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Role of acamprosate in traumatic brain injury: A study in *Drosophila melanogaster* using high impact trauma model

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Abstract
Traumatic brain injury is major neurodisorder mainly caused by road accidents, sports related events or any violence. This study was used to determine the role of acamprosate in traumatic brain injury. High impact trauma device used to induce head injury in *Drosophila melanogaster*. Behavioral and biochemical parameters were studied to evaluate the pharmacological potential of acamprosate in head injury. Acamprosate was found to be effective results in decreasing mortality. Our results suggest that acamprosate show antioxidant activity as it decreases the level of MDA and NO and increase the level of SOD and catalse.

Keywords: *drosophila melanogaster*, acamprosate, high impact trauma device, and head injury

1. Introduction
Traumatic brain injury (TBI) is major cause of death and disability in children and young adults has been identified as an important public health problem in the worldwide [1]. It is a result of an outside force causing immediate mechanical disruption of brain tissue and delayed pathogenic events which collectively mediated widespread neurodegeneration [2]. There are two stages in TBI: first occur at the moment of impact, exclusively sensitive to preventive, that is primary injury then pathological process initiated at the moment of injury with delayed clinical presentation, sensitive to therapeutic interventions, that is secondary injury [3]. The prevalence of patients with TBI in India is estimated to be 1.6 million, out of which 200,000 die due to TBI in each year [4]. Approximately, 2 million persons suffer TBI in united state annually and out of these about 70,000 to 90,000 have permanent long term disability, creating a socioeconomic and emotional burden on the families and society [1].
On the behalf of many achievements in neurodegenration field in the past decades we have used *Drosophila melanogaster* in the current study. Response of flies to many drugs that act on the CNS is similar to the effect observed in CNS in mammalian systems [5].
Here, we evaluated the pharmacological potential of acamprosate in high impact trauma model in *Drosophila melanogaster*. Acamprosate (N-acetyl homotourine) is NMDA receptor modulator approved by FDA in 2004 as pharmacological treatment for alcohol dependence [6]. Also in different study acamprosate was shown effective results in various CNS disorders. It inhibit nitric oxide synthase [7], exhibit anti-inflammatory activity by reduction of TNF-α level in blood [8], and found to block voltage gated calcium channel [9]. So here we hypothesized that acamprosate may have pharmacological potential in TBI.

2. Materials and Methods
- **Fly strain:** Oregon R^+ strains were used for the proposed studies which have obtained from *Drosophila* stock center, Mysore (India). The flies were reared on a standard food medium containing cornmeal, yeast, agar, sugar and added propanoic acid as antimouldant (Standard Drosophila Medium = SDM) and maintained at 25ºC on natural light/dark cycle in glass bottles. Flies of either sex were used in the studies.
- **Drug treatment:** Fifteen days old flies were placed in empty vial for 18h starvation before drug treatment then flies were placed in standard food medium supplemented with acamprosate (20, 100, and 200µg/ml) for 24h. The control and vehicle group flies were fed on standard food medium.

Establishment of high impact trauma model in laboratory
High impact trauma (HIT) device reproducibly inflicts closed head traumatic brain injury in flies. It consists of metal spring clamped at one end on a wooden board with the free end positioned over a polyurethane pad [10].
A standard plastic vial containing unanaesthetized flies that are confined to the above quarter of the vial by stationary cotton ball were connected to the free end of the spring (Fig 1). After that 1-10 hit were given to unanaesthetized flies by deflecting the spring from 90º with an interval of 5min between each hit on the behalf of standardization of model in our laboratory followed by mortality index data.

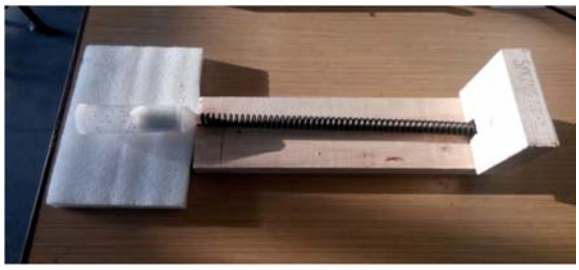

Fig-1: High Impact Trauma Device

Incapacitated flies data

Number of incapacitated flies was observed under microscope after injury at 4h and 6h.

Climbing assay

Locomotor activity was determined by using negative geotaxis assay as described by Bland *et al.*, with minor modifications. To access the locomotor activity on vertical climbing single fly in empty glass vials were placed without medium for an hour. The flies were gently tapped to the bottom of the vial to stimulate a negative geotactic climbing response and the time required to climb up 8cm of the vial wall was recorded. Each fly were tested 4 times at 1 minute intervals [11]. For each experiment, climbing mean was calculated. Climbing assay was performed at 4h and at 6h after injury.

Mortality index

Mortality of flies was examined at 4-6h and at 24h after TBI. Number of dead flies was recorded. Then mortality index was calculated.

At 6h of injury, legs and wings of all flies were separated from the body with sharp edge of blade and then homogenized in sodium phosphate buffer (0.1M, pH8.0) and was centrifuged at 2500g for 10min at 4^0C. The supernatant was filtered through nylon mesh and was used for following biochemical parameters.

Total protein content [12]

The principle behind the Lowry method of determining protein concentrations is the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin Ciocalteau phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10-10.5. The phenolic group of tyrosine and trytophan residues (amino acid) in a protein was produce a blue purple colour complex, with maximum absorption in the region of 660nm wavelength.

Superoxide dismutase (SOD) estimation [13]

Superoxide dismutase activity was assayed according to the method of Kono *et al.,* where in the reduction of nitazoblue tetrazolium (NBT) was inhibited by the superoxide dismutase is measured at 560nm using UV/visible spectrophotometer. Briefly, the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and tissue homogenate. The results were expressed as units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50%.

Catalase estimation [14]

In this method, dichromate in acetic acid will be reduced to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of per chromic acid as an unstable intermediate. The reaction mixture (1.5ml vol.) was containing 1ml of 0.01M (pH-7.0) phosphate buffer, 0.1ml of tissue homogenate (supernatant), and 0.4ml of 2M hydrogen peroxide. The reaction was stopped by the addition 2.0ml of dichromate acetic acid reagent (5%potassium dichromate and glacial acetic acid will be mixed in 1:3 ratio). Catalase was assayed by colorometrically at 620nm.

Malondialdehyde (MDA) estimation [15]

Lipid peroxidation is a free radical mediated event. The primary products of such damage are a complex mixture of peroxides which then breakdown to produce carbonyl compounds. The malondialdehyde (MDA) is one such carbonyl compound, which forms a characteristic chromogenic adduct with two molecules of thiobarbituric acid to give a pink colour, the absorbance of which were determined at 540nm. The colorimetric reaction of TBA with MDA, a secondary product of lipid peroxidation has been widely accepted for measuring lipid peroxidation. It is also known as TBARS (Thiobarbituric acid reactive substance) estimation.

Nitric oxide (NO) estimation [16]

The presence of NO was assayed colorimetrically by greiss reagent. The supernatants (100µl) collected was mixed with an equal volume of greiss reagent and left for 10min at room temperature. The absorbance of the reaction was measured at 550nm against suitably prepared blank solution (100µL of distilled water was be used). The amounts of NO produced was determined by calibrating a standard curve using sodium nitrite.

3. Results

Incapacitated flies data

In vehicle group, number of incapacitated flies were observed to be more as a compared to treated group (Fig 2). Acamprosate was found to be decreased percentage incapacitation at different concentration. The number of incapacitated flies were found to be significantly decreases in Ac 100 and 200 ($p<0.01$) groups (Fig 2a). Acamprosate was found to be decreasing the number of incapacitation flies at 6h of injury but not significantly.

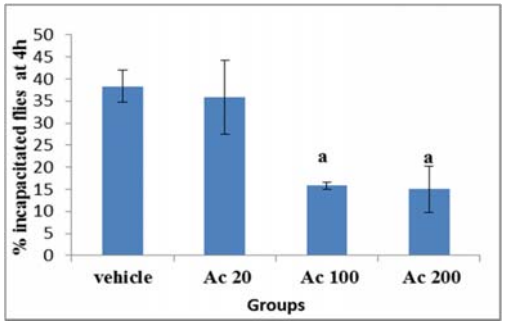

Fig 2: The figure shows effect of acamprosate on % incapacitation, of flies at 4h and 6h after injury. Values were expressed as Mean ± SEM. Data was analysed by one way ANOVA followed by turkey test.

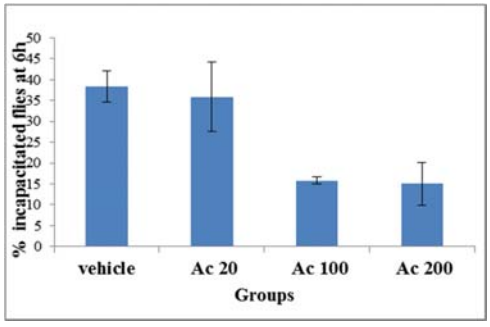

Fig 2a: P<0.01 vs vehicle. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Climbing assay

In vehicle group no fly was climb in 10 sec at 4h compared to control group. At different concentration of drug percentage climbing of flies was improved. Injured flies take more time to climb while at different drug concentration more flies climb in 10sec and in 30sec and the no. of flies climbs in 60sec got decreases. At 6h, in vehicle group percentage climbing of flies were improved compared to 4h. At 4h Ac 100 and 200µg/ml ($p<0.001$, $p<0.01$) concentrations were found to be significantly increasing % climbing in 10sec as compared to vehicle group. Same results were observed in 30sec climbing. At 6h Ac 100 and 200µg/ml concentrations were found to be significantly increasing % climbing in 10sec and 30sec ($p< 0.01$, 0.01 and $p<0.05$, $p<0.05$) respectively as compared to vehicle group. Acamprosate doesn't show any significant effect on % climbing in 60sec (Fig-3).

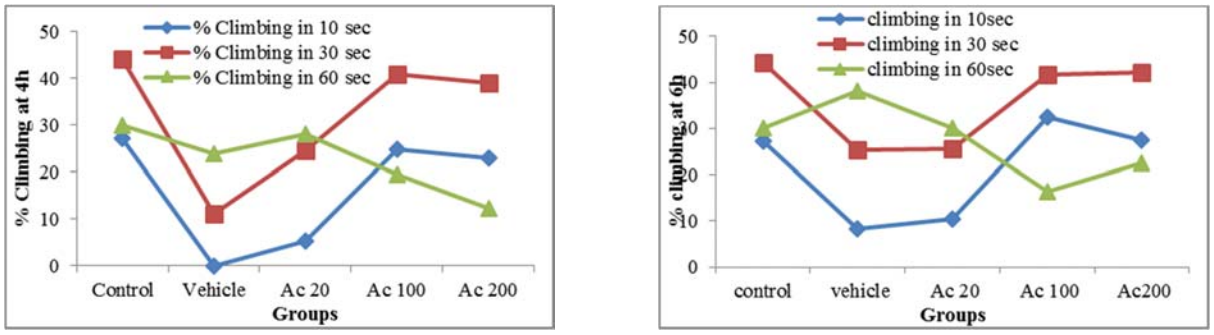

Fig 3: The figure shows effect of acamprosate on climbing response of flies in10, 30, and 60 sec at 4h and 6h after traumatic brain inury. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by Tukey test. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Effect of acamprosate on percentage mortality

Mortality index

After injury, the mortality of flies was started at 4-6h. In vehicle group, 14.16% mortality was shown. The percentage mortality reduces at 4-6h with respect to different drug concentrations. Maximum mortality was observed at 24h after injury which was 63.5 % in vehicle group. Acamprosate 200µg/ml ($p<0.05$) shows significant reduction in mortality at 4-6h (fig-4a). Mortality at 24h was significantly decreased by acamprosate at 100 and 200µg/ml ($p<0.01$, 0.01 respectively) concentrations (fig-4b).

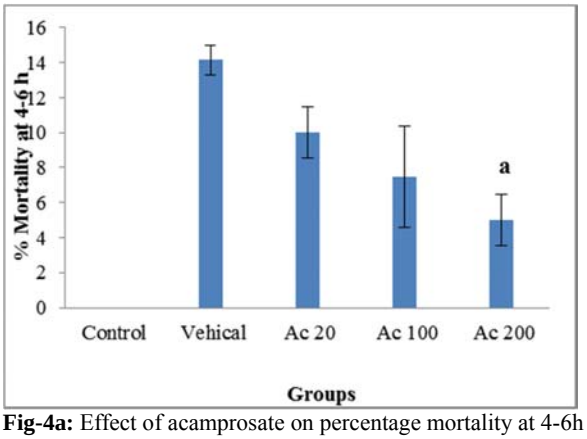

Fig-4a: Effect of acamprosate on percentage mortality at 4-6h a. $p<0.05$ vs vehicle. The F-value was found to be F (4, 10) F=10.711.

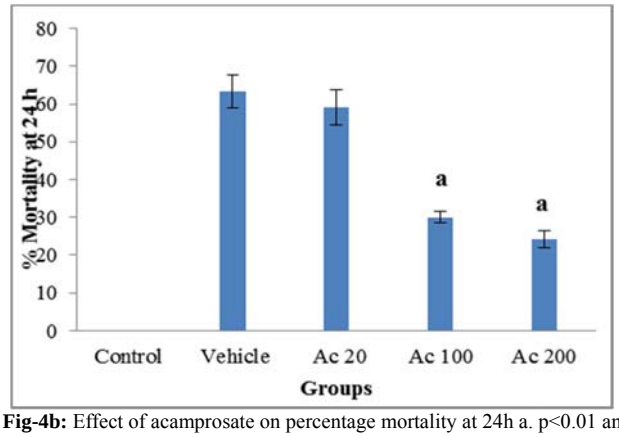

Fig-4b: Effect of acamprosate on percentage mortality at 24h a. $p<0.01$ and $p<0.001$ vs vehicle. The F-value was found to be F (4, 10) F = 72.078. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Fig 4: The figure shows effect of acamprosate on mortality response of flies. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by tukey test.

Effect of acamprosate on level of total protein content

In traumatic brain injury, total protein content level decreases. In present study, it was found to be protein level in vehicle group was significantly decreased as compared to control group. Acamprosate does not shown any significant effect on total protein content level (fig 5a).

Effect of acamprosate on activity of superoxide dismutase

SOD is an enzyme that catalyses partitioning of superoxide radical into hydrogen peroxide. Figure 5(b): After injury, SOD level showing decreased in vehicle group as compared to control group ($p<0.01$). Acamposate 100 and 200µg/ml ($p<0.05$) was shown significant increase in level of SOD compared to vehicle group. Acamprosate 100 and 200µg/ml shows non-significant result vs control. It was shown in our result Ac 100 and Ac 200µg/ml enhances the level of SOD near about to level in control group.

Effect of acamprosate on level of catalase

Catalase is an enzyme which catalyzes the decomposition of hydrogen peroxides to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive

oxygen species. In tramatic brain injury, there is depletion of this antioxidant enzyme. Figure 5(c): The catalase level was found to be decreases in vehicle group compared to control group ($p<0.001$). Acamposate 100 and 200µg/ml ($p<0.01$) was shown significant increase in catalase level compared to vehicle group while when compared to each other no significant effect was observed between acamprosate treated group.

Effect of acamprosate on level of malondialdehyde (MDA)

MDA is a naturally occurring product of lipid peroxidation; lipid peroxidation is a free radical event. Figure 5(d): After injury, MDA level was found to be increased in vehicle group as compared to control group. Changes in MDA level in flies and its modulation by acamprosate was recorded. Acamposate 100 and 200µg/ml ($p<0.05$, $p<0.05$ respectively) show significant reduction compared to vehicle group while when compared to each other no significant effect was observed between acamprosate treated group.

Effect of acamprosate on level of nitric oxide

Under normal physiological conditions, the level of nitric oxide is low. However, excessive NO can bind with superoxide and formed peroxynitrite which is highly toxic to proteins and membrane lipids. Figure 5(e): After injury, NO level was found to be increased in vehicle group as compared to control group ($p<0.01$). Acamposate 100 and 200µg/ml ($p<0.05$, $p<0.05$ respectively) show significant decrease in nitric oxide level compared to vehicle group while when compared to each other no significant effect was observed between acamprosate treated group.

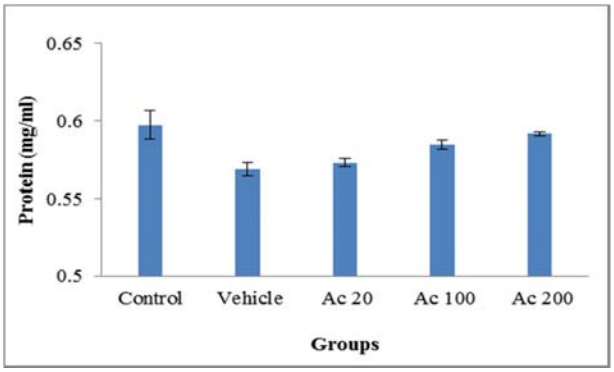

Fig-5a: Effect of acamprosate on level of total protein content. Acamprosate 20, 100 and 200µg/ml.

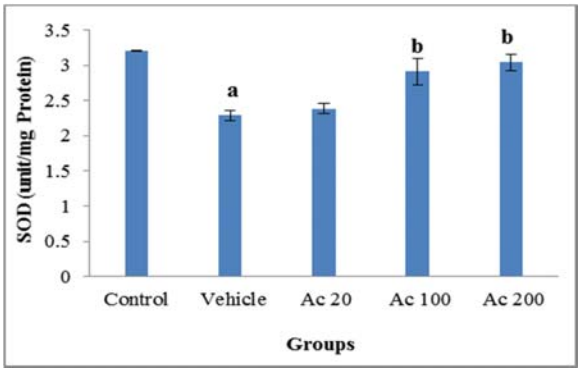

Fig-5b: Effect of acamprosate on activity of superoxide dismutase (SOD) level a. $p<0.01$ vs control, b. $p<0.05$ vs vehicle, vehicle. The F-value was found to be F (4, 10) F=13.986.

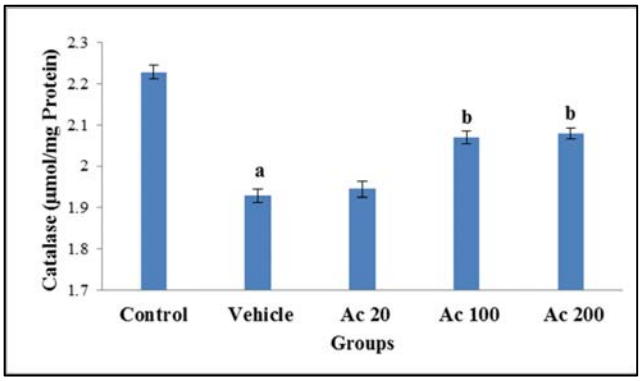

Fig-5c: Effect of acamprosate on level of catalase a. $p<0.001$ vs control, b. $p<0.01$ vs vehicle. The F-value was found to be F (4, 10) F = 64.898.

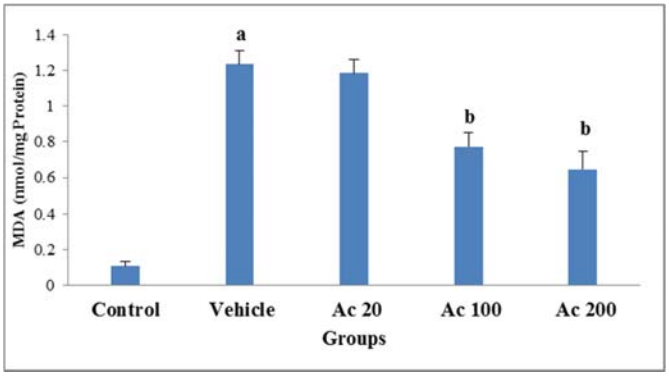

Fig-5d: Effect of acamprosate on level of malondialdehyde (MDA) a. $p<0.001$ vs control, b. $p<0.05$ vs vehicle. The F-value was found to be F (4, 10) F = 36.340.

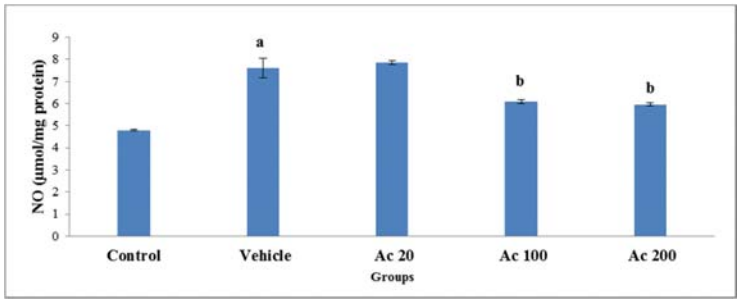

Fig-5e: Effect of acamprosate on level of nitric oxide (NO) a. $p<0.01$ vs control, b. $p<0.05$ vehicle, The F-value was found to be F (4, 10) F = 36.504. . Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Fig-5: The figure shows effect of acamprosate on biochemical parameter in flies' homogenate. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by tukey test.

4. Discussion

Drosophila has already proved to be an extremely useful model for studying human neurodegenerative disorders. The inflicting mechanical injury on flies by rapid acceleration and deccleration produces outcomes that are similar to outcomes characterstics of closed head traumatic brain injury in humans [10]. Closed head traumatic brain injury is most common form of traumatic brain injury. For that reason we choose high impact trauma device which reproducibly inflict closed head traumatic brain injury in flies. We establish high impact trauma device in our laboratory according to laboratory conditions. We facilitated three springs with different spring strength, we observed the mortality index data using these springs at different strikes. On the basis of MI_{24}, we select 10 hits at 5min time interval with impact force 3.65Nm. From the observation data, we were observed that the MI_{24} and MI_{48} values have not shown any significant difference so we were proceed our study by estimating the mortality index parameter at 24h. At single strike, the temporary incapacitated flies fell to the bottom but did not contribute to any external damage like head, body and appendages most of incapacitated flies were recovered in 5min interval. We examined that recovery of incapacitated flies was initiated within 4h after 10 hits.

During hit, we observed the legs and wings of flies were totally affected firstly from the whole body in which leg shivering, wings broadening are commonly observed. Due to affected legs after injury most of flies were failed to climb or took more time to climb up. This shivering and broadening effect of legs of *Drosophila melanogaster* affect the climbing percentage directly.

The death from exceed threshold primary injury was observed to be complete by 24h because the percent survival at 24h was not substantially different from the percent survival at 48h. Additional strikes increases mortality at mortality index at 24h but not significantly. If the primary injury exceeds to specific threshold it causes mortality within 24h [10]. In present study, we observed that the immediate death of flies was only 2 or 3 for that reason we were not consider them in our result data. The death of flies due to secondary injury started at 4-6h and maximum death was observed at 24h.

Oxidative stress play a significant role in neuronal death

following injury due to the progressive compromise of endogenous antioxidant (such as SOD and catalase) defence system. The neutralization of reactive oxygen species by endogenous or exogenous antioxidant has a protective effect on the brain [17]. After injury, non ischaemic events occur such as the increase of intracellular free calcium concentration (through receptor such as glutamate and ion channels) may also induce release of oxygen free radicals from mitrochondria. Stimulation of enzymetic activities of COX, monoamine oxidase, nitric oxide synthase can produce free oxygen radicals. The highly reactive oxygen free radicals can cause damage by lipid peroxidation in cell membrane and oxidation of intracellular proteins and nucleic acids. TBI may activate phospholipase A2 and C to hydrolyze membrane phospholipids releasing arachidonic acid. Formation of pathogenic compounds such as free fatty acids from the arachidonic acid cascade has been found in experimental TBI [18]. Free radical acting on polyunsaturated fatty acid leads to the formation of highly reactive aldehydes including MDA which are the most aboundant product. Reactive aldehydes are noxious byproduct of lipid peroxidation. Lipid peroxidation causes damage to biological membrane and neurons [19]. The peak level of endogenous molecule appears at 24h. The endogenous molecules are responsible for the activation of innate immune response by stressed or injured cells. The production of proinflammatory cytokines, such as TNF is a component of this response. Antimicrobial gene (AMP) is responsible for TNF-α production. In previous study, it was observed that AMP gene was activated after 1h of primary injury and reaches to maximum level within 24h [10]. In present study we observed that acamprosate significantly reduces the level of nitric oxide. It was previously demonstrated that acamprosate inhibit NADPH-diapharase which are responsible for nitric oxide synthesis [7]. Present, result had shown that acamprosate significantly improving the endogenous antioxidant system by increasing the level of catalase and SOD. As acamprosate inhibit nitric oxide synthesis further level of MDA was also observed to be decreased because peroxynitrite or free radical formed from nitric oxide further cause lipid peroxidation.

Our drug is a NMDA modulator which inhibits the receptor in its overactive state [20]. As excitotoxicity is major problem in head injury all oxidative damage are started from after excitotoxicity [21]. So our findings suggest that acamprosate shows its action through NMDA modulator activity.

5. Acknowledgements

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