travelled in the open field apparatus. AA is involved in the synthesis and release of neurotransmitters (Shah et al. 2015) such as dopamine (Chen et al. 2017) and Gamma aminobutyric acid (GABA) (Calero et al. 2011) which are excessively release during TBI. Neurotransmitters are involved in regulating motor activity and their increase activation causes motor deficits (McAllister 2011). The scavenging and neutralizing effect of AA, on ROS, can prevent the

motor control areas of the brain from free radical damage and loss of motor function.

The result from the OFT (Figure 4) indicated that, TNT rats significantly have decreased central distance total distance ratio in the open box. This anxiety like behavior was abolished by AA indicating increased distance ratio in the treated rats. There are many studies showing increased ROS in anxiety (Guney et al. 2014). This study also supports this fact since AA treatment has attenuated anxiety that was observed in the TNT group. The antioxidant effect of AA could be the reason for this.

On the other hand, disruptions of neurotransmitters like GABA, serotonin and noradrenalin as well as an impairment of the hypothalamic-pituitary-adrenal axis regulation are the major pathophysiology in anxiety disorders. Several studies have shown that patients with anxiety have vitamin c deficit. AA has been found in large amount in brain structures associated with anxiety such as amygdala (Mefford et al. 1981; Millby et al. 1982). It has inhibitory effect on N-methyl D- aspartate (NMDA) receptor (Figueroa-Méndez and Rivas-Arancibia 2015) and normalizes the level of serotonin in brain (Puty et al. 2014). Thus, it can serve as a modulator of the behaviors linked to these neuro transmitters (Hansen et al. 2014). The role of AA on hypothalamic pituitary adrenal axis is reflected in its ability to attenuate cortisol activitby (de Oliviera et al. 2015). Our result has been in consistent with the literature (de Oliviera et al. 2015) that the administration of vitamins C decreases anxiety.

Evidence supporting the involvement of OS mediated by reactive oxygen species (ROS) as a contributory factor to the pathology of TBI is accumulating; several findings substantiate the susceptibility of the brain to OS (Chen et al. 2017).

Oxidative stress in this study was evaluated by measuring the levels of SOD, CAT, GPx and MDA as indicators of enzymatic antioxidant activity and lipid peroxidation respectively. The significant decrease in the activities of SOD, CAT, GPx and increase concentration of MDA observed in the brain tissue homogenate of TNT rats as compared to NTNT rats (Figure 5-9) suggest the occurrence of OS due to the induced TBI. Supplementation with AA ameliorated the induced OS (Figure 5-9). This is shown in the group treated with AA which has significantly increase ($P< 0.05$) activities of SOD, CAT and GPx compared to TNT group (Figure 5 – 9). Concentrations of MDA have been reduced to the level of the NTNT group indicating that lipid peroxidation could be normalized by AA.

This might be due to the ability of AA to quench free radicals and reduce their oxidative activity on lipids, proteins and nucleic acid which leads to the suppression of the antioxidant system and accumulation of MDA. It can also be due to the regeneration of vitamin E and glutathione which are very effective against ROS. Findings of this work are in agreement with the result of Devi et al. (2007), WHO reported that ascorbate is highly efficient in trapping free radicals, and preventing them from forming lipid hydroperoxide that can be generated during TBI.

The result of S100B observed in this study indicates that there is persistence of injury in the TNT group evidenced by the high concentration of the protein. In the AA treated rats, the protein level was observed to decrease significantly compared to the TNT rats. This indicates that the injury has reduced and the release of the protein is inhibited. S100B has been known to be a reliable biomarker of TBI owing to its release by activated glial cells during injury. It has been correlated with injury severity in hospitalized human patients (Hendoui et al.2013). Its function is dose dependant with low level supporting neuronal growth and survival. On the other hand, high level can stimulate inflammatory injury (Reali et al. 2005). S100b has cytokine like activities and can interact with the receptor for advance glycation end product (RAGE) (Mori et al. 2008). Therefore, the decreased concentration of S100B observed in the AA treated rats could be due to its anti-oxidative damage by ROS which damage glial cell membrane and release S100B. AA also has anti-inflammatory effect which can prevent astrocytic damage and activation due to inflammatory mediated damage by ROS.

The result of the gene expression studies revealed that the antioxidants enzymes genes (SOD, GPX, CAT) were up-regulated in the treated groups and down-regulated in the TNT group. This is in agreement with the increased expression of the enzymes in the treated rats and decreased expression in the TNT rats recorded. This can be explained by the fact that antioxidants enzymes are suppressed by oxidative stress and the compensatory mechanism which is boosted by AA in this case. ROS can alter gene expression in various stages of protein

synthesis. They can operate as signal molecules in the modulation of gene expression (Palmer and Paulson, 1997). Conversely, antioxidants can obstruct or attenuate cellular signaling by ROS. ROS can react with nucleic acid affecting gene expression which can be prevented by antioxidants. Antioxidants can increase gene expression by activating transcription factors (Susana *et al* 2013). Transcription factors such as nuclear factor erythroid related factor (Nrf2), nuclear factor kappa B (NF-kB) and AP-1 are activated by antioxidants (Müller et al. 2003). These factors subsequently activate antioxidants genes transcription and increase their expression. Nrf2 once activated, binds to the antioxidant responds element (ARE) located at the regulatory regions of the antioxidant enzymes genes and induce transcription of the antioxidant genes (Tonelli *et al*. 2018). Therefore, these could be the possible mechanism for the increased expression of the antioxidants enzymes genes by AA observed in this study.

The photomicrographs of the brain tissue slide from the TNT group indicated the occurrence of injury on the brain ranging from rupture and sloughing off of the meninges to congestion, hemorrhage and inflammation. These lesions were observed in the cerebrum, cerebellum and meninges indicating the induction caused diffuse brain injury. Slough off and ruptured meninges observed were indicative of primary injury while inflammation indicates secondary injury process as describe by Andre et al. (2014). These lesions observed in the TNT were however not observed in the group treated with AA possibly indicating normalization of the brain tissue

The hippocampus is a vital brain section that assists in a number of essential functions, such as cognition, learning and memory. In several animal models, such as hypoxia and ischemia, the hippocampus has high vulnerability to injury because of the over-activation of glutamate receptors and caspases (Shah et al. 2015). Therefore, the hippocampal lesion observed in this study (Plate 4) might be responsible for the impairment in memory and learning exhibited by the TNT rats and the protective effect of AA may perhaps be responsible for the enhancement of learning and memory seen in the treated group.

Axonal swelling is the major characteristic of diffuse brain damage due to TBI and causes severe disability in TBI patient. Rapid acceleration/deceleration induces shearing forces on the axons which cause stretching and tearing damage. Impaired transport of axonal protein and intracellular protein buildup at the point of axonal breakage forms retraction bulbs/swelling (Risdall and Menon, 2011), Beta amyloid precursor protein (β-APP) accumulates at the point of axonal severance (axonal bulb), indicating impaired axonal transport post-TBI (Lifshitz et al. 2007). Axonal injury is associated with impairment in memory, sensorimotor and learning functions (Sidaros et al. 2007).

In this work, axonal swelling and axonal break were observed (Plate 3) in the TNT group while restoration of the injury was observed in the AA treated group. The deficit in learning, memory and motor functions observed in the TNT rats which have been attenuated by the intervention may as well be due to the axonal injury.

From the study it can be concluded that induction of traumatic injury by weight drop method causes neurological deficits and oxidative stress. However, treatment with AA improves functional recovery in rats after TBI, enhanced antioxidant defense system, suppresses lipid peroxidation, reduced oxidative stress and neuropathology. These neuro-therapeutic effects can be as a result of the enhanced antioxidant defense which is mechanistically associated with the up-regulation of the SOD, GPx, and CAT genes. These promising results suggest that antioxidants supplementation can be a useful neuro-therapeutic strategy in targeting secondary injury of TBI

Acknowledgement

The current sudy was performed at Department of Human Anatomy, Collage of Health Science, University Putra, Salengor, Malaysia

Conflict of interest

The authors declare that there is no conflict in the current study.

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