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Role of acamprosate in anoxia-reoxygenation injury in *Drosophila melanogaster*

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Abstract
Anoxia damage is major cause of morbidity and mortality in humans. In anoxia, oxygen supply decreased relative to metabolic demand. The response of *Drosophila* flies to many drugs that act within the central nervous system is similar to the effect observed in mammalian tissue. So, here we evaluate pharmacological potential of acamprosate in anoxia-reoxygenation (AR) injury in *drosophila melanogaster*. Anoxia reoxygenation injury was induced by introducing the pure nitrogen to the vial containing flies then flies were placed in room air for reoxygenation. Acamprosate shows survival advantage against AR injury. Acamprosate was observed to be protective against mortality due to anoxia reoxygenation injury at three concentrations 20, 100 and 200µg/ml. It was observed from our results that acamprosate increased the level of endogenous antioxidants (e.g. superoxide dismutase and catalase) and decrease the lipid peroxidation and level of nitric oxide.

Keywords: *drosophila melanogaster*, acamprosate, anoxia reoxygenation, cerebral stroke

1. Introduction
Stroke is the second most common cause of death worldwide (Mathers *et al*. 2009) [16]. Approximately 15 million people worldwide are suffering from stroke each year (WHO 2004). According to the Indian fact sheet on stroke updated in 2012 the estimated prevalence rate for stroke is 90-220/100,000 (Dalal *et al*. 2007) [8]. 1.44-1.64 million cases of new acute strokes occur in India in every year (www.who.int/chp/chronic_disease_report/en/).
So far studies done over acamprosate in alcohol dependence indicate that it is quiet safe and effective NMDA receptor modulator. It inhibits nitric oxide synthase and exhibit anti-inflammatory activity. It also found to block voltage gated calcium channels (Nassila *et al*. 1988; Sepulveda *et al*. 2013; Sternberg *et al*. 2012; Allgaier *et al*. 2000) [18, 20, 22, 2]. As per literature survey acamprosate was shown effective results in various central nervous system disorders through these mechanisms. So, here we will evaluate the pharmacological potential of acamproste in cerebral stroke.
WHO define stroke as "rapidly developing clinical signs of focal disturbance of cerebral function, lasting more than 24h or leading to death, with no apparent cause other than vascular origin". There are two main types of stroke on the basis of its aetiology as either ischemic (87%) or haemorrhagic (13%). In humans ischemic stroke in which blood supply disrupted is caused by blocked blood vessels results in formation of thrombus that occlude an artery (Aggarwal *et al*. 2010) [1]. Brain is highly metabolic organ which needs regular glucose and oxygen supply through the blood. Interruption of blood supply leads to failure in supply of oxygen and glucose; it leads to energy failure in brain. With this mechanism *in vitro* models of cerebral stroke have been developed by oxygen and glucose supply to the cells (Singh *et al*. 2008). Experimental models of focal cerebral ischemia have been developed in an attempt to closely mimic the changes that occur during and after human ischemic stroke. Ischemia in animals like rats and mice are most commonly produced by multiple cervical vessel occlusions. Blood supply to the brain is interrupted by occluding the blood vessels that supply to the brain. The middle cerebral artery is the most commonly occluded vessel, the vessel can be occluded either permanently or transiently and damage results from both ischemia and reperfusion as in humans. In similar fashion cerebral stroke is produced in *Drosophila melanogaster* flies by introducing them in an anoxic environment maintained with oxygen level below 1% for 3h and then reoxygenation. The fruit fly *Drosophila melanogaster* presents a possible source of new discoveries since it shares fundamental oxygen regulation pathways with humans (Haddad 2000) [11]. The restored blood flow reintroduces oxygen within cells that damages cellular proteins, DNA and the plasma membrane which in turns cause oxidative damage by production of free radicals (Divya *et al*. 2013) [9]. Reperfusion/Reoxygenation of hypoxic/anoxic tissues induces a massive production of reactive oxygen species causing important oxidative damage. The elevated apoptosis after reoxygenation resembles the effect of reperfusion after cerebral ischemia in mammals, where reperfusion accelerates the rate of cell death.

2. Materials and Methods
Fly strain
Oregon R^+ strains were used for the proposed studies which have obtained from *Drosophila* stock center, Mysore. The flies were reared on a standard food medium containing cornmeal, yeast, agar, sugar and added propanoic acid as antimouldant and maintained at 25ºC on natural light/dark cycle in glass bottles. Flies of both sexes were used in the study.

Apparatus

We designed the anoxic chamber as suggested by Vigne and Colleagues as a model of AR injury Figure1 & 2 (Caraball *et al*. 2014) [4]. An outlet was made available for air for replacement with nitrogen (to create the anoxic environment). 10 days old flies were placed in standard plastic vial having wire mesh on bottom. Nitrogen was supplied in vial through flow meter at a flow rate of 200ml/min Figure 1. Air outlet was at the top of vial containing flies. After 162.6min of continuous flow of nitrogen, oxygen concentration in vial got dropped below 1%.

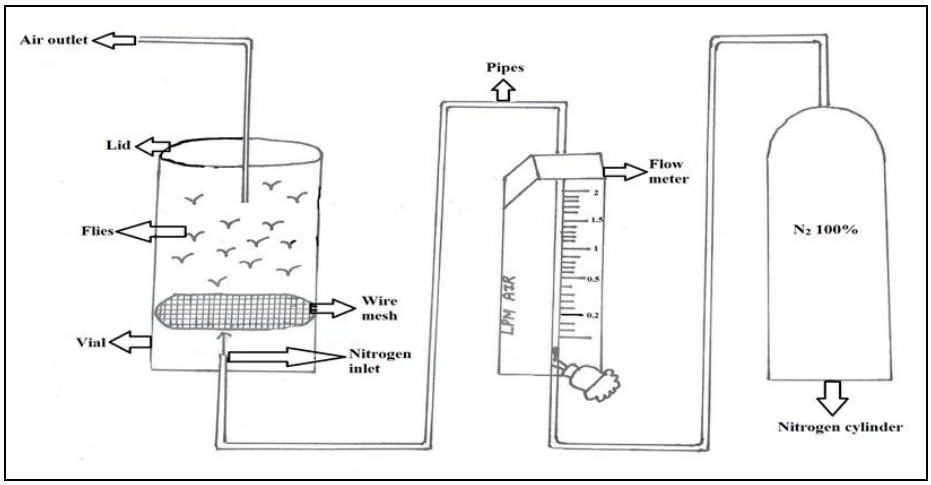

Fig 1: Anoxia Reoxygenation Injury Model

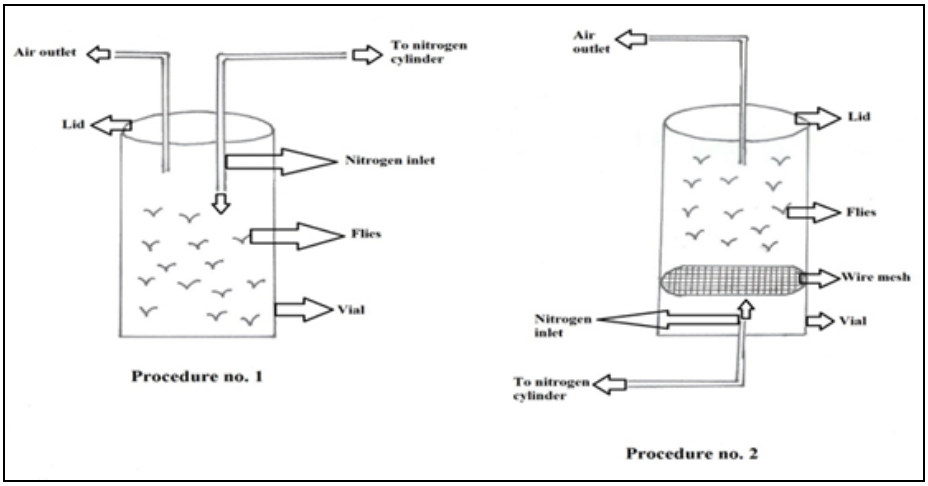

Fig 2

Time required to achieve oxygen concentration ‹1% was calculated using following formula

$$\% \text{ oxygen concentration} = \frac{100 \times 0.2095 (V_v - V)}{V_v}$$

V_v = Vial volume (ml)

V = Volume of liquid nitrogen required to replace air in the above vial (ml)

$$1\% = \frac{100 \times 0.2095 (50 - V)}{50}$$

V = 47.61ml

Where

$V_g = V \times X$

V_g = Volume of nitrogen gas required to replace air in the above vial (ml)

X = Gas expansion factor

X for Nitrogen is = 683

V_g = 47.61 x 683 = 32517.63ml

Time required to achieving oxygen concentration <1% is:

Flow rate of flow meter = 200ml/min

∴ 200ml gas flow in 1min

$$\Rightarrow \text{32517.63ml gas flow in } \frac{1}{200} \times 32517.63$$

Standardization of anoxia-reoxygenation injury model

We were designed a model for anoxia reoxygenation injury. In this model we were used a vial of 50ml capacity connected to nitrogen cylinder through a flow meter. We were used two procedures to inject nitrogen in vial. In procedure no.1 pure nitrogen was introduced from top of the vial through an inlet attached to the vial lid. In procedure no. 2 we were used same vial of 50ml capacity with wire mesh fixed 2cm above from the bottom of vial Figure 2. The inlet for pure nitrogen was fixed from the bottom of the vial in this procedure. The time required to achieve oxygen concentration <1% was calculated by

formula given above. Different experiments were conducted using these two procedures at different anoxic periods. The mortality at 24h after reoxygenation was observed.

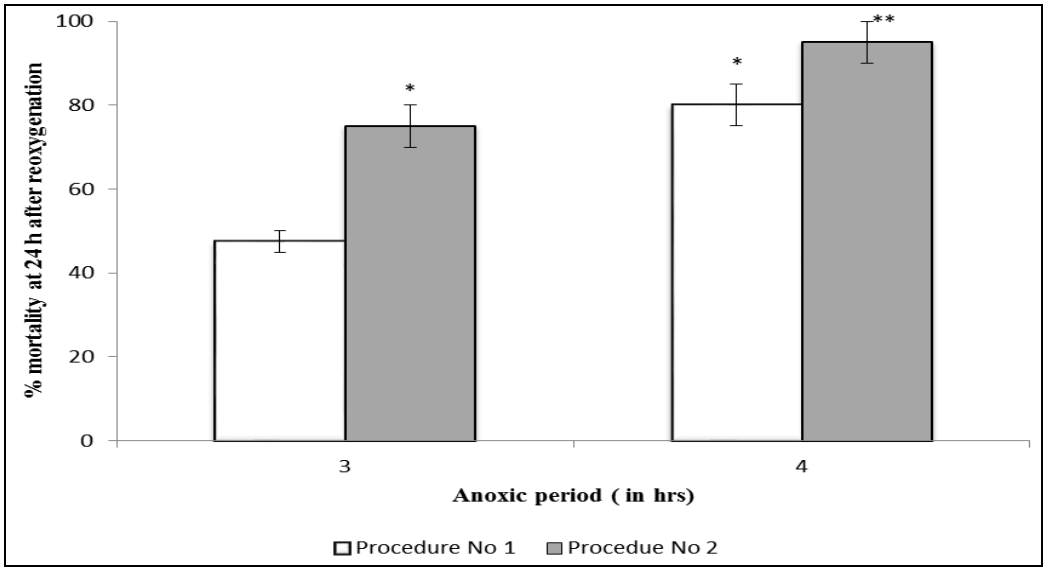

Fig 3: The figure was showing% mortality at 24h after reoxygenation in different anoxic period by using two different procedures. Values were expressed as Mean±SEM, Where, n=20. Data was analyzed by one way ANOVA followed by Tukey test. * indicates significance difference with $P<0.05$ vs 3h anoxic period in Procedure No1, As shown in figure mortality was increased with increase of anoxic time period. It was observed from figure% mortality in procedure No 2 is high as compared in procedure No1.

Drug treatment

10 days old flies were placed in empty vial for 18h starvation before drug treatment then flies were placed in standard food medium supplement with acamprosate (20µg/ml, 100 µg/ml, and 200µg/ml) for 24h. The control group flies were fed on standard food medium.

Recovery from stupor state after anoxia reoxygenation

Flies were examined every 10min after exposure to room air (reoxygenation) till 1h and number of active flies were calculated. All flies that remained motionless at the bottom of the flask were considered inactive, and all flies that were moving or at the wall of the flask were considered active.

Mortality

Number of dead flies were observed at 24h after reoxygenation. Then percentage mortality was calculated.

Climbing assay

Locomotor activity was determined by using negative geotaxis assay as described by Bland *et al* (2009) [3] with minor modifications. To access the locomotor activity on vertical climbing single fly was placed in empty glass vial (without food medium) for an hour. The flies was 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 was tested 3 times at 1min intervals (Bland *et al*. 2009) [3]. For each experiment, climbing mean was calculated. Climbing assay was performed at 2h after reoxygenation.

At 24h of reoxygenation, legs and wings of all flies were separated from the body with sharp edge of blade and then homogenized in sodium phosphate buffer (0.1 M, pH 8.0) and were centrifuged at 2500rpm for 10min at 4ºC. The supernatant was filtered through nylon mesh and then used for following biochemical parameters.

Total protein content

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen [s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu 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 tryptophan residues (amino acid) in a protein will produce a blue purple colour complex, with maximum absorption in the region of 660nm wavelength (Lowry *et al*. 1951) [14].

Superoxide dismutase (SOD) activity

Superoxide dismutase activity was assayed according to the method of Kono *et al.,* where the reduction of nitroblue tetrazolium (NBT), inhibited by the superoxide dismutase was measured at 560nm using UV/visible spectrophotometer. The reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and tissue homogenate (Kono 1978) [12]. 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 activity

In this method, dichromate in acetic acid has been reduced to chromic acetate, when heated in presence of hydrogen peroxide with the formation of per chromic

acid as an unstable intermediate. The reaction mixture (1.5ml vol.) was containing 1.0ml 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 of 2.0ml of dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid was mixed in a 1:3 ratio). Catalase (CAT) was assayed colorimetrically at 620nm (Chandramohan *et al*. 2009) [5].

Malondialdehyde (MDA) estimation

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 was 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 (Okhawa *et al*. 1979) [19].

Nitric oxide (NO) estimation

The presence of NO will be assayed colorimetrically by Greiss reagent. The supernatant (100 µl) collected will be 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 (100 µl) of distilled water. The amounts of NO produced was determined by calibrating a standard curve using sodium nitrite (Green *et al*. 1982).

Statistics

Data was represented as Mean ± SEM. The significance of differences between groups was assessed using one-way ANOVA followed by tukey test. Differnences were considered significant at $p<0.05$.

3. Results

Stadadization of model

Mortality index data was observed at 24h after reoxygenation using both the procedures described earlier at different anoxic periods. The% mortality rate was observed after a period of 24h of reoxygenation. Anoxic period of 4h duration was too long in both the procedures. At anoxic period of 3h,% mortality rate was observed to be below 50% in procedure No.1, as it was 47.5% whereas in procedure No.2,% mortality was observed to be 77.5%. As optimum mortality rate was observed by procedure No.2 in anoxic period of 3h, so we decided to precede our study following procedure No.2 with anoxic period of 3h.

Effect of acamprosate on recovery from stupor state

20 flies in each group were exposed to anoxia of 3h. Then flies were exposed to room air for reoxygenation. During anoxia all flies were in stupor state. After reoxygenation flies first started to move then climb at the walls of the vial. Percentage recovery was calculated by recording numbers of flies recovered from anoxic stupor state at each 10min difference till 1h after reoxygenation. Acamprosate at concentrations of 100µg/ml and 200 µg/ml ($p<0.05$) showing significant increase in% recovery (Figure 4) of flies at 1h after reoxygenation as compared to vehicle group. When compared with each other, non significant difference was observed between acamprosate (100 and 200µg/ml) treated groups.

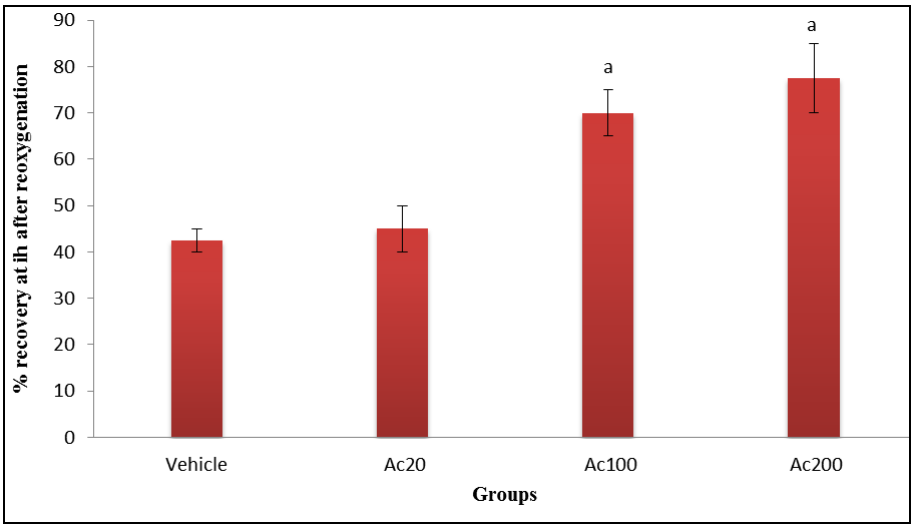

Fig 4: Effect of acamprosate on% recovery at 1h after reoxygenation. Values were expressed as Mean±SEM, Where n= 20. Data was analysed by one way ANOVA followed by Tukey test. a. $p<0.05$ vs vehicle. The F-value was found to be F (4, 5) =25.472. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Effect of acamprosate on mortality by AR injury

Mortality was found to be maximum at 24h after reoxygenation and remained the same at 48h after reoxygenation. Flies in different groups were exposed to low oxygen concentration and after anoxic period, flies were placed at room air for reoxygenation. Mortality was

recorded at 24h after AR stress. Acamprosate at 100 and 200µg/ml (p<0.01) concentrations was found to be showing significant decrease in percentage mortality (Figure 5) as compared to vehicle group. When compared with each other, non-significant difference was observed between acamprosate (100 and 200µg/ml) treated groups.

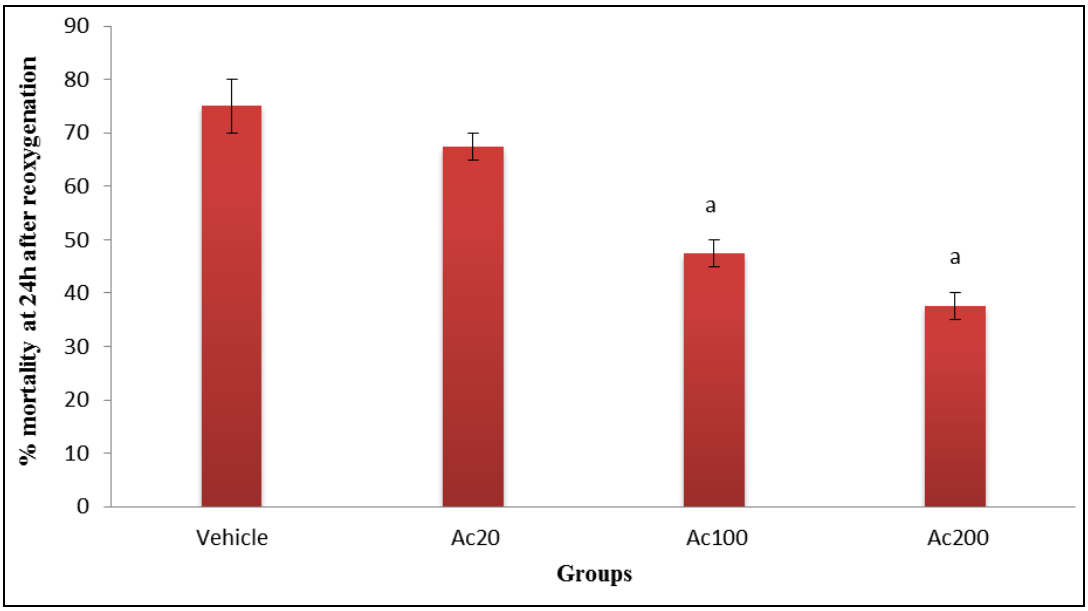

Fig 5: Effect of acamprosate on% mortality at 24h after reoxygenation. Values were expressed as Mean±SEM, Where, n= 20. Data was analysed by one way ANOVA followed by Tukey test. a. p<0.01 vs vehicle. The F-value was found to be F (4, 5) = 99.786.**Ac** 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standarfood medium, respectively.

Effect of acamprosate on climbing assay

After AR injury maximum flies were taken more time to climb so climbing% in 60sec was higher as in 30 and 10sec in vehicle group. Acamprosate at different concentration (20, 100, 200 µg/ml) increase the climbing% in 10sec and 30sec, but the climbing% in 60sec got decreased with acamprosate. Acamprosate does not show any significant effect on climbing response in 10 sec and 30sec (Figure 6) Acamprosate 200µg/ml (p< 0.05) was found to be significantly decreasing climbing% of flies in 60sec (Figure 6) as compared to vehicle group.

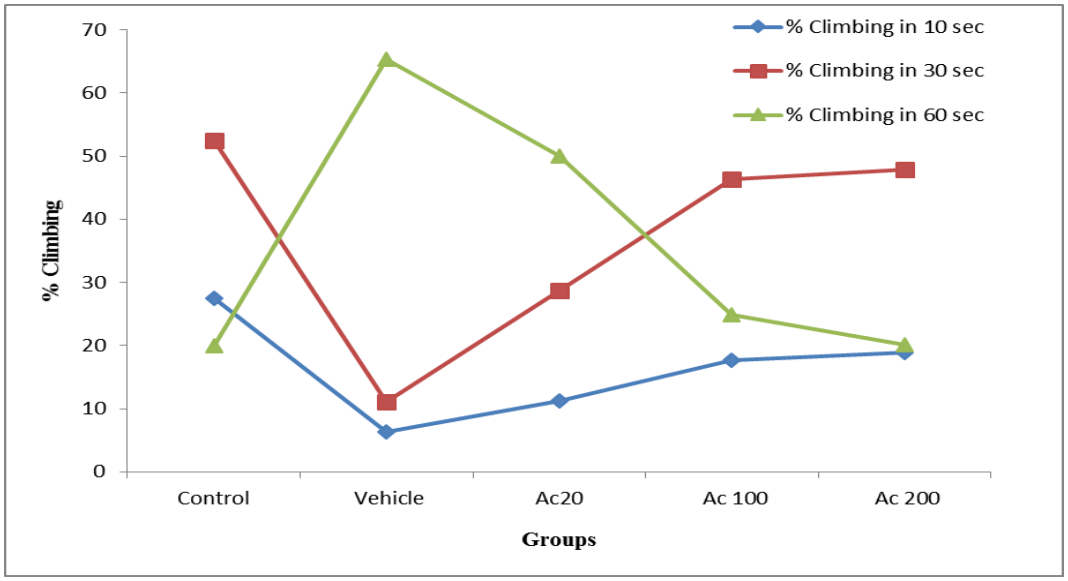

Fig 6: The figure showing effect of acamprosate on climbing response of flies in 10, 30, and 60sec at 2h after reoxygenation. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by Tukey test. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Effect of acamprosate on total protein content

In anoxia reoxygenation injury there is decrease in protein content in flies' homogenate as (Figure7) vehicle group showing significant decrease as compared to control group. There was concentration dependent and significant increase in protein level (Figure 7) by two different concentrations 100 and 200µg/ml of acamprosate (p<0.01).

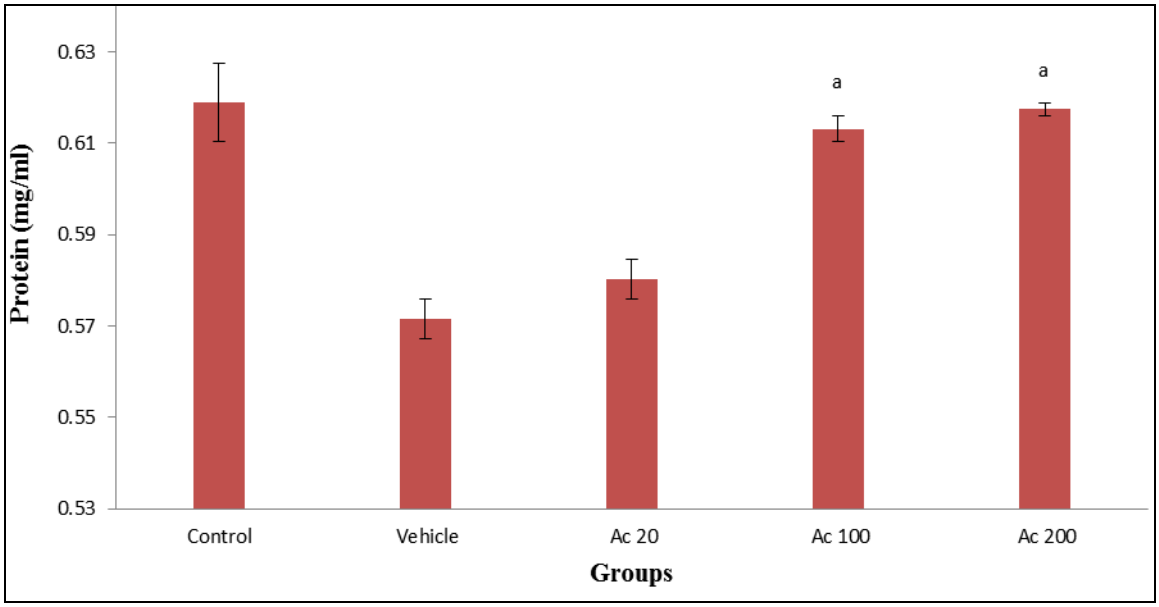

Fig 7: The figure showing effect of acamprosate on total protein content in flies's homogenate at 24h after reoxygenation. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by Tukey test. a. $p<0.01$ vs vehicle. The F-value was found to be F (4, 5) = 21.048. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively

Effect of acamprosate on activity of superoxide dismutase (SOD)

Superoxide dismutase (SOD) is a family of metalloproteins that catalyze the dismutation of 2 molecules of O_2 (super oxides) to form hydrogen peroxide. The level of SOD was found to be significantly decreased in vehicle group ($p<0.05$) as compared to control group (Figure 8). When compared with drug treated groups, non-significant difference was observed by all concentrations of acamprosate (20,100, 200µg/ml).

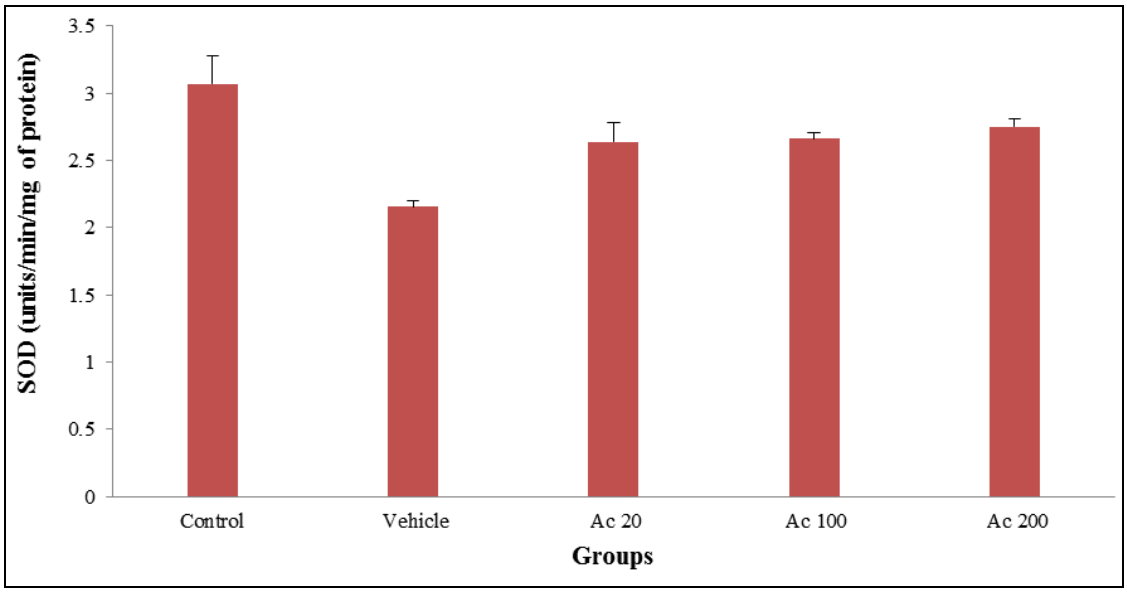

Fig 8: The figure showing effect of acamprosate on activity of SOD in fly's homogenate at 24h after reoxygenation. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by Tukey test. The F-value was found to be F (4, 5) = 7.892. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Effect of acamprosate on Catalase level

Catalase detoxifies the reactive hydrogen peroxide molecules and converts it into water and diatomic oxygen thus preventing through harmful effects of reactive oxygen species. Catalase levels were found low after anoxia reoxygenation injury. In present study in comparison to control group catalase level (Figure 9) was found to be decreased in vehicle group. Acamprosate 100 and 200µg/ml ($p<0.001$) were shown significant increase in catalase level (Figure 9) as compared to vehicle group. When compared to each other both the concentrations were found to be non significant.

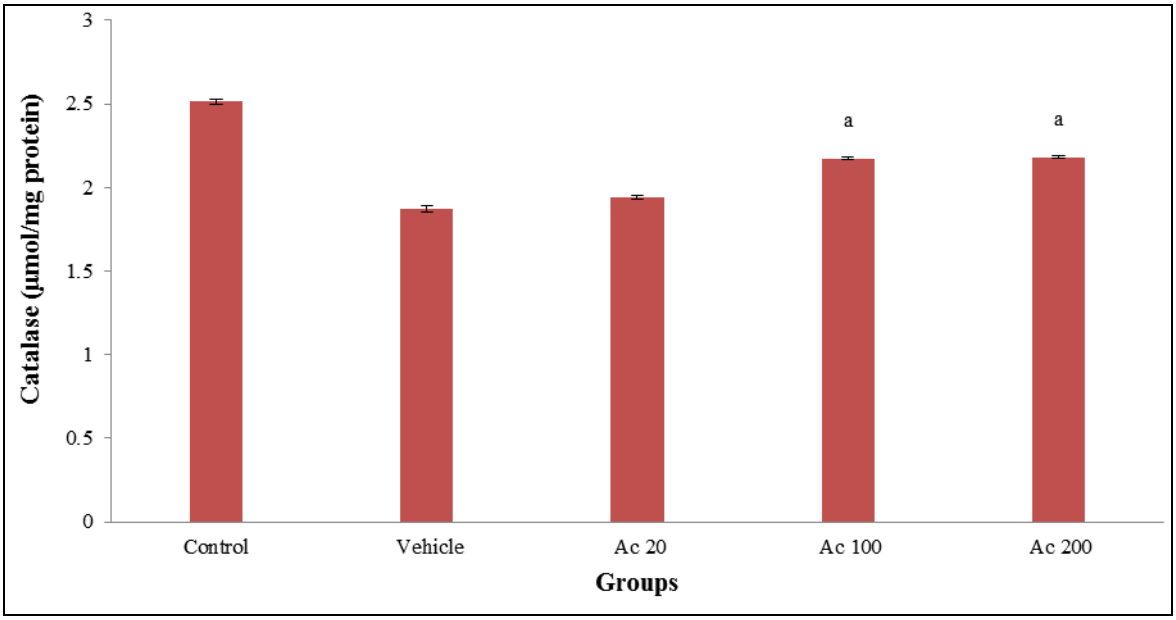

Fig 9: The figure showing effect of acamprosate on level of catalase in flies homogenate at 24h after reoxygenation. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by Tukey test. a. $p<0.001$ vs vehicle. The F-value was found to be F (4, 5) = 373.47. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Effect of acamprosate on MDA level

Malondialdehyde (MDA) is an end product of lipid peroxidation. Lipid peroxidation is a free radical mediated event. Therefore, MDA is a measure of free radical generation. MDA level was observed to be significantly increasing in vehicle group as compared to control group (Figure 10). Acamprosate 200µg/ml ($p<0.05$) was shown significant decrease in level of MDA (Figure 10) as compared to vehicle group.

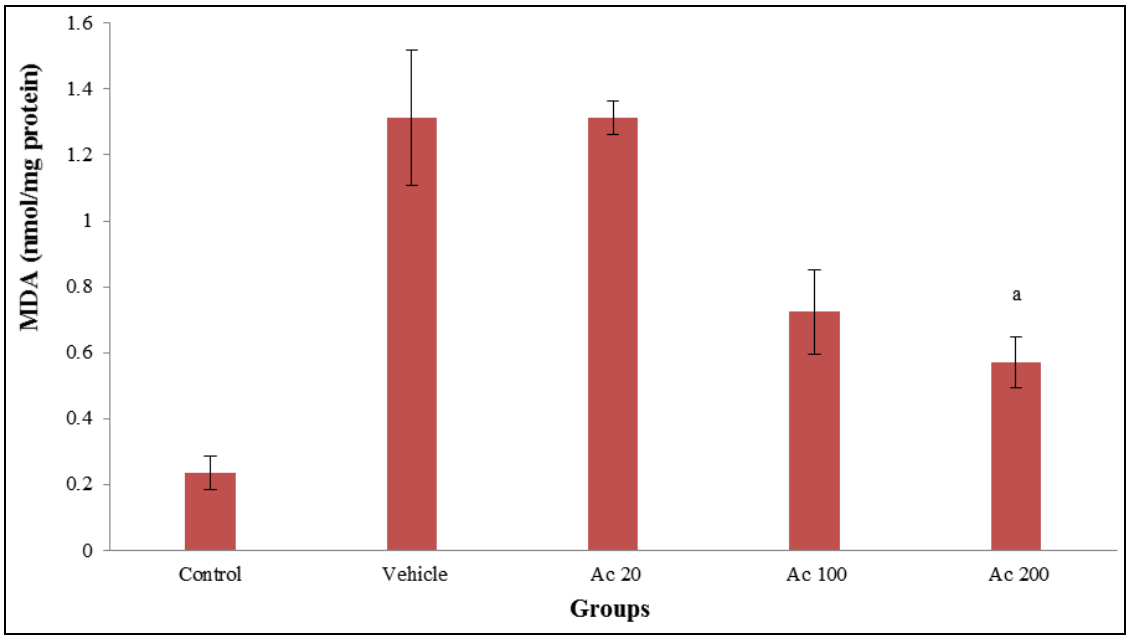

Fig 10: The figure showing effect of acamprosate on level of MDA in flies' homogenate at 24h after reoxygenation. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by Tukey test. a. $p<0.05$ vs vehicle. The F-value was found to be F(4, 5) = 16.119. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

Effect of acamprosate on nitrite level

Increased NO generation in AR injury is associated with cytotoxic effects. NO cause free radical damage by formation of peroxynitrite may have an important role in anoxia reoxygenation injury. Peroxynitrite decomposes to other reactive oxygen species. In present study vehicle group show significant increase in nitrite level as compared to control group (Figure 11). In comparison to vehicle treated group there was concentration dependent significant decrease in nitrite level (Figure 11) by two concentrations 100 and 200µg/ml of acamproste ($p<0.05$). When compared to each other, a non-significant difference was observed between the acamprosate 100 and 200µg/ml treated groups.

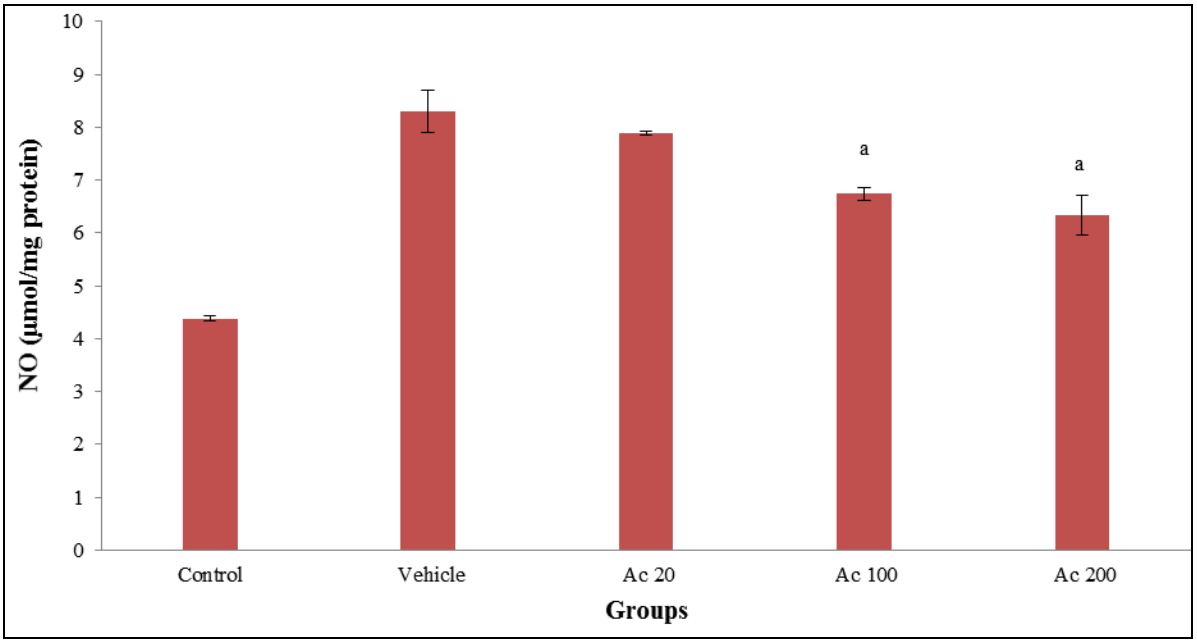

Fig 11: The figure showing Effect of acamprosate on level of nitrite in flies' homogenate at 24h after reoxygenation. Values were expressed as Mean±SEM. Data was analysed by one way ANOVA followed by Tukey test. a. $p<0.05$ vs vehicle. The F-value was found to be F (4, 5) = 37.287. Ac 20, Ac 100, Ac 200: Acamprosate 20, 100 and 200µg/ml, in standard food medium, respectively.

4. Discussion

Each year millions of individuals in the United States die or become morbidly ill because of conditions or diseases that actually reduce oxygen supply to hypoxia-sensitive tissues, such as myocardium, central nervous system and kidneys. *Drosophila* definitely senses the lack of oxygen, as it responds quickly in a way similar to mammals. These flies develop anoxic stupor state when the oxygen level is very low. They show a physiological response that is proportionate with their behavioural response. In low oxygen condition flies seems to behave phenotypically in a manner that is similar to mammals. The only difference is that *drosophila* can tolerate anoxia for hours unlike mammals, as they suffer from irreversible damage after a few minutes of anoxia (Haddad 2000) [11]. *Drosophila* is anoxia tolerant because of abundant amount of trehalose present in its head and oxygen stored in trachea (Chen *et al.* 2002; Krishnan *et al.* 1997) [7, 13]. Thus we have taken advantage of this model system to study effect of acamprosate on anoxia reoxygenation (AR) injury. In this study, we determined the biochemical and behavioural alterations in *Drosophila melanogaster* in responses to anoxia reoxygenation injury and in responses to AR injury with drug treatment. Acamprosate is an NMDA modulator. It is an FDA approved drug for alcohol dependence. Here we evaluate its pharmacological potential in anoxia reoxygenation injury.

The effect of anoxic periods on *Drosophila melanogaster* has been previously studie (Caraball *et al.* 2014; Krishnan *et al.* 1997) [4, 13]. As in previous models vial containing flies were placed in a chamber saturated with pure nitrogen, in these models concentration of oxygen in chamber was recorded using oxygen sensors. The uniqueness of our work was that we were kept flies in direct contact of pure nitrogen in a vial without using any chamber and we were studied the pharmacological effect of acamprosate on anoxia reoxygenation injuy which was not yet published. We were designed a model to study anoxia reoxygenation injury in *Drosophila melanogaster* using a standard vial of capacity 50ml attached to nitrogen cylinder through a flow meter, which regulates the flow of nitrogen. We were used two procedures to introduced nitrogen in vial. In procedure No.1 nitrogen was introduced from top of the vial while in procedure No.2 nitrogen was introduced from bottom of the vial and the nitrogen reached to the flies through a wire mesh fixed 2cm above from the bottom of vial. The flies were remained on the wire mesh during the anoxic stage. The wire mesh was used to avoid the direct contact of nitrogen with the flies, it was observed in second procedure that due to wire mesh immediate death of flies due to anoxia got decreased as compared in first procedure. Anoxia reoxygenation injury was induced in flies using both procedures at different anoxic periods and mortality index data was observed at 24h after reoxygenation. Oxygen concentration of <1% was achieved after 162.6min of introducing 100% nitrogen into vial at a flow rate of 200ml/min. In procedue No.1 the immediate death of flies due to anoxia was noted and the mortality due to reoxygenation was less as compared in procedure No.2. So we were decided to precede our study using procedure No.2.

Within 1min of achieving anoxic level, flies stop flying and lay at the bottom of vial, motionless. They remained stuporous throughout the anoxic time. After reoxygenation, flies become active from the anoxia induced stupor and start flying. With different concentrations of acamprosate% recovery of flies was observed to be increased. Acamprosate at concentrations of 100µg/ml and 200µg/ml ($p<0.05$) showing significant increase in% recovery (Figure 4) of flies at 1h after reoxygenation as compared to vehicle group. It had been reported previously by Vinge and collegues, that

mortality increases in *Drosophila* after AR exposure (Caraball *et al*. 2014) [4]. Neuron excitotoxicity, free radical damage, neuroinflammation, and immune system activation are major toxic events in anoxia reoxygenation injury. As acamprosate is NMDA receptor modulator, it inhibits NMDA receptor in its overactive state, it also inhibit excessive calcium release, NO production and release of TNF-α (Nassila *et al*. 1988; Allgaier *et al*. 2000; Sepulveda *et al*. 2013; Sternberg *et al*. 2012) [18, 20, 22, 2]. Generation of reactive oxygen species (ROS) is major cause of death in *Drosophila* after anoxia reoxygenation injury. Acamprosate decrease mortality in *drosophila melanogaster* after reoxygenation from anoxic environment, may be by combating excitotoxic effects of glutamate through NMDA receptor (Nassila *et al*. 1988) [18] and by preventing formation of free radicals as it inhibit NO synthesis (Sepulveda *et al*. 2013) [20]. It was also reported in previous studies that acamprosate inhibit release of TNF-α (Sternberg *et al*. 2012) [22], so it can control neuroinflammation also. All the above are suggested mechanism helpful in decreasing mortality in *drosophila melanogaster* suffer from AR injury. Our results suggest that acamprosate at concentrations 100 and 200µg/ml was found to be significantly decreased the mortality at 24h after reoxygention (Figure 5).

It was observed that co-ordination of movement got impaired in some flies when exposed to anoxic periods. The muscle compound action potential ceased to occur within 30sec of onset of anoxia (Krishnan *et al*. 1997) [13]. In present study after 3h of anoxia recovery is initiated after 5min of reoxygenation but complete recovery was not observed till 1h. So climbing response of flies got affected after anoxia. Climbing assay was performed only on the flies that were found active at 2h of reoxygenation. The flies in vehicle group took much more time to climb up as compared to control group. Some flies after reoxygenation were not fully active they were shown some body movement but unable to climb up on the walls of the vial. Acamprosate 200µg/ml ($p<0.05$) showing significant decrease in% clmbing in 60sec. In acamprosate treated groups% climbing in 10sec and 30sec got improved but acamprosate does not show any significant effect on climbing at10sec and 30sec (Figure 6).

Anoxia reoxygenation, as well as ischemia reperfusion injury is mainly mediated by the overproduction of reactive oxygen species (ROS). Oxidative stress is an essential element of ischemia reperfusion injury, where perfusion of anoxic tissues proceed increase of ROS production (beyond the antioxidant capability of the organism) causing oxidative damage of cell membrane by lipid peroxidation. This increase in oxidative stress is due to excitotoxicity and exhausation of the endogenous antioxidant system (e.g. superoxide dismutase (SOD), glutathione peroxidase (GSH), catalase (Chen *et al*. 2011) [6]. After anoxia when flies are re-introduced to normoxic environment, oxygen is reintroduced to cells. The accumulation of reactive oxygen species (ROS) increases which in turns damage lipids, proteins and DNA (Meszaros *et al*. 2015) [17]. In the present study, increase in oxidative stress following anoxia reoxygenation injury was evidenced by significant increase in MDA, NO level and decrease in SOD, Catalase level. NO cause free radical damage by formation of peroxynitrite which further decomposes to other free radicals such as hydroxyl and nitrogen dioxide, which causes lipid peroxidation. MDA is end product of lipid peroxdation (Aggarwal *et al*. 2010) [1]. Lipid peroxidation products are the stable markers of membrane lipid damage and oxidative stress. In present study we observed that acamprosate 100 and 200µg/ml ($p<0.05$, $p<0.05$) significantly inhibit the formation of nitric oxide (NO) (Figure 11). As it was previously demonstrated that acamrosate inhibit NADPH-diphorase, which is responsible for calcium/calmodulin-dependent synthesis of guanylylcyclase activator nitric oxide (NO) from L-arginine (Sepulveda *et al*. 2013) [20]. Present results were shown that acamprosate significantly improving the endogenous antioxidant system by increasing the level of catalase and super oxide dismutase (SOD). As acamprosate inhibit nitric oxide synthesis further level of MDA was also observed to be decreased because peroxynitrite and other free radicals formed from nitric oxide further cause lipid peroxidation. Our drug, acamprosate is an NMDA modulator which inhibits the receptor in its overactive state (Nassila *et al*. 1988) [18]. As excitotoxcity is major problem in ischemic injury. All the oxidative damage events are started after excitotoxicity (Won *et al*. 2002) [23]. So our findings suggest that acamprosate shows its action through NMDA modulation property.

Our results suggest that acamprosate may be responsible for protection against the oxidative stress, possibly by reducing the lipid peroxidation, NO production and increasing the endogenous defensive capacity by increasing level of catalase and superoxide dismutase.

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