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Time-dependent effects of ethanol on blood oxidative stress parameters and cytokines

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Alcohol consumption is implicated in the genesis of a spectrum of liver abnormalities, which are associated with a number of factors. In the present study, time-dependent effects of ethanol on cytokines (TNF-α, IL-2, IL-4, IL-10, IFN-γ, VEGF-A and TGF-β1) in serum, and blood oxidative stress parameters such as reduced glutathione content, TBARS level and activities of GPx, GR, GST, catalase and SOD in 8-10 weeks-old male BALB/c mice have been investigated. Ethanol administered @1.6 g/kg body wt/day significantly increased the activities of liver marker enzymes AST, ALT and ALP. Serum nitrite levels and haemolysate TBARS level also increased, while total antioxidant status in serum and GSH content in whole blood hemolysate decreased from 4th week onwards of exposure. Inspite of the increased serum nitrite level and GST activity in the haemolysate, albumin level in serum, GPx and GR activities in haemolysate decreased after 12 weeks of exposure. Chronic ethanol treatment did not show any effect on IL-2, but IL-4 level was reduced and other cytokines such as IL-10, TNF-α, IFN-γ, TGF-β1 and VEGF-A levels were increased significantly after 12 weeks. The study indicates a relationship between free radical generation and immune response, and suggests that ethanol-induced liver damage is associated with oxidative stress and immunological alterations in a time-dependent manner.

Keywords: Cytokines, Ethanol, Interleukin, Liver, Oxidative stress

Alcoholic beverages have been used in human societies since the beginning of recorded history. The relation between alcohol consumption and health outcomes is complex and multi-dimensional[1]. Many processes related to consumption or breakdown of alcohol that contribute to alcohol-induced liver disease are mediated by cytokines[2]. Ethanol-associated endotoxaemia and subsequent release of inflammatory mediators may cause lethal hepatocyte injury via oxyradical-dependent or -independent mechanisms[3] and may eventually result in cirrhosis[4]. Tissue repair plays a critical role in determining the final outcome of toxicity, i.e., recovery from injury or progression of injury, leading to liver failure and death. It also encompasses regeneration of hepatic extracellular matrix and angiogenesis, the processes necessary to completely restore the structure and function of the liver[5].

In the present study, we have investigated time-dependent effects of ethanol on cytokines in serum and blood oxidative stress parameters along with liver marker enzymes in mice.

Materials and Methods

Materials

Ethanol from Bengal Chemicals, Kolkata; thio-barbituric acid from Loba Chemie; and 4-amino-phenazone, 3,5-dichloro-2-hydroxybenzenesulfonic

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-s-transferase; H_2O_2, hydrogen peroxide; IFN, interferon; IL, interleukin; $NO^{\bullet}$, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

acid (3,5-DCHBS), glutamate dehydrogenase and yeast glutathione reductase (GR) from Sigma Chemical Co., St. Louis, USA were purchased. All other chemicals were purchased from Sisco Research Laboratory, India. Total antioxidant status (TAS) kit from Randox Laboratories Ltd, UK; transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF)-A ELISA kits from Bender Medsystems, Austria; and other cytokine kits from BD Biosciences (Becton, Dickinson & Company, USA) were used.

Animals and treatment

Six male BALB/c mice (8-10 weeks old, weighing 20-30 g) were housed in plastic cages inside a well-ventilated room under standard conditions[4,6]. All mice had free access to standard diet[4,6] and water *ad libitum*. The animals were weighed daily and their general condition was recorded including their daily intake of liquid. A dose of 1.6 g ethanol/kg body wt/day was selected for administration to mice as it found tolerable and simultaneously caused maximum liver damage in male Wistar rats in our previous dose-dependent study[7]. Ethanol was diluted with distilled water to get desired concentration and fed orally. The Animal Ethics Committee of the Institution approved the procedures in accordance with the CPCSEA guideline.

Blood samples were collected from retroorbital plexus of mice, prior to start of the ethanol feeding (0 week), and at the end of 4th and 12th week of ethanol treatment. Serum was separated and used for protein[8], albumin[9], aspartate transaminase (AST)[10], alanine transaminase (ALT)[10], alkaline phosphatase (ALP)[11], nitrite[12], and total antioxidant status (TAS)[13]. Cytokines [tumor necrosis factor (TNF)-α, interleukins (IL-2, IL-4, IL-10), interferon (IFN)-γ, VEGF-A and TGF-β1] were estimated using Sandwich ELISA, according to manufacturer's instruction. Haemolysate, prepared from whole blood[14] was used for the estimation of reduced glutathione (GSH)[15], thiobarbituric acid reactive substance (TBARS)[16], nitrite[12] levels and the activities of glutathione peroxidase (GPx)[17], glutathione reductase (GR)[18], glutathione-s-transferase (GST)[19], catalase[6] and superoxide dismutase (SOD)[20].

Statistical analysis

All data were analyzed using the statistical package SPSS (version 11.0, SPSS Inc., Chicago, IL). Results were expressed as mean ± SD (standard deviation). The sources of variation for multiple comparisons were assessed by the Analysis-of-variance (ANOVA), followed by Post-hoc test with Bonferroni's and Tukey's multiple comparisons test. The difference were considered significant at $P<0.05$.

Results

Time-dependent effects of ethanol on mice blood were demonstrated in this study. Though there was no significant change in serum protein level due to ethanol exposure, albumin level decreased significantly after 12th week of ethanol exposure, compared to the control or 4 weeks of ethanol-exposed groups (Table 1). AST, ALT, ALP activities and nitrite level were elevated significantly, whereas total antioxidant status decreased after 4th week, and further down after 12th week of ethanol exposure (Table 1).

The GSH level decreased and TBARS level increased significantly after 4th week of ethanol exposure (Table 2). Nitrite level in haemolysate was significantly increased after 12th week, compared to the control group (Table 2). No significant change was observed in GPx, GR, GST, SOD and catalase activities after 4th week of ethanol exposure (Table 2). However, the activities of GPx, GR and catalase decreased significantly, while the activity of GST increased in animals treated with ethanol for 12 weeks, when compared with 4 weeks of ethanol exposure or control group (Table 2). Interestingly, SOD activity decreased significantly after 12 weeks of ethanol exposure, compared to the 4 weeks of ethanol-exposed group, but not compared to the control group (Table 2).

Ethanol treatment showed no effect on interleukin (IL)-2, but IL-4 level was reduced, and other cytokines (IL-10, TNF-α, IFN-γ, VEGF-A and TGF-β1) levels increased significantly after 12 weeks of ethanol exposure (Table 3).

Discussion

The present study demonstrated that oxidative stress and cytokine response patterns were involved in immune responses and disease processes. Increased activities of liver marker enzymes viz., AST, ALT and ALP in response to the chronic ethanol exposure, when compared with control group in a time-dependent manner (Table 1) indicated that these animals suffered from liver damage. Though protein deficiency is often associated with liver disease due to decreased dietary intake or deficiencies in digestion

Table 1—Effect of ethanol on serum protein fractions and liver enzyme activities

[Values represent mean ± SD of 6 rats in each group]

	Protein (g%)	Albumin (g%)	Globulin (g%)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Nitrite (μ mole)	TAS (m*M*)
Control	4.4 ± 0.23	3.23 ± 0.16	1.16 ± 0.08	18.17 ± 2.31	18.17 ± 1.6	41.3± 2.16	8.5± 1.37	3.41± 0.14
4th week	4.37 ± 0.18	3.1 ± 0.1	1.26 ± 0.13	58 ± 3.22[a]	51.17 ± 2.3[a]	61.67 ± 2.58[a]	50 ± 12.76[a]	2.78 ± 0.49[c]
12th week	4.11 ± 0.17	2.77 ± 0.18[ae]	1.34 ± 0.15	124 ± 7.9[ad]	87 ± 6[ad]	94.57 ± 4.31[ad]	140.4 ± 14.24[ad]	1.56 ± 0.24[ad]
F-variance	4.03	15.354	3.045	654.853	470.072	448.604	234.425	49.415
Significance	0.058	<0.001	0.076	<0.001	<0.001	<0.001	<0.001	<0.001

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to control group; [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to 4th week of ethanol-exposed mice group

Table 2—Effect of ethanol on reduced glutathione (GSH) content, thiobarbituric acid reactive substances (TBARS) level, nitrite level, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-s-transferase (GST) activities in haemolysate

[Values represent mean ± SD of 6 rats in each group]

	GSH content (μg/mg protein)	TBARS (nmol/ml)	Nitrite (n*M*)	GPx (U/g haemoglobin)	GR (nmol NADPH oxidized/min/ mg protein)	GST (nmol CDNB conjugate formed/ mg protein/min)	SOD (U/mg haemoglobin)	Catalase (mmol H_2O_2 decomposed/ mg) protein/ min
Control	3.25 ± 0.23	3.2 ± 0.09	0.19 ± 0.03	2.95 ± 0.18	0.28 ± 0.04	0.17 ± 0.01	3.38 ± 0.48	0.31 ± 0.02
4th week	2.83 ± 0.14[b]	4.29 ± 0.26[a]	0.22 ± 0.03	2.65 ± 0.18	0.26 ± 0.02	0.19 ± 0.02	3.5 ± 0.26	0.3 ± 0.02
12th week	1.88 ± 0.17[ad]	5.92± 0.41[ad]	0.26 ± 0.03[b]	2.18 ± 0.22[ae]	0.18 ± 0.02[ae]	0.27 ± 0.03[ad]	2.95 ± 0.21[f]	0.26 ± 0.01[be]
F- variance	4.617	137.591	7.126	23.545	17.223	26.456	4.888	12.809
Significance	<0.001	<0.001	0.006	<0.001	<0.001	<0.001	0.022	<0.001

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to control group; [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to 4th week of ethanol-exposed mice group

Table 3—Effect of ethanol on different cytokines

[Values represent mean ± SD of 6 rats in each group]

	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)	VEGF-A (pg/ml)	TGF-β (ng/ml)
Control	20.3 ± 3.5	176.3 ± 33.2	191.6 ± 69.0	477.3 ± 76.2	885.8 ± 169.8	134.6 ± 29	0.32 ± 0.06
4th week	20.3 ± 3.1	164.1 ± 34.8	183.5 ± 46	445.5 ± 69.6	737.5 ± 120.3	213.2 ± 23.3	0.35 ± 0.04
12th week	19.5 ± 3.4	118.8 ± 21.0[c]	485 ± 75.5[be]	1832.3 ± 510.3[cf]	1698.3 ± 429.7[cf]	364.3 ± 96.0[ae]	0.56 ± 0.09[ad]
F- variance	0.122	5.997	12.926	6.782	5.971	22.93	22.606
Significance	0.886	0.012	0.001	0.008	0.012	<0.001	<0.001

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to control group; [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to 4th week of ethanol exposed mice group.

and absorption[21], but no significant change in serum protein level of ethanol-treated mice was observed (Table 1). Progressive hypoalbuminemia, a common feature of chronic alcoholic liver disease[22,23] was also observed in this study (Table 1). A significant decrease in total antioxidant status in serum from 4th week of ethanol exposure (Table 1) indicated that oxidative stress could be one of the plausible causes for abnormal liver function, due to ethanol treatment.

There was a significant increase in nitrite level in serum from 4th week (Table 1) and in haemolysate after 12th week (Table 2) of ethanol exposure. Nitric oxide ($NO^{\bullet}$) appears to play a multitude of roles in ethanol-mediated liver disease[24]. Its production may play a dual role, mediating protective effects at lower concentrations and tissue damage by over production[24]. $NO^{\bullet}$ significantly contributes to the prosurvival/proangiogenic process of capillary formation and maturation by triggering and transducing cell growth and differentiation[25]. It enhances proliferation and migration of endothelial cells *in vitro*[26] and plays vital role in vasodilation[25]. Much of the toxicity of $NO^{\bullet}$ may be mediated by forming more potent oxidants like peroxynitrite [$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$][24].

GSH is a major non-protein thiol in living organisms, which plays a key role in coordinating the body's antioxidant defense processes. Depletion of GSH (Table 2) renders the cell more susceptible to oxidative stress[27]. Elevated lipid peroxidation or TBARS (Table 2), mediated by free radicals is considered to be a primary mechanism of cell membrane destruction and cellular damage[28]. GSH is also known to function as a substrate for GPx and GST[29]. Decreased activity of GPx in the mice after 12th week of ethanol exposure might be due to inactivation of the enzyme by reactive oxygen species (ROS)[30]. Decreased GR activity after 12th week of ethanol exposure might be a predominant cause for GSH depletion within the RBC, leading to serious consequences like increased lipid peroxidation and haemolysis[7]. GST plays an essential role in liver by eliminating toxic compounds by conjugating them with GSH[7] and its activity increased after 12th week of ethanol exposure (Table 2). Alterations in GPx, GR and GST activities, followed by thiol depletion are important factors sustaining a pathogenic role for oxidative stress[7].

SOD catalytically dismutes superoxide radical anion ($O_2^{\bullet-}$) to H_2O_2, while catalase and GPx render H_2O_2 harmless within cells by converting it into water and oxygen[31]. However, prolonged ethanol exposure (12 weeks) decreased SOD and catalase activities in the present study (Table 2), indicating further that chronic ethanol treatment was associated with severe oxidative stress. Decreased catalase activity might be due to loss of NADPH or generation of superoxide or increased activity of lipid peroxidation or combination of all[7].

Cytokines are multi-functional proteins that play a critical role in cellular communication, activation, inflammation, cell death, cell proliferation and migration as well as healing mechanisms[2]. Though IL-2 stimulates the proliferation of activated B lymphocytes[32] and promotes the induction of immunoglobulin secretion[33], no significant change was found in IL-2 level due to ethanol exposure in this study (Table 3). Ethanol inhibits IL-4-induced B-cell proliferation and IgG class switching[34] and IL-4 level was reduced due to ethanol exposure in this study (Table 3).

Downregulation of anti-inflammatory cytokines may additionally exacerbate liver injury. Though IL-10 with anti-inflammatory and immunosuppressive activities may limit alcohol-induced liver damage[35], but interestingly, long-term (12 weeks) ethanol exposure enhanced IL-10 level in this study (Table 3). Long-term ethanol consumption also caused alteration in IFN-γ production and a high concentration of TNF-α in serum (Table 3). IFN-γ has been reported to induce proinflammatory cytokine TNF-α[36]. These alterations in immune status may lead to impairment of host defenses against infections, which are frequent complications of alcoholic cirrhosis[37].

Transforming growth factors (TGF)-β are pleiotropic, multi-functional cytokines with a wide range of biological effects, which include cell-cycle control, regulation of early development, haematopoiesis, angiogenesis, immune functions, and induction of apoptosis[24]. The complexity and diversity of TGF-β mediated effects are demonstrated through its multiple roles in immune system suppression, wound healing, and fibrosis. Three structurally similar isoforms of TGF-β (TGF-β1, 2, and 3), encoded by three distinct genes have been identified[24]. TGF-β1 is the prevalent isoform and is found almost ubiquitously, whereas other isoforms are expressed in a more limited spectrum of cells and tissues[24]. In the present study, TGF-β1 level increased with duration

of ethanol exposure (Table 3), which is believed to be involved in hepatic fibrosis[36].

Vascular endothelial growth factor (VEGF), a glycosylated peptide with multiple isoforms potently induces endothelial proliferation[38] and increases vascular permeability[24]. VEGF-A and B participate in the regulation of normal and pathological angiogenesis, while VEGF-C and D are involved in lymphangiogenesis[24]. In the present study, VEGF-A level increased significantly after 12 weeks of ethanol exposure (Table 3), indicating that long-term ethanol consumption might be associated with physiological or pathological angiogenesis.

Alcohol is recognized as an important immunomodulator because of its ability to regulate the functions of immunocompetent cells. During chronic alcohol intoxication, upregulation of chemokine release is time-dependent[39]. Increased cytokines expression was observed after 12 weeks, but no significant alteration was observed after 4 weeks of ethanol feeding (Table 3). Concomitantly, lipid peroxidation was increased as observed in other study[40]. ROS, such as super oxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are involved in the signaling pathways mediating stress and growth responses including angiogenesis[41]. Nitric oxide ($NO^{\bullet}$) is an upstream and downstream regulator of VEGF-mediated angiogenesis[42]. Depletion of GSH is the most important sensitizing mechanism rendered by ethanol feeding to TNF-α induced cell death[43]. TNF-α and other cytokines may be associated with releasing TGF-β[36]. Alteration of these biologically active substances during alcohol consumption contributes to pathogenesis of alcoholic liver disease[39].

In conclusion, the present study demonstrated that chronic ethanol-induced liver damage was associated with oxidative stress and immunological alterations in a time-dependent manner.

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