Effect of lecithin with vitamin-B complex and tocopheryl acetate on long term effect of ethanol induced immunomodulatory activities

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The alcoholic liver disease usually causes overall immunological alterations which might be attributed to hepatic disease, to ethanol action, and/or to malnourishment. In the present study, efficacy of lecithin with vitamin-B complex to treat ethanol induced immunomodulatory activity was compared with the effect of lecithin alone and tocopheryl acetate (vitamin E). Ethanol (1.6 g/kg body wt/day for 12 weeks) exposure increased thiobarbituric acid reactive substance (TBARS) level, while decreased superoxide dismutase (SOD) activity and reduced glutathione (GSH) content in whole blood hemolysate of 8-10 week-old male BALB/c mice (weighing 20-30 g). The activities of transaminase (AST and ALT) enzymes, interleukin (IL)-10 and gamma interferon (IFN-γ) elevated, while IL-2 and IL-4 reduced in mice serum due to ethanol exposure. These suggested that oxidative stress and immunomodulatory activities were interdependent and associated with ethanol induced liver damage. Lecithin treatment significantly reduced AST (32.44%), ALT (32.09%), IL-10 (25.63%) activities and TBARS content (12.76%) compared to ethanol treated group. However, lecithin with vitamin-B complex treatment, significantly reduced AST (62.83%); ALT (61.96%); IL-10 (35.88%); IFN-γ (22.55%) activities and TBARS content (31.58%), while significantly elevated GSH content (36.49%) and SOD activity (61.21%). Tocopheryl acetate treatment significantly reduced AST (62.83%); ALT (61.54%); IL-10 (36.35%); IFN-γ (23.28%) activities and TBARS content (35.84%), while significantly elevated GSH content (28.76%) and SOD activity (62.42%) compared to ethanol treated group. These findings persuasively argued that lecithin with vitamin-B complex was a new promising therapeutic approach in controlling ethanol induced immunomodulatory activities involving liver damage processes. Prevention of oxidative stress with correction of nutritional deficiency caused alteration in the ethanol-induced immunomodulatory activities and associated liver diseases.

Keywords: Cytokines, Ethanol, Glutathione, Interleukin, Lecithin, Liver, Vitamin-B complex

Alcoholic liver disease (ALD) develops as a consequence of priming and sensitizing mechanisms rendered by cross interactions of primary mechanistic factors, and secondary risk factors. The alcoholic liver disease usually causes overall immunological alterations which might be attributed to the hepatic disease itself, to ethanol action and/or to malnourishment. Many processes related to the consumption or breakdown of alcohol that contribute to alcohol-induced liver disease are mediated by small proteins known as cytokines, which are produced and secreted by liver cells and many other cells throughout the body. Cytokines are multifunctional proteins that play a critical role in cellular communication and activation. Cytokines have been classified as being proinflammatory (T helper 1, Th1) or anti-inflammatory (T helper 2, Th2) depending on their effects on the immune system. However, cytokines impact a variety of tissues in a complex manner that regulates inflammation, cell death, cell proliferation and migration as well as healing mechanisms. Ethanol is known to alter cytokine levels in a variety of tissues. In the liver, persistent cytokine secretion resulting in chronic inflammation leads to conditions such as hepatitis, fibrosis, and cirrhosis. Because of their diverse functions, cytokines might make attractive targets in the prevention or treatment of alcoholic liver disease.
Th1- and Th2-associated cytokines tend to be reciprocally regulatory; IFN-γ inhibits Th2-associated functions, and IL-4 and IL-10 inhibit Th1-associated functions. In extreme cases, primary or secondary immune responses may develop exclusively in either a Th1 or Th2 response pattern and thus impair the body’s overall ability to combat infection.

Prevention of oxidative stress along with correction of nutritional deficiency is one of the proposed mechanisms of therapeutic approach. Glutathione depletion is considered to be the most important sensitizing mechanism. One of the contributing factors is decreased methionine metabolism. Ethanol feeding induced alterations in mitochondrial membrane phospholipid and fatty acid composition with resulting impairment in GSH uptake. Phospholipid methylation is required for maintenance of normal membrane fluidity and function. Therefore, administration of phosphatidyl choline has been advocated for prevention and treatment of alcoholic liver disease.

Malnutrition is a constant accompaniment of ALD, it is logical to question whether the prognosis of ALD can be improved by provision of nutritional support along with the source of antioxidants. We have already shown the efficacy of lecithin with vitamin-B complex to treat ethanol induced oxidative stress and compared with lecithin treatment alone. Tocopheryl acetate (vitamin E) is well-accepted nature’s most effective antioxidant. Effectiveness of lecithin with vitamin B complex was further compared with tocopheryl acetate (vitamin E). Further, efficacy of these treatments was also evaluated in alcohol induced immunomodulatory activities specifically in glutathione (GSH) levels, thiobarbituric acid reactive substances (TBARS) levels, aminotransferase activities, superoxide dismutase activities and Th1/Th2 cytokine response pattern.

Materials and Methods

Chemicals—Ethanol was purchased from Bengal Chemicals, Kolkata. Cytokine kits from BD Biosciences (Becton, Dickinson and Company, USA) were used. Other chemicals were purchased from E. Merck, Sisco Research Laboratory (SRL), India; and Sigma Chemical Co., St. Louis, USA.

Animal selection—Male BALB/c mice (8-10 weeks old, 20-30 g) were housed in plastic cages inside a well-ventilated room at 25±2°C, 60-70% RH, and under 12 hr light/dark cycle. All mice had free access to standard diet and water ad libitum. The animals were weighed daily and their general condition was recorded including their daily intake of liquid. The Animal Ethics Committee of the Institution approved the procedures in accordance with the CPCSEA guideline.

The mice were divided into 5 groups having 6 individuals in each group. Group I served as control and were fed normal diet and water; Group II mice were treated with ethanol (1.6 g ethanol/kg body wt/day for 12 weeks); Group III mice were treated with lecithin + ethanol (1.6 g ethanol and 500 mg lecithin together/kg body wt/day for 12 weeks); Group IV mice were treated with lecithin + vitamin B-complex + ethanol (1.6 g ethanol and 500 mg lecithin mixed with vitamin-B complex together/kg body wt/day for 12 weeks); and Group V mice were treated with tocopheryl acetate + ethanol (1.6 g ethanol and 80 mg tocopheryl acetate/kg body wt/day for 12 weeks).

Vitamin-B complex consisting of thiamine mononitrate (10 mg), riboflavine (10 mg), pyridoxine hydrochloride (3 mg), cyanocobalamin tritrate (15 mcg), nicotinamide (45 mg), and calcium pantothenate (50 mg) was supplemented per kg body weight. Tocopheryl acetate was suspended in distilled water; and lecithin or vitamin-B complex were freshly dissolved in distilled water during treatment. Ethanol was diluted with distilled water to get desired concentration and fed orally by intragastric infusion technique.

Methods

After 12 week, blood samples were collected from retoroorbital plexus. Serum was separated and used for aminotransferases (aspartate, AST and alanine, ALT) activities and cytokines levels (IL-2, IL-4, IL-10) and IFN-γ estimation. Cytokines were estimated using Sandwich ELISA according to manufacturer’s instruction. Hemolysate, prepared from whole blood was used for reduced glutathione (GSH) content, thiobarbituric acid reactive substance (TBARS) level, and superoxide dismutase (SOD) activity estimation.

Statistical analysis—all data were analyzed using the statistical package SPSS (version 11.0, SPSS Inc., Chicago, IL). Results are expressed as mean ± SE (standard error). The sources of variation for multiple comparisons were assessed by the analysis of variance (ANOVA), followed by Post Hoc test. The difference were considered significant at P<0.05.
In the present study, group I served as normal control, while group II served as experimental control. Level of significance mentioned in the text is compared to group II values. Detailed analysis is given in the corresponding tables.

**Results**

Effect of lecithin, lecithin with vitamin B-complex and tocopheryl acetate was observed on transaminases (AST and ALT) activities, superoxide dismutase (SOD) activity, reduced glutathione (GSH) content and thiobarbituric acid reactive substances (TBARS) level (Table 1). Results showed that ethanol feeding sharply increased transaminases activities and TBARS levels, while diminished GSH content and SOD activity. Although lecithin could significantly reverse transaminases activities and TBARS content; lecithin with vitamin B complex and tocopheryl acetate significantly reversed all parameters.

Serum cytokine (IL-2, IL-4, IL-10 and IFN-γ) levels of mice exposed to different treatments protocol has been presented in Table 2. Ethanol treatment significantly reduced IL-2 and IL-4 levels, while significantly increased IL-10 and IFN-γ levels. Although ethanol with lecithin treatment could reduce IL-10 activity significantly, lecithin with vitamin B complex or tocopheryl acetate treatment significantly reduced IL-10 and IFN-γ levels compared to ethanol treated group.

**Discussion**

Present study demonstrated that GSH levels, TBARS levels, transaminase (AST and ALT) activities, SOD activities and Th1 or Th2 cytokine response patterns were involved in immune responses and disease processes. Ethanol treatment significantly increased AST activity, ALT activity and TBARS level, while diminished GSH content and SOD activity in serum or whole blood; indicating that these animals might be suffering from liver damage and oxidative stress could be one of the plausible causes for these abnormal features. The long term effects of ethanol induced liver damage were further evaluated through immune pattern and disease processes and also different treatment options were evaluated.

Interleukin-2 (IL-2) was originally described as “T-cell growth factor” present in lymphocyte conditioned medium which was able to maintain the growth of cytotoxic T cells for relatively long periods. IL-2 stimulates dramatic proliferation of activated (antigen or lectin) T lymphocytes. It also stimulates cytolytic activity of subsets of T lymphocytes, enhances T cell motility, and induces secretion of other cytokines such as IFN-γ, IL-4, and tumor necrosis factor (TNF). It is therefore a T cell differentiation factor. It stimulates the proliferation of activated B lymphocytes, and promotes the induction of immunoglobulin secretion. In the present study, IL-2 level was significantly reduced in serum of ethanol treated group.

**Table 1**—Effects of lecithin, lecithin with vitamin B-complex and tocopheryl acetate on aspartate aminotransferase (AST) activity, alanine aminotransferase (ALT) activity, reduced glutathione (GSH) content, thiobarbituric acid reactive substances (TBARS) level and superoxide dismutase (SOD) activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
<th>GSH (μg/mg protein)</th>
<th>TBARS (nmol/ml)</th>
<th>SOD (U/mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18 ± 1.23</td>
<td>20.17 ± 1.64</td>
<td>66.5± 2.47</td>
<td>3.21 ± 0.11</td>
<td>3.41 ± 0.21</td>
</tr>
<tr>
<td>Ethanol</td>
<td>226 ± 20.18a</td>
<td>202 ± 23.96a</td>
<td>38.83±3.17a</td>
<td>6.11 ± 0.2a</td>
<td>1.65±0.07a</td>
</tr>
<tr>
<td>Lecithin</td>
<td>152.67±20.53bc</td>
<td>137.17±21.29bc</td>
<td>45.67±1.76bc</td>
<td>5.33±0.2af</td>
<td>2.15±0.07a</td>
</tr>
<tr>
<td>Lecithin + vitamin</td>
<td>84 ± 7.55cdi</td>
<td>76.83 ± 6.54cd</td>
<td>53.0±1.36be</td>
<td>4.18±0.17bdi</td>
<td>2.66±0.07bdj</td>
</tr>
<tr>
<td>B complex</td>
<td>(-62.83)</td>
<td>(-61.96)</td>
<td>(+36.49)</td>
<td>(-31.58)</td>
<td>(+61.21)</td>
</tr>
<tr>
<td>Tocopheryl acetate</td>
<td>84 ± 3.97cdi</td>
<td>77.67 ± 4.04di</td>
<td>50.0±1.57af</td>
<td>3.92±0.11cfd</td>
<td>2.68±0.07bdi</td>
</tr>
<tr>
<td>F value</td>
<td>34.668</td>
<td>21.985</td>
<td>22.274</td>
<td>49.063</td>
<td>31.474</td>
</tr>
<tr>
<td>Significance</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Significant at P: a< 0.001, b< 0.01, c< 0.05 compared to control group and; d< 0.001, e< 0.01, f< 0.05 compared ethanol treated group; g< 0.001, h< 0.01, i< 0.05 compared to lecithin with ethanol treated group.

Figures in the parentheses are % increase (+) or decrease (-) compared to experimental control group (Group II)
treated rats compared to the control group. It has been reported that IL-2 activity is significantly reduced in patients with autoimmune chronic active hepatitis, primary biliary cirrhosis and alcoholic hepatitis with or without cirrhosis, may be due to low IL-2 production or presence of an IL-2 antagonist(s)\textsuperscript{25}. Such an abnormality may occur, not only as a result of liver damage, but may also be important in determining immunological disturbances involved in the pathogenesis of the liver disease.

Interleukin-4 (IL-4) acts as a costimulator of B cell proliferation with anti-Ig M\textsuperscript{26}. This molecule also increased the expression of class II MHC molecules on resting B cells. The most striking features of the biological activity of IL-4 are the number of cell types on which IL-4 acts, the numerous activities it induces, and the pleiotropic manner in which the activities occur. Ethanol addition to B lymphocytes \textit{in vitro} has been shown to inhibit antigen-induced antibody secretion and antigen-induced cell proliferation\textsuperscript{26}. Ethanol inhibits IL-4-induced B-cell proliferation and IL-4-induced Ig class switching\textsuperscript{27}. In this study, IL-4 level was reduced in mice serum due to ethanol exposure. In another study, IL-4 mRNA were lower in the group exhibiting inflammatory liver injury\textsuperscript{28}. Down regulation of anti-inflammatory cytokines may additionally exacerbate liver injury.

Interleukin-10 (IL-10) is a pleiotropic cytokine with anti-inflammatory and immunosuppressive activities. Ability of endogenous IL-10 to modulate inflammatory response and to limit hepatotoxicity has been shown in several models of liver injury\textsuperscript{29}. Pro-inflammatory cytokines play an important role in alcohol-induced liver injury. Alcohol exposure results in predominantly pro-inflammatory cytokine secretion leading to liver injury. This anti-inflammatory cytokine, known for its hepatoprotective effects, is secreted simultaneously with pro-inflammatory cytokines. IL-10 may also limit alcohol-induced liver damage by counteracting the effects of pro-inflammatory cytokines\textsuperscript{29}. The IL-10 level was elevated in mice serum due to ethanol treatment for 12 weeks in the present study. Upregulation of pro- and anti-inflammatory cytokine system and simultaneous desensitisation of effector cells could explain the restricted systemic inflammatory response to chronic endotoxaemia. Evidence suggests that ethanol treatment resulted in elevated interleukin IL-10 (Th2 cytokine) production\textsuperscript{30}. Ethanol can induce a dysbalance of monocyte-derived mediator production at the expense of Th1 cytokines\textsuperscript{30}. This alteration in immune status may lead to impairment of host defences against infections, which are frequent complications of alcoholic cirrhosis\textsuperscript{31}.

Most work with mice has involved 2-week ethanol exposures or less, which result in decreased IFN-\(\gamma\) responses. In the present work, we administered 1.6g ethanol/kg body wt/day per animal for 12 weeks. Chronic alcohol consumption polarizes the immune response away from Th1-mediated cell-mediated immunity\textsuperscript{32} and functional alteration in Th1-mediated IFN-\(\gamma\) production occurred in the population fed

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-10</th>
<th>IFN-(\gamma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22 ± 1.31</td>
<td>169 ± 16.32</td>
<td>210.67 ± 14.55</td>
<td>336.33 ± 11.66</td>
</tr>
<tr>
<td>Ethanol (\text{+10.68})</td>
<td>15.45 ± 1.14(\text{a})</td>
<td>107.67 ± 4.99(\text{b})</td>
<td>353 ± 28.69(\text{a})</td>
<td>476.67 ± 19.82(\text{a})</td>
</tr>
<tr>
<td>Lecithin (\text{+25.63})</td>
<td>17.1 ± 0.74(\text{b})</td>
<td>123.67 ± 5.75(\text{c})</td>
<td>262.5 ± 27.74(\text{c})</td>
<td>423.5 ± 11.73(\text{b})</td>
</tr>
<tr>
<td>Lecithin + vitamin B complex(\text{+14.86})</td>
<td>19.41 ± 0.64(\text{b})</td>
<td>120 ± 9.89(\text{c})</td>
<td>226.33 ± 8.74(\text{c})</td>
<td>369.17 ± 12.14(\text{d})</td>
</tr>
<tr>
<td>Tocopheryl acetate(\text{+16.25})</td>
<td>19.01 ± 0.49(\text{b})</td>
<td>125.17 ± 9.19(\text{c})</td>
<td>224.67 ± 7.75(\text{c})</td>
<td>365.67 ± 8.61(\text{d})</td>
</tr>
<tr>
<td>F value</td>
<td>7.212</td>
<td>5.376</td>
<td>8.606</td>
<td>17.523</td>
</tr>
</tbody>
</table>

Significant at \(P: \text{a} < 0.001, \text{b} < 0.01, \text{c} < 0.05\) compared to control group and; \(\text{d} < 0.001, \text{e} < 0.01\), \(\text{f} < 0.05\) compared ethanol treated group; \(\text{g} < 0.001, \text{h} < 0.01\), \(\text{i} < 0.05\) compared to lecithin with ethanol treated group

Figures in the parentheses are % increase (+) or decrease (-) compared to experimental control group (Group II)
ethanol-containing liquid diets. In this study, long-term ethanol consumption by mice caused an increase in IFN-γ production. This result is in agreement with the study carried out by others. The overall results support the concept that in humans as well as in mice, chronic alcohol exposure of sufficient duration results in T cell activation or sensitization in vivo and consequently there is an increased percentage of the effector/memory subset.

Chronic ethanol intake enhances the damaging consequences. Alcohol consumption can lead to increased endotoxin levels in the blood and liver. When activated, Kupffer cells produce signaling molecules (i.e., cytokines) that promote inflammatory reactions as well as molecules called reactive oxygen species (ROS), which can damage liver cells. Agents that deplete GSH have been shown to alter immune function. Ethanol, for example, down-regulates cell-mediated immune responses and up-regulates humoral immune responses. Subtle changes in GSH levels may have profound effects on the immune response.

In this study, we have shown that treatment with lecithin, lecithin with vitamin-B complex and tocopheryl acetate could reverse ethanol induced alterations. The methionine pool is partially conserved at the expense of lecithin as studied by change in body weight and biochemical parameters. Although folate and B12 deficiency contribute to the fall in methionine synthase activity, the enzyme is also postulated to be inactivated by possibly forming adducts with acetaldehyde or nitrosylated derivative. Prevention of oxidative stress with correction of nutritional deficiency is one of the proposed mechanisms for the therapeutic approach.

From the present study, it can be concluded that oxidative stress and immunomodulatory activities are interdependent and associated with ethanol induced liver damage. Tocopheryl acetate supplementation is a potential therapeutic agent. However, lecithin with vitamin-B complex supplementation significantly reversed immunomodulatory activity in ethanol induced liver damage. Together, these findings persuasively argue that lecithin with vitamin-B complex is a new promising therapeutic approach in controlling ethanol induced immunomodulatory activities and liver damage processes.

References


