Combined *In-Vivo* and *In-Silico* Studies Shed Insights into the deterioration of Oxidative Damage with Vitamin E Supplementation in Ethanol-Induced Myocardial Infarction in Rats.

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ABSTRACT

Oxidative stress plays an indispensable role in the development of cardiac disorders. In the present study, we intend to determine whether the administration of vitamin E ameliorates the antioxidant system in ethanol-induced myocardial infarction (MI) in rats by *in vivo* and *in silico* studies. We found a significant (P<0.05) reduction in cardiac antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glutathione (GSH) while in glutathione-s-transferase activity (GST) and MDA levels were increased in ethanol alone treated rats (6g/kg b.w. orally) compared to control. Vitamin E treatment (100mg/kg b.w. orally) significantly (P<0.05) elevated SOD, CAT, GSH-Px, GR and GSH content while in GST and MDA levels were decreased. The present results suggested that Vitamin E could act as cardio protective agent under ethanol induced MI by rehabilitation of antioxidant defense system and conserve the cardiac architecture.

Keywords: Ethanol, Vitamin E, Antioxidant enzymes, Myocardial infarction
INTRODUCTION

Alcohol is one of the most abused drugs in all over the world. It is well known that alcohol-induced oxidative stress can play import role in tissue damage [1, 2]. The oxidative stress could form due to over production of free radicals during extensive ethanol metabolism is trigger the lipid peroxidation and derangement of antioxidant system [3]. The previous reports demonstrated that ethanol decrease the GSH levels and induce the myocardial injury [4]. In contrast, the earlier reports demonstrated that moderate alcohol consumption is beneficial for cardiac health [5,6]. However, Vitamin E and selenium levels were decrease during chronic alcohol consumption play a main role in the muscle damage [7].

Vitamin E is a lipid soluble vitamin and it used in food supplements and cosmetic products. It has been proven that Vitamin E an effective antioxidant to reduce the oxidative stress [8]. Vitamin E has been shown to slow or inhibit the progress of atherosclerosis in human and animals [9, 10]. In addition vitamin E reduces the smooth muscle cell proliferation and isoproterenol - induced cardiac damage [11,12]. Epidemiological data indicated an inverse association between cardiovascular risk and vitamin E intake from dietary sources or supplements [13].

Our study is mainly focused on to evaluate the potential cardio protective effect of vitamin E on ethanol - induced myocardial infarction in rats by in vivo and in silico studies.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the present study were Analar Grade (AR) and obtained from the following scientific companies: Sigma (St. Louis, MO, USA), Fisher (Pittsburgh, PA, USA), Merck (Mumbai, India), Ranbaxy (New Delhi, India), Qualigens (Mumbai, India).

Experimental design

Pathogen free Wistar strain male albino rats aged 18 months, weighing 250 ± 20 g were obtained from Indian Institute of Science, Bangalore. The rats were housed in clean polypropylene cages having six rats per cage and maintained under temperature controlled room (27 ± 2 °C) with a photoperiod of 12 h light and 12 h dark cycle. The rats were given standard pellets diet (Sai Durga feed, Bangalore) and water ad libitum throughout the experimental period. The experiments were carried out in accordance with guidelines and protocol approved by the Institutional Animal Ethics Committee (Reg No: 10/i/a/CPCSEA/IAEC/SVU/KSR-GVS/dt 15/11/2010).

After acclimatization 24 rats were divided into four groups, six rats in each group and treated as follows.
Group I: Normal control (NC): rats received normal saline (0.9 % NaCl);
Group II: Vitamin E (ViEt): rats received vitamin E (100 mg/kg body wt) orally for 7 weeks;
Group III: Ethanol treatment (Et): rats received alcohol at dose of 6g/kg for 7 weeks. The dose was selected by doing dosage studies (2g/kg, 3g/kg, 4g/kg, 6g/kg);
Group IV: Ethanol + Vitamin E (Et +ViEt): rats received vitamin E+ ethanol for 7 weeks as followed by group-II and group-III.

At the end of 7 weeks treatment period, the rats were sacrificed by cervical dislocation and heart tissues were isolated, washed with ice cold saline, immersed in liquid nitrogen and stored in deep freezer at -80°C for further biochemical analysis.

Analytical procedures

Superoxide dismutase (SOD) activity was assayed in the mitochondrial fraction by the method of Misra and Fridovich at 480 nm for 4 min on a Hitachi U-2000 spectrophotometer. Activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 U per milligram of protein. Catalase (CAT) activity was determined at room temperature by using the modified version of Aebi (1984) and absorbance of the sample was measured at 240 nm for 1 min in a UV-spectrophotometer. Activity of Glutathione peroxidase (GPx) was determined by the method of Flohe and Gunzler (1984) in the presence of NADPH and absorbance was measured at 340 nm using cumene hydrogen peroxide. GR enzyme activity was determined according to the method of Carlberg and Mannervik. The concentration of reduced Glutathione (GSH) was measured as described by Akerboom and Sies. Glutathione-S-transferase, (GST) were estimated in cardiac tissue by the methods of Ohkawa et al. The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive product MDA by using the method of Ohkawa et al. All the enzyme activities were expressed per mg protein and the tissue protein was estimated according to the method of Lowry et al. using bovine serum albumin (BSA) as a standard.

Histopathology

Cardiac tissues were isolated from the animal and washed with ice-cold saline. They were then fixed in 10% formalin solution. Sections of 3 µm thickness were stained with Haematoxylin` and Eosin (H & E) for histopathological examination in the light microscope and a part of tissue was given for TEM (Transmission Electron Microscope) examination fixed in 2% glutaraldehyde to Ruska labs R’Nagar Hyderabad-30. In silico studies

Targeted antioxidant protein sequences of rats such as SOD (NP_058746), CAT (AAB2378), GPx (CAA30928), GR (NP_446358) and GST (AAA41290) were retrieved from Genbank database (http://www.ncbi.nlm.nih.gov/genbank/). The structural homology entries for SOD, CAT, GPx, GR and GST were selected from blast search and which are downloaded from PDB (Protein Data Bank). Targeted protein sequences and its respected homology entries were used to build initial 3D structures with MODELLER 9.10 homology modeling software.
Target protein initial models validated in SAVES (Structural Analysis and Verification Server) server http://nihserver.mbi.ucla.edu/SAVES/). Valid protein models used to dock with α-tocopherol with AutoDock vina tool in PyRx (http://pyrx.scripps.edu) software. The binding site of α-tocopherol within target proteins, its interaction with amino acid residues and amino acids surrounded to it has visualized in PyMOL visualization tool.

**Statistical analysis**

Statistical analysis of data was represented as mean ± SD. All the statistical analyses were carried out by SPSS software. Dunnett’s multiple comparison test and one-way analysis of variance (ANOVA) were used to assess the differences. P Values <0.05 were considered as highly statistically significant.

**RESULTS**

**Antioxidant profile and lipid peroxidation**

Fig.1 represents the activities of SOD, CAT, GPx and GR in the different experimental groups. There was significant decline in the SOD, CAT, GPx and GR activities in the cardiac tissues of the ethanol treated group when compared to control group and vitamin E alone treated group (P< 0.05), whereas in ethanol + vitamin E group SOD, CAT, GPx and GR activities were significantly increased (P<0.05) when compared to ethanol alone treated group.

**Figure 1:** Effect of vitamin E and Ethanol on SOD, CAT, GPX and GR activities in the cardiac tissue of normal and experimental rats

Data are expressed as means ± SD (n = 6). The groups are not shared same letters are significant to the control (abc p < 0.05), ethanol (Ψp < 0.001) (Dunnett’s multiple comparison test).
GST activity and GSH, MDA levels were measured in all experimental groups. Significant increase (p<0.05) in GST activity and MDA levels while GSH content was decreased in ethanol alone treated group when compared to control. There was a significant decrease (P<0.05) of GST activity and MDA levels while in GSH content was increased were observed in vitamin E treated ethanolic rats (Fig-2).

**Histopathological studies**

The cardiac tissue was observed under light microscopy and TEM (Transmission Electron Microscope). In light microscopy studies, we observed necrosis of cardiac cells, hemorrhages, degenerative changes in myofibrils, degeneration nucleus and complete loss of intercalate disc in alcohol treated rats. TEM studies shown complete degeneration of myofibrils and thrombosis was observed in coronary vessel. The treatment of ethanolic rats with vitamin E, we observed regeneration of all degenerative changes in cardiac tissue (Figu-3).

![Figure 2: Effect of vitamin E and Ethanol on GSH, and MDA levels, GST activity in the cardiac tissue of normal and experimental rats.](image)

Data are expressed as means ± SD (n = 6). The groups are not shared same letters are significant to the control (abc p < 0.05), ethanol (Ψ p < 0.001) (Dunnett’s multiple comparison test).
Figure: 3 Histopathological Studies

E. Ethanol treatment (Et) TEM: 1. Thrombosis formation in coronary vessel, 2. Coronary vessel, 3. Necrosis of cardiac cells
F. Ethanol +Ginger treated (Et+ViEt) TEM: 1. Recovery of nucleus, 2. Regeneration of myofibril structure.

In silico studies

Modeller produced 100 protein homology model structures, in which the first class protein model selected based on DOPE score value. The DOPE scores for preferred model of SOD, CAT, GPx, GR and GST were -14088.10645, -53833.30078, -21149.61133, -42322.25000 and -27335.67578. The modeled protein structures and their validated parameters such as Ramachandran Plot, Verify 3D Errat, values were given in Table1. Binding affinity of tochopherol with target proteins were -4.5, -7.4, -6.2, -7.0, and -7.0, for SOD, CAT, GPx, GR and GST respectively. In protein and ligand interaction studies (Figure 4) tochopherol O\textsubscript{30} atom hydrogen bonded with O (oxygen) atom of Tyr\textsubscript{358} amino acid in Catalase with bond length 3.1 Å, bond angles 96.1°. H\textsubscript{32} atom hydrogen bonded with O atom of Leu\textsubscript{82} with bond angle 2.3 Å and bond length 98.5° in glutathione peroxidase the remaining proteins not shown any hydrogen bond interactions. Amino acids that surrounded to the tochopherol binding site in proteins were given in table 2.
Table 1: Homology modeling antioxidant proteins validated using SAVES server

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Ramachandran Plot</th>
<th>Verify 3d</th>
<th>Errat</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>91.7%</td>
<td>100.00%</td>
<td>98.630</td>
</tr>
<tr>
<td>CAT</td>
<td>91.6%</td>
<td>68.56%</td>
<td>82.056</td>
</tr>
<tr>
<td>GPx</td>
<td>92.8%</td>
<td>91.44%</td>
<td>91.011</td>
</tr>
<tr>
<td>GR</td>
<td>93.8%</td>
<td>84.43%</td>
<td>85.139</td>
</tr>
<tr>
<td>GST</td>
<td>93.5%</td>
<td>93.72%</td>
<td>86.449</td>
</tr>
</tbody>
</table>

Table 2: The surrounding amino acids of antioxidant enzymes at Tocopherol binding site

<table>
<thead>
<tr>
<th>S No</th>
<th>Protein Name</th>
<th>Surrounding Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SOD</td>
<td>His81, Asn66, Lys70, Ser69, Lys71, His72, His64, Pro63, Lys137, Thr136, Glu133</td>
</tr>
<tr>
<td>2</td>
<td>CAT</td>
<td>Val74, His75, Asn148, Phe153, Ala158, Met159, Phe161, Pro162, Ile165, Met350, Gly353, Arg354, Phe356, Ala357, Tyr358, Asp360, Thr361</td>
</tr>
<tr>
<td>3</td>
<td>GPx</td>
<td>Pro12, Leu13, Leu82, Asn83, Leu85, Phe94, Glu95, Pro96, Asn97, Phe98, Met99, Leu100</td>
</tr>
<tr>
<td>4</td>
<td>GR</td>
<td>Trp2, Val6, His7, His14, Pro308, Asp373, Pro307, His306, Glu9, Phe10, Tyr339, Thr347, Gly371</td>
</tr>
<tr>
<td>5</td>
<td>GST</td>
<td>Tyr9, Phe10, Ala12, Gly14, Ile107, Met208, Val110, Ile111, Ile213, Ala216, Arg217, Phe222, Phe220, Val219</td>
</tr>
</tbody>
</table>

Figure 4: Structures of Tocopherol
DISCUSSION

The present study was unveiled that ethanol drinking deteriorated the free radical scavenging system and this was reversed by supplementation with vitamin E in cardiac tissue. It was noticed that vitamin E could reduce oxidative stress which was mainly cause to cardiac damage by improvement of antioxidants activity. Oxidative stress was mainly emerged due to depletion of free radical scavenging system resulted from over production of free radicals in ethanol metabolism [15] [16]. Alcohol related cardiac disorders were mostly occurred as a result of elevated Reactive Oxygen Species (ROS) during extensive ethanol metabolism because of the antioxidant enzyme activities in the heart are relatively lower than in other tissue [17]. MI is nothing but myocardial necrosis due to free radical mediated reperfusion injury in cardiac tissue [18]. It has been reported that ROS enhance the oxidative damage of membrane lipids, proteins and DNA [19].

The cells have well equipped with antioxidant defense system for detoxification of free radicals which are mainly toxic to the cell [20]. SOD and CAT enzymes are antioxidant enzymes which are detoxify the superoxide anions and H$_2$O$_2$ [21]. However, chronic ethanol ingestion altered the antioxidant defense system [22]. It was also observed in the previous studies SOD and CAT activity was decreased in ethanol intoxication [23] [24]. In the present study we found similar results in ethanol treated rats shown significantly declined SOD and CAT activity (Fig-1). This reduction of SOD and CAT may attribute to ROS induced enzyme degradation or reduced synthesis in ethanol intoxication [25]. However, SOD and CAT activities were recovered when treatment with vitamin E to ethanolic rats. It was suggested that Vitamin E might contribute to increase the glutathione content this can be reduce the free radicals and improved the antioxidants activity [26] [27].

Glutathione peroxidase (GPx), selenocysteine-containing enzyme catalyze the reduction of H$_2$O$_2$ and lipid peroxides via oxidation of GSH [28]. Chronic ethanol consumption decreases the GPx activity due to deplete their substrates such as GSH and NADPH [29]. Similar results were also observed in the previous studies on the lung tissue [30]. In the present study, we observed significantly decreased GPx activity in ethanol treated group due to similar consequence may occur in GPx activity during ethanol ingestion and it may be resulting off accumulation of H$_2$O$_2$ and reduced selenium levels in cardiac tissue [31]. However, Vitamin E supplementation significantly increased the GPx activity in ethanolic rats. It has been demonstrated that Vitamin E is an effective chain breaking antioxidant and protect cell membranes from the oxidative damage [32] and it can also reduce the coronary artery disease in relevance of age and gender in humans [33]. In the present investigation Vitamin E reduce the oxidative stress by increased the GPx activity it may be attribute to increase the availability of GSH and selenium which could be enhance the antioxidants activity.

Glutathione reductase (GR) catalyzes the reduction of glutathione from oxidized glutathione and detoxify the hydrogen peroxide into water, it’s necessary to maintain the cellular redoxstate in the cell [34]. When deplete the GSH levels can be lead to forma the oxidative stress by altered the cellular redoxstate, it could be indirect effect to the GR activity. It
has been reported in the previous studies that glutathione levels were decreased in ethanol intoxication which reflects on the GR activity was observed in rat liver, kidney and lung [35] [36]. In the present study ethanol alone treated rats have shown significantly decreased the GR activity may be due to decrease the utilization of GSSG or may NADPH deficiency. However, GR activity was significantly increased when supplement with the Vitamin E to ethanolic rats. Recuperation of antioxidant enzymes activity was observed in isoproterenol (ISO) induced myocardial infarcted rats with vitamin E supplementation [37][27].The GR activity in cardiac tissue was increased with supplementation of vitamin E may be resulted from detoxified the free radical which are generated during ethanol intoxication due to its free radical scavenging activity.

GSH plays a main role in the cell defense system due to it thiol compound acts in cells as an antioxidant. GSH, as a co-factor of glutathione peroxidase participates in the reduction of peroxides with concomitant formation of oxidized glutathione disulfide (GSSG) [38] [39]. The ethanol intoxication studies on rats reported that exacerbates mitochondrial ROS production in liver and kidney and decrease the levels of GSH and cause to tissue damage [40] [5] [41]. In our study GSH levels were down regulated in ethanol alone treated rats. Similar findings were also observed in the previous study GSH content decrease in cardiac tissue during ethanol intoxication [4]. Ethanol metabolites increase the binding and inhibition of biosynthesis of GSH have might be suggested to the mechanism accounting for the GSH depleted during ethanol intoxication [42] [5]. However, GSH levels were significantly increased in vitamin E treated ethanolic rats. GSH levels were recovered with supplementation of vitamin E in DOX induced myocardial infarcted rats. In our results, GSH levels were increased when treated with vitamin E to ethanolic rats may have resulted from antioxidant activity of vitamin E can cause to enhance the GR activity results GSH content increase in the cardiac tissue.

Inconsistency of other antioxidant enzymes, GST activity was up regulated in ethanol alone treated rats. It has been demonstrated that the oxidants may activate gene expression through the antioxidant responsive elements via electrophilic thiol modification [43] and it might be over expression of enzyme protein occur during ethanol induced stress condition [44]. In contrast, Vitamin E supplementation significantly reduced the GST activity in ethanolic rats. It has been demonstrated that Vitamin E protect from ethanol induced oxidative damage in mice liver and in liver cancer due to decrease the GST activity [30] [45]. In our study similar findings were observed with supplementation of vitamin E in ethanolic rats might be its free radical scavenging activity.

It has been reported that chronic alcohol ingestion enhanced the level of thiobarbituric acid reactive substances which reflect extensive lipid peroxidation process in the liver, heart and kidney of rats [46] and it results in the loss of membrane fluidity, resulting in tissue damage and eventual cell death [47] [48]. MDA levels were increased in ethanol ingestion due to reduced in the antioxidant levels [16] [44]. Similar consequences were observed in the present study during ethanol ingestion in myocardial infarcted rats. In discrepancy, MDA levels were significantly decreased with administration of vitamin E to the ethanolic rats. The previous reports were also demonstrated that vitamin E has reduced the lipid peroxidation during
diabetic condition and ISO induced myocardial infarction in rats due to their antioxidant activity [36] [49]. In this study, we found that ethanol induce the lipid peroxidation in cardiac tissue reversed by vitamin E supplementation may be due to vitamin E reduced lipid peroxides which are mainly produced in lipid peroxidation resulted from the ROS chain reaction with cell membrane under ethanol intoxication condition could be restricted by its free radical scavenging activity and reduce the cell damage.

The histopathological studies were carried out by light microscopy and TEM. The heart tissue in ethanolic rats showed the necrosis along with hemorrhages and degenerative changes occur in myofibrils resulting that nucleus were scattered in the myoplasm. TEM slides showed complete degeneration of myofibrils, fat droplets in mitochondria, complete loss of interstitial disc, and coronary thrombosis. These observations confined that ethanol can initiate the myocardial infarction due to their toxicity on cardiac tissue. However, with vitamin E supplementation to the ethanolic rats shows regeneration of myofibrils structure, intercalate disc, decreasing of fat droplets in mitochondria and reduce the coronary thrombosis. These results suggested that vitamin E protected the cardiac tissue from ethanol induced myocardial infarction.

In our results, in vivo study was reported that vitamin E reduce the oxidative damage in ethanol induced myocardial infarction rats and it was strongly supported by the in silico studies. In the docking simulation of vitamin E (α-tocopherol) probable binding affinity shows with, SOD, CAT, GPx, GR and GST along with its interactions. α-tocopherol shows H (Hydrogen) - bond interaction with Tyr358 amino acid residue of CAT and Leu92 of GPx (Figure-4) to induce the conformational changes and increased the enzyme activity, while remaining SOD,GR and GST not showing any H-bond interactions but showing binding affinity to change their mode of action.

CONCLUSION

In conclusion, in the in vivo and in silico results demonstrated that Vitamin E exerts significant protective effect against ethanol induced oxidative cardiac damage by augmenting the antioxidant defense mechanism. Thus, vitamin E is promising agent for the prevention of ethanol induced toxicity through enhancing the antioxidants activity as well as lowering the extent of lipid peroxidation and conserve the cardiac architecture.

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