Effect of Vitamin E on alcohol-induced changes in oxidative stress and expression of transcription factors NFκB and AP-1 in mice brain cerebral hemispheres

Jasmeet Kaur & M P Bansal*
Department of Biophysics, Panjab University, Chandigarh 160 014, India

Received 8 August 2007; revised 12 June 2008

Redox sensitive transcription factors nuclear factor κB (NF-κB) and activator protein-1 are involved in the pathogenesis of alcohol-induced disorders. Because of its antioxidative properties, vitamin E may help prevent oxidative stress-induced disorders. The aim of the present study was to delineate the molecular mechanisms associated with alcohol-induced oxidative stress and to see whether vitamin E supplementation counters the alcohol-induced adverse effects. The results showed that vitamin E supplementation restored the redox status and thus prevented the alcohol-induced oxidative stress. Further measurements of the mRNA expressions of cjun, cfos, p65 (NFκB) indicated an increase in their expression during oxidative stress. Although Vit E inhibited NFκB activation, it stimulated AP1 expression. The results support the findings that alcohol induces oxidative stress in nervous tissue. The data further show that vitamin E can mitigate the toxic effects of alcohol and thus can be suitable as a potential therapeutic agent for alcohol-induced oxidative damage in brain.

Keywords: Alcohol, AP1, Cerebral hemispheres, Neurotoxicity, NFκB, Vit. E

Metabolism of alcohol is tightly connected with oxidative stress1. After chronic and excessive consumption, alcohol may accelerate oxidative mechanisms both directly via increased production of reactive oxygen species2 and indirectly by impairing protective mechanisms against them3. Alteration of the redox status of cells following chronic alcohol consumption may have profound effects on cellular function and viability leading to cell death and tissue damage4. These changes linked to pathologic processes in the organism are related to alteration of intracellular signaling pathways associated with protein kinases and transcription factors activation4. Mainly mitogen activated protein kinase family (MAPK) family transcription factors—nuclear factor κB (NF-κB) and activator protein 1 (AP-1) are involved in the deterioration of cells and organs4.

Chronic and excessive abuse causes many biochemical and physiological changes in the brain like alterations of specific neurotransmitter systems5 and intricate signaling pathways6. A recognized mechanism of alcohol action is its ability to enhance oxidative stress in the brain7. Increased NF-κB activation has been shown by both acute8 and chronic9 alcohol treatment.

Because of its antioxidative properties, vitamin E may help prevent diseases/disorders associated with oxidative stress, such as cardiovascular disease, cancer, chronic inflammation and neurologic disorders10. Vitamin E is a potent antioxidant and its role as an inhibitor (chain breaker) of lipid peroxidation is well established11,12.

Keeping these facts in view, the present study has been carried out to investigate the oxidative stress status and expression of AP1 and NFκB in cerebral hemispheres after chronic alcohol treatment of mice. Further, modulation of alcohol-induced oxidative stress by vitamin E was also studied.

Materials and Methods

Animals—Male Balb/c mice (25-30 g body weight, 8-10 weeks of age) were obtained from the Central Animal House, Panjab University, Chandigarh. The animals were kept in plastic cages under hygienic conditions and were provided standard animal feed.

Treatment protocol—The animals (18) were randomly divided into following 3 groups of 6 animals each: Group I control, Group II alcohol treated and Group III alcohol + vitamin E treated. Group II animals were given 8% ethanol (in tap
water) as a sole source of drinking fluid during the entire period of treatment (15 days). Group III animals received orally daily 0.2 ml of 5 IU/kg body weight of vitamin E (in olive oil) along with drinking water containing 8% ethanol. Control group animals received only 0.9% saline.

Body weight of animals and their daily intake of fluids were recorded on alternate days. At the end of the treatment period, the mice were killed by cervical dislocation after ip injection of sodium phenobarbitone (50 mg/kg body weight) as approved by the Institutional Animal Ethics Committee. Brains were immediately taken out and cerebral hemispheres including basal ganglia, diencephalon and mesencephalon from each mouse were taken separately. To obtain post mitochondrial fraction (PMF)\textsuperscript{15}, 10% homogenate prepared in ice cold 50 m\text{M} Tris-HCl buffer (pH 7.4) was centrifuged at 10,000 rpm for 30 min at 4°C. Further assays were carried out in PMF.

**Lipid peroxidation**—Lipid peroxidation was determined by the method in which development of pink color due to TBA-MDA (thiobarbituric acid-malondialdehyde) chromophore was taken as an index of lipid peroxidation\textsuperscript{16}.

**Activity of antioxidant enzymes**—Catalase activity was estimated directly by the method in which the decrease in the absorption of light at 220-240 nm due to decomposition of hydrogen peroxide by the enzyme (catalase) gives a measure of catalase activity\textsuperscript{17}. GST activity was measured by using 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate\textsuperscript{18}. Superoxide dismutase (SOD) activity was estimated by the method based on the principle of inhibition of the rate of oxidation of nitro blue tetrazolium (NBT) using hydroxylamine hydrochloride. The units of SOD activity was expressed as Units/mg protein\textsuperscript{19}.

**Redox status**—Total and oxidized (GSSG) glutathione were quantitated by the fluorimetric method\textsuperscript{20}. The fluorescence intensity for the OPT-GSH product at pH 8.0 was directly related to glutathione concentration. Reduced glutathione (GSH) levels were quantitated by subtracting GSSG/mg protein from total GSH/mg protein. Redox ratio was determined by taking the ratio of reduced GSH to oxidized GSSG.

Protein concentration in the PMF was determined by the method using bovine serum albumin (BSA) as standard\textsuperscript{21}.

RNA isolation and RT-PCR—Total RNA isolation was done using TRI REAGENT (Molecular Research Centre Inc. Ohio, USA). RT–PCR was done by using the one step method of RT–PCR kit (QIAGEN, Germany). RNA template (3 µg) from different groups was used in RT-PCR reaction. Optimal oligonucleotide primer pairs for RT-PCR of genes viz. c-jun, c-fos, p65 (Nuclear Factor-κB), Inhibitory-κB factor (IκB) and γ-glutamylcysteinyl synthetase (γGCS) are listed in Table 1.

After an initial denaturation step of 1 min at 94°C, 35 amplification cycles were performed. Each cycle included an initial denaturation step at 94°C for 45 sec, annealing at 56°C (γGCS, NF-κB and IκB), or 58°C (cjun, cfos) for 45sec and extension at 72°C for 45 sec. A final extension step of 5 min at 72°C was performed in order to complete the PCR reaction. The amplified product was analyzed on 1.5% agarose gel electrophoresis and densitometric analysis of bands was done by Image J Software (NIH, USA).

**Statistical analysis**—Statistical analyses were performed using the Student’s ‘t’ test. Biochemical data are expressed as mean ± SD of 6 observations for each group. Values of $P < 0.05$ were considered significant.

**Results**

Alcohol administration caused a significant increase in lipid peroxidation (MDA level), activities of catalase and glutathione-S-transferase, and a

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>γGCS</td>
<td>Forward 5'-CCT TCT GGC ACA GCA CGT TG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TAA GAC GGC ATC TCG CTC CT-3'</td>
</tr>
<tr>
<td>c-fos</td>
<td>Forward 5'-ATG GGC TCT CCT GTC AAC AC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ATG ATG CCG GAA ACA AGA AG-3'</td>
</tr>
<tr>
<td>c-jun</td>
<td>Forward 5'-ATG GGC ACA TCA CCA CTA CA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TGA GTT GGC ACC CAC TGT TA-3'</td>
</tr>
<tr>
<td>p65 (NF-κB)</td>
<td>Forward 5'-TGG CGA GAG AAG CAC AGA TA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TGT TGG TCT GGA TTC GCT G-3'</td>
</tr>
<tr>
<td>IκB</td>
<td>Forward 5'-CTG CAG GCC ACC AAC TAC AA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GGC CTC CAA ACA CAC AGT CA-3'</td>
</tr>
</tbody>
</table>

Table 1—Primer pairs
decrease in the levels of superoxide dismutase, total glutathione, oxidized glutathione, and reduced glutathione. Co-administration of vit. E with alcohol reversed the above observed changes (Table 2).

A significant decrease in redox ratio was observed in alcohol-treated mice brains. The ratio increased in mice co-administered with vitamin E in comparison to alcohol-treated mice (group II) (Table 2).

RT PCR analysis—Activator Protein 1 (AP1): A significant increase in the expression of c-jun and c-fos was observed in alcohol-treated mice brains (group II) (Table 3). Vitamin E co-administration resulted in a further increase in the expression of both in comparison to alcohol-treated mice brains of group II (Fig. 1a and b).

Nuclear Factor-kappaB (NF-κB): Alcohol treatment of mice resulted in a significant increased expression of p65 subunit of NF-κB as well as of IκB in comparison to controls. Coadministration of vitamin E in alcohol-treated mice caused a significant decrease in the expression of both p65 and IκB in comparison to alcohol-treated mice of group II (Fig. 1c and d).

γGlutamyl cysteine synthetase (γGCS): The brains of alcohol-treated mice showed a significant decrease in the expression of γGCS in comparison to the brains of controls. A significant increase in the expression of γGCS was observed in the brains of mice coadministered with vitamin E (group III) in comparison to the brains of alcohol-treated mice (Fig. 1e).

Discussion
Significantly increased levels of MDA were found in alcohol-treated mice cerebral hemispheres. This reflects alcohol-induced oxidative stress in the brain and production of oxygen-derived free radicals.

<table>
<thead>
<tr>
<th>Table 2—Effect of alcohol treatment and vitamin E supplementation on various biochemical parameters</th>
<th>Group I (Control)</th>
<th>Group II (Alcohol-treated)</th>
<th>Group III (Alcohol + Vit E-treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxidation (nmole MDA/mg protein)</td>
<td>1.35±0.03</td>
<td>1.59±0.03c</td>
<td>1.36±0.03a</td>
</tr>
<tr>
<td>Catalase (μmole H$_2$O$_2$ decomposed/min/mg protein)</td>
<td>4.72±0.28</td>
<td>8.72±0.49c</td>
<td>7.89±0.17c</td>
</tr>
<tr>
<td>Glutathione S-transferase (μmole CDNB conjugated/min/mg protein)</td>
<td>6.35±0.43</td>
<td>9.05±0.66c</td>
<td>6.16±0.36c</td>
</tr>
<tr>
<td>Superoxide dismutase (Units/mg protein)</td>
<td>2.91±0.12</td>
<td>1.99±0.13b</td>
<td>3.14±0.09b</td>
</tr>
<tr>
<td>Total glutathione (nmole/mg protein)</td>
<td>45.1±1.59</td>
<td>22.4±0.73c</td>
<td>38.7±1.06c</td>
</tr>
<tr>
<td>Oxidized Glutathione (GSSG) (nmole/mg protein)</td>
<td>10.05±0.26</td>
<td>16.03±0.15c</td>
<td>11.01±0.2c</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH) (nmole/mg protein)</td>
<td>35.10±1.4</td>
<td>6.43±0.81c</td>
<td>27.7±1.2c</td>
</tr>
<tr>
<td>Redox ratio (GSH/GSSG)</td>
<td>3.48±0.09</td>
<td>0.39±0.05c</td>
<td>2.52±0.16c</td>
</tr>
</tbody>
</table>

P values: *< 0.05, **< 0.01, ***< 0.001 as compared to Gr. I; **< 0.01, ***< 0.001 as compared to Gr. II

<table>
<thead>
<tr>
<th>Table 3—Densitometry analysis of effects of alcohol treatment and vitamin E supplementation on mRNA expression by RT-PCR in mice cerebral hemispheres</th>
<th>Group I (Control)</th>
<th>Group II (Alcohol-treated)</th>
<th>Group III (Alcohol + Vit E-treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-jun</td>
<td>106.38±4.72</td>
<td>130.22±2.66c</td>
<td>208.42±7.96c</td>
</tr>
<tr>
<td>c-fos</td>
<td>56.88±2.37</td>
<td>104.08±3.99g</td>
<td>155.04±4.05</td>
</tr>
<tr>
<td>p65 (NF-κB)</td>
<td>80.64±2.41</td>
<td>114.72±12.9g</td>
<td>72.05±7.88</td>
</tr>
<tr>
<td>IκB</td>
<td>58.81±3.71</td>
<td>84.32±7.69h</td>
<td>45.93±4.29</td>
</tr>
<tr>
<td>γGCS</td>
<td>80.07±3.59</td>
<td>41.82±4.66e</td>
<td>61.52±3.88</td>
</tr>
</tbody>
</table>

P values: *<0.05, **<0.01, ***<0.001 as compared to Gr. I; **<0.01, ***<0.001 as compared to Gr. II
Increases in the activities of catalase and glutathione S-transferase (GST) were found in alcohol-treated mice cerebral hemispheres. Located largely in peroxisomes, the catalase pathway catabolizes $H_2O_2$, which results largely from the metabolism of fatty acids via peroxisomal $\beta$-oxidation\(^{22,23}\). In the brain, catalase has a dual action; on one hand it generates acetaldehyde from alcohol and on the other hand it plays a protective role against oxidative stress\(^{24}\). The protective increase in the activity of catalase observed in the present study could also be supported by the studies which suggested a competition between ethanol and inhibitors for the $H_2O_2$-catalase compound\(^{25}\).

GST is a detoxifying enzyme induced by a variety of electrophilic drugs and toxins\(^{26}\). It is mainly involved in the free radical scavenging, peroxide reduction and detoxification of GSH-S-Conjugates\(^{27,28}\). The relatively easy inducibility of this enzyme by alcohol is shown to indicate its role in detoxification of alcohol\(^{29}\). Thus increased activities of both catalase and GST as observed in our study may be explained as an adaptive response against ROS overproduction.

Superoxide dismutase catalyses the dismutation of superoxide anion ($O_2^-$) to hydrogen peroxide in cell cytosol and mitochondria\(^{30}\). Decreased SOD levels may be responsible for the increased levels of toxic oxygen radicals in cerebral hemispheres. Inhibition of SOD activity by alcohol further indicates alcohol-induced oxidative stress.

Glutathione (GSH) is one protective mechanism that serves to limit the oxidative damage. Therefore, depletion of intracellular GSH and accumulation of GSSG is a biochemical hallmark of oxidative stress\(^{31}\). In the present study a significant decrease in GSH levels, a significant increase in GSSG levels and a decrease in redox ratio (GSH/GSSG) following alcohol administration were observed. The brain maintains a high ratio of GSH/GSSH for antioxidant defense and thus depletion of total glutathione and a decreased GSH/GSSH ratio are markers for oxidative stress in the brain\(^{22,23}\). With above facts in view the mRNA expression of $\gamma$-glutamyl cysteine synthetase ($\gamma$GCS, a regulating enzyme involved in the maintenance of GSH content) was studied during alcohol treatment. A significant decrease in the mRNA expression of $\gamma$GCS in cerebral hemispheres of alcohol-treated mice was observed. In light of the above information it may be proposed that alcohol-

---

**Fig. 1**—Levels of mRNA expression of (a) c-jun, (b) c-fos (c) p65 subunit (NFkB), (d) IxB and (e) $\gamma$GCS in mice cerebral hemispheres after 15 days of respective treatments. [Values are Mean ± SD of 4 observations. $P$ values: $^a<0.05$, $^b<0.01$, $^c<0.001$ as compared to Gr. I; $^e<0.01$, $^f<0.001$ as compared to Gr. II]
induced GSH depletion via a decrease in the mRNA expression of γGCS contributes to lipid peroxidation during alcohol treatment.

The alcohol-induced changes in MDA levels, antioxidant enzymes, and non-enzymatic antioxidant GSH during alcohol treatment were reversed by vitamin E co-administration. Thus, our study demonstrates the protective effect of vitamin E against chronic alcohol exposure.

In order to investigate the influence of alcohol-induced oxidative stress on transcription factors, mRNA expressions of p65 subunit of NF-κB, IκB, c-jun and c-fos in cerebral hemispheres of mice were studied. Alcohol-treated mice cerebral hemispheres showed significantly increased mRNA expression of both IκB and p65 subunit of NFκB. IκB controls the activity of NF-κB. The expression of IκB therefore reflects the activity of NF-κB and represents a well accepted and powerful tool to investigate the regulation of this transcription factor. As IκB becomes phosphorylated and degraded upon extracellular stimulation, its increased mRNA expression could be possibly due to replacement of degraded IκB by activated NF-κB through an autoregulatory loop. Vitamin E supplementation reduced the expression of both NFκB (p65) and IκB. Increased NF-κB activation has been shown in the rat brain cerebral cortex, showing thereby an upregulation of the production and expression of inflammatory mediators in the brain, and activation of signaling pathways and transcription factors (involved in inflammatory damage and cell death) by chronic ethanol treatment. As NF-κB is redox sensitive, its increased expression in the brain is indicative of the disturbed redox status in cells.

Superoxide and hydrogen peroxide induce the expression of several early response genes including c-fos and c-jun. Perturbation of cellular thiol redox status has also been suggested to be a signal that may be implicated in the induction of c-fos and c-jun expression. The present study showed an increased mRNA expression of both c-jun and c-fos in alcohol-treated mice cerebral hemispheres. Vitamin E coadministration, however, potentiated the expression of both c-jun and c-fos. This observation can be explained on the basis of previous studies which showed that both antioxidant and prooxidant conditions induce AP-1. It is thus possible that in the present study vitamin E exerted its antioxidant effect against alcohol-induced oxidative stress-related toxicity by the increased expression of c-jun and c-fos.

In conclusion, the present study strengthens the evidence that alcohol-promoted oxidative stress was the major cause of alcohol toxicity in cerebral hemispheres. The results further showed that alcohol-induced changes in the activities of enzymatic and non-enzymatic defenses, mRNA expressions of NFκB, AP-1 and γGCS are reversed by vitamin E which mitigates the toxic effects of alcohol on the brain. Vitamin E thus may be a potential therapeutic agent for alcohol-induced oxidative damage in brain.

References


