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EFFECTS OF CHRONIC ETHANOL CONSUMPTION IN BLOOD: A TIME DEPENDENT STUDY ON RAT

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ABSTRACT

Alcohol consumption and health outcomes are complex and multidimensional. Ethanol (1.6g / kg body weight/ day) exposure initially affects liver function followed by renal function of 16-18 week-old male albino rats of Wistar strain weighing 200-220 g. Chronic ethanol ingestion increased in thiobarbituric acid reactive substances level and glutathione s-transferase activity; while decreased reduced gluatathione content and activities of catalase, glutathione peroxidase and glutathione reductase in a time dependent manner in the hemolysate. Though superoxide dismutase activity increased initially might be due to adaptive response, but decreased later. Elevation of serum nitrite level and transforming growth factor-b_1 activity indicated that long-term ethanol consumption may cause hepatic fibrosis and can elicit pro-angiogenic factors. However, no alteration in vascular endothelial growth factor-C activity indicated that ethanol consumption is not associated with lymphangiogenesis. Therefore, we conclude that long-term ethanol-induced toxicity is linked to an oxidative stress, which may aggravate to fibrosis and elevate pro-angiogenic factors, but not associated with lymphangiogenesis.

KEY WORDS

Ethanol, Glutathione, Liver function, Nitric oxide, Oxidative stress, Transforming growth factor,Vascular endothelial growth factor.

INTRODUCTION

Alcoholic beverages and the problems they engender, have been familiar in human societies since the beginning of recorded history. Alcohol is causally related to more than 60 medical conditions and overall, 3.5% of the global burden of disease is attributable to alcohol (1). Ethanol associated endotoxaemia and subsequent release of inflammatory mediators may cause hepatocyte injury via oxyradical-dependent and -independent mechanisms. Cells are protected against oxidation by the action of certain enzymes, vitamins, and other substances, known collectively as antioxidants. When the balance between the ROS (reactive oxygen species) production and antioxidant defence is lost, "oxidative stress" results, which through a series of events deregulates the cellular functions leading to various pathological conditions (2). Acute and chronic ethanol consumption causes hypoxia (3). Local low levels of oxygen have been postulated to induce several factors involved in angiogenesis (4).

Therefore, in this study we focussed on examining the expression and function of pro-angiogenic molecules along with oxidative stress in the setting of chronic alcoholic liver diseases.

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MATERIALS AND METHODS

Ethanol was purchased from Bengal Chemicals, Kolkata. Chemicals were purchased from Sisco Research Laboratory (SRL), India; Sigma Chemical Co., St. Louis, USA; and E. Merck. Transforming growth factor (TGF)-b and vascular endothelial growth factor (VEGF)-C ELISA kits were purchased from Bender Med Systems, Austria.

Six male albino Wistar strain rats of 16-18 weeks-old weighing 200- 220 g were used. The animals were housed in plastic cages inside a well-ventilated room. The room was maintained under standard husbandry condition. All rats had free access

of standard diet (5). Food and water were given *ad libitum*. The animals were weighed daily and its general condition was recorded including their daily intake of liquid. 1.6 g ethanol/ kg body weight/ day was administered. 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 was collected from retero-orbital plexus of animals prior to start the ethanol feeding (0 week), and at the end of 4, 12 and 36 weeks of ethanol treatment. Serum was separated.

Total protein (6), albumin (7), creatinine (8), nitrite (9) in serum and ascorbic acid in plasma (10) were estimated. Enzyme activities such as transaminases (alanine aminotransferases (ALT, EC 2.6.1.2) and aspartate aminotransferases (AST, EC 2.6.1.1)) (11), alkaline phosphatases (ALP, EC 3.1.3.1) (12), γ-glutamyl transpeptidase (GGT, EC 2.3.2.2) (13) were also monitored. Agarose gel electrophoresis was used to separate rat serum proteins and quantitated by densitometry. Serum VEGF and TGF-β_1 were estimated following manufacturer's instruction. Hemolysate were prepared from venous blood samples, which were collected in EDTA containing vacutainers (14) and were used for estimation of reduced glutathione (GSH) content (15), lipid peroxidation (16) and activities of catalase (EC 1.11.1.6) (5), glutathione reductase (GR; EC 1.6.4.2) (17), glutathione S-transferase (GST; EC 2.5.1.18) (18), glutathione peroxidase (GPx; EC 1.11.1.9) (19) and superoxide dismutase (SOD; EC 1.15.1.1) activity (20).

All data were analyzed using the statistical package SPSS (version 11.0, SPSS Inc., Chicago, IL). Results are 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. The differences were considered significant at $P<0.05$.

RESULTS

Urea and creatinine levels were elevated significantly after 12 weeks of ethanol exposure compared to the control group (Table 1). Though serum protein and albumin levels were decreased significantly due to ethanol exposure compared to the control group (Table 1), but showed no effect on globulin level (Table 1). Serum protein electrophoretogram (Fig 1), subsequent scanning densiometric analysis (Fig 2 and Fig 3) and a comparative study (Table 2) of ethanol fed and control animals also revealed it. Activities of liver specific enzymes such as AST, ALT, ALP and GGT increased significantly in response to duration of ethanol exposure (Table 3).

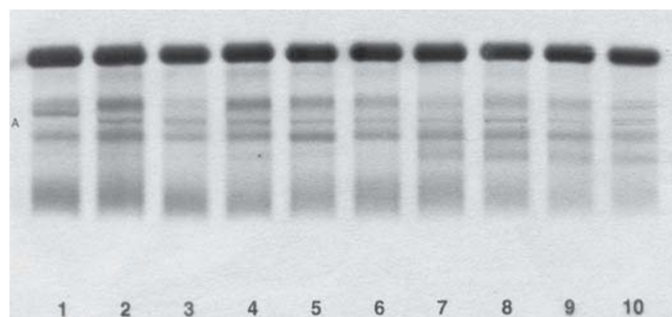

Fig 1: Serum protein electrophoretogram. Lane 1 & 2: Control rats, Lane 3 & 4: 4 weeks ethanol treated rats; Lane 5 & 6: 12 weeks ethanol treated rats; Lane 7 & 8: 24 weeks ethanol treated rats; Lane 9 & 10: 36 weeks ethanol treated rats.

Compared to the control group, reduced glutathione content in the hemolysate decreased significantly after 12 weeks of ethanol exposure, while thiobarbituric acid reactive substances (TBARS) level elevated significantly after 4 weeks of ethanol exposure (Table 4). Although 12 weeks of ethanol exposure

Table 1: Effects of ethanol on protein, albumin, globulin and creatinine levels in serum of rats for different time period [Values are mean ± SD of 6 rats in each group]

	Protein (g %)	Albumin (g %)	Globulin (g %)	Urea (mg %)	Creatinine (mg %)
Control	7.33 ± 0.15	4.25 ± 0.21	3.08 ± 0.13	30.0 ± 1.51	0.43 ± 0.05
4 weeks	6.55 ± 0.31^{a}	3.53 ± 0.25^{a}	3.01 ± 0.14	30.8 ± 1.44	0.49 ± 0.02
12 weeks	6.18 ± 0.28^{a}	3.1 ± 0.12^{ae}	3.08 ± 0.19	39.3 ± 4.08^{bf}	0.51 ± 0.02^{c}
24 weeks	6.06 ± 0.17^{af}	3.06 ± 0.1^{ae}	2.98 ± 0.07	44.8 ± 5.2^{ad}	0.57 ± 0.04^{af}
36 weeks	5.91 ± 0.25^{ae}	2.85 ± 0.1^{ad}	3.06 ± 0.17	54.5 ± 6.0^{adgk}	0.71 ± 0.05^{adgj}
F value	32.59	64.669	0.529	37.27	36.584
Significance	<0.001	<0.001	0.715	<0.001	<0.001

P values: ^{a}< 0.001, ^{b}< 0.01, ^{c}< 0.05 compared to control group and; ^{d}< 0.001, ^{e}< 0.01, ^{f}< 0.05 compared to 4 weeks ethanol treated group; ^{g}< 0.001, ^{h}< 0.01, ^{i}< 0.05 compared to 12 weeks ethanol treated group; ^{j}< 0.001, ^{k}< 0.01, ^{l}< 0.05 compared to 24 weeks ethanol treated group.

Table 2: Serum Protein Electrophoresis Report for 36 weeks of ethanol treated and control animal.

Fraction	%	
	Control (n=6)	Alcoholic (n=6)
Albumin	61.2 ± 1.5	51.5 ± 2.3*
α_1	5.8 ± 0.5	4.4 ± 0.3
α_2	3.9 ± 0.4	6.7 ± 0.3
β	9.2 ± 0.8	12.5 ± 0.8
γ	19.9 ± 1.9	24.9 ± 1.2

Values are mean ± SD of n number of rats.
P value: *<0.05 compared to the control group.

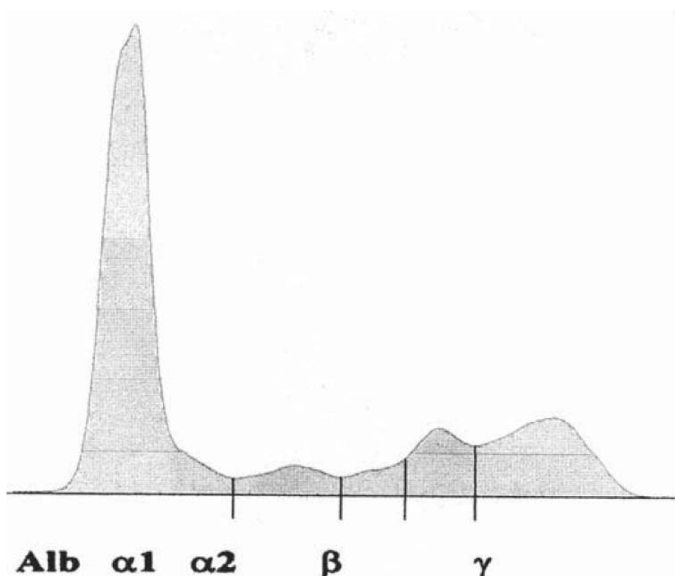

Fig 2: Serum protein electrophoretogram for a Control animal

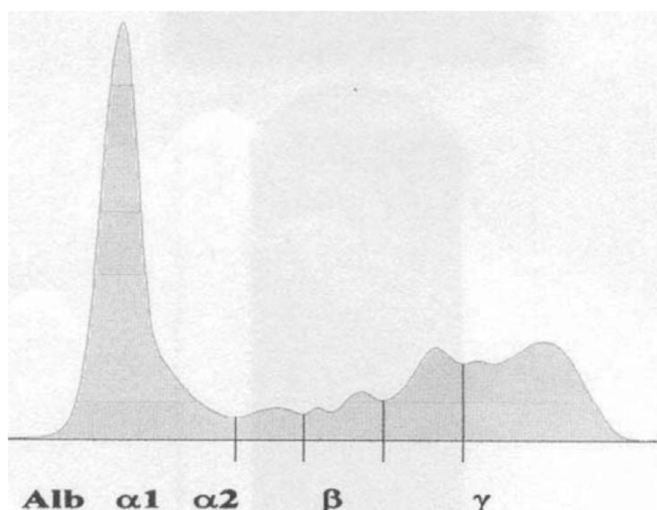

Fig. 3. Serum protein electrophoretogram for alcoholic rat (Lane 5)

caused significant change in GR and GST activities; GPx activity reduced significantly after 36 weeks of ethanol exposure compared to the control group (Table 4). While catalase activity reduced significantly due to ethanol exposure, interestingly, superoxide dismutase (SOD) activity elevated significantly up to 12 weeks of ethanol exposure and then decreased (Table 4).

Ethanol exposure reduced ascorbic acid content compared to the control group, and elevated nitrite level in the blood in a time dependent manner (Table 5). Though serum TGF-β1 level elevated significantly after 12 weeks of ethanol exposure (Table 5) but no significant change in VEGF-C level was observed due to ethanol exposure (Table 5).

DISCUSSION

Decreased serum protein and albumin levels and elevated activities of serum enzymes like AST, ALT, ALP and γGT,

Table 3: Effect of ethanol on liver specific enzymes activities (IU/L) in serum of rats for different time period [Values are mean ± SD of 6 rats in each group]

	AST	ALT	GGT	ALP
Control	29.63 ± 2.26	33.57 ± 1.88	7.67 ± 0.82	89.72 ± 6.49
4 weeks	57.83 ± 5.64[b]	53.66 ± 5.27[c]	39.5 ± 7.69[a]	118.5 ± 7.34[a]
12 weeks	143.66 ± 17.31[ad]	111 ± 6.84[ad]	91.83 ± 10.46[ad]	139.17± 11.44[af]
24 weeks	164.83 ± 34.36[ad]	138.66 ± 16.05[adh]	105.17 ± 12.89[ad]	154.67± 14.26[ad]
36 weeks	231.83 ± 23.45[adgj]	209.17 ±19.29[adgj]	123.83 ±16.8[adi]	180.83± 8.82[adgk]
F value	97.759	208.282	113.718	71.052
Significance	<0.001	<0.001	<0.001	<0.001

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to control group and; [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to 4 weeks ethanol treated group; [g]< 0.001, [h]< 0.01, [i]< 0.05 compared to 12 weeks ethanol treated group; [j]< 0.001, [k]< 0.01, [l]< 0.05 compared to 24 weeks ethanol treated group.

Table 4. Effect of ethanol on reduced glutathione (GSH) content, thiobarbituric acid reactive substance (TBARS) level, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, glutathione s-transferase (GST) activity, catalase activity and superoxide dismutase (SOD) activity in hemolysate of rats for different time period

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

Parameters	Control	4 weeks	12 weeks	24 weeks	36 weeks	F value	Significance
TBARS (nmol/ ml)	0.638 ± 0.012	0.926 ± 0.022[a]	1.016 ± 0.1[a]	1.05 ± 0.094[af]	1.288 ± 0.066[adgj]	68.942	<0.001
GSH (mg / ml)	40.95 ± 4.15	37.57 ± 4.03	33.18 ± 3.4[bf]	30.05 ± 1.64	27.88 ± 2.77[ah]	15.525	<0.001
GPx (U/ g haemoglobin)	33.33 ± 6.25	29.83 ± 3.43	27.5 ± 3.78	25.5 ± 2.88	22.67 ± 2.16[bf]	6.384	0.001
GR (nmol NADPH oxidized/min/mg protein)	1.7 ± 0.14	1.62 ± 0.12	1.47 ± 0.09[c]	1.37 ± 0.07	1.32 ± 0.1[ae]	13.221	<0.001
GST (nmol CDNB conjugate formed/ mg protein/ min)	1.16 ± 0.07	1.22 ± 0.12	1.37 ± 0.12[c]	1.38 ± 0.09	1.5 ± 0.14[ae]	8.746	<0.001
Catalase (mmol H_2O_2 decomposed/ mg protein / min)	38.76 ± 0.56	32.63 ± 0.81[a]	30.22 ± 2.47[a]	29.76 ± 1.28[a]	27.91 ± 3.52[ae]	25.158	<0.001
SOD (U/mg haemoglobin)	6.33 ± 0.15	9.07 ± 0.21[a]	9.15 ± 0.69[a]	7.47 ± 0.73[bde]	6.08 ± 0.19[dgek]	57.184	<0.001

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to control group and; [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to 4 weeks ethanol treated group; [g]< 0.001, [h]< 0.01, [i]< 0.05 compared to 12 weeks ethanol treated group; [j]< 0.001, [k]< 0.01, [l]< 0.05 compared to 24 weeks ethanol treated group.

indicated that liver damage starts after 4 weeks of ethanol exposure. Significant elevation in blood urea and creatinine levels after 12 weeks of ethanol exposure compared to the control group indicated initiation of kidney damage. This is presumably because of the presence of higher concentration of alcohol dehydrogenase in the liver than kidney, which catalyzes alcohol to its corresponding aldehyde (21). Common features of chronic alcoholic liver disease are progressive hypoalbuminemia (22). Ethanol inhibits the secretion of protein from the liver. Chronic ethanol abuse results in intrahepatic accumulation of export-type proteins and decreased plasma levels. These effects appear to be mediated by acetaldehyde (23).

Ethanol-induced liver injury may be linked, at least in part, to an oxidative stress resulting from increased free radical production and/ or decreased antioxidant defence. Though erythrocytes are prone to oxidative damage due to presence of polyunsaturated fatty acids (PUFA), heme, iron and oxygen; but these can counteract the oxidative stress due to the presence of antioxidant enzymes (24).

Elevated Lipid peroxidation mediated by free radicals is considered to be a primary mechanism of cell membrane destruction and cell damage (25). Depletion of GSH renders the cell more susceptible to oxidative stress (26). Increased GST activity and decreased GPx and GR activities, followed by thiol depletion are important factors sustaining a pathogenic role for oxidative stress (14). The increase in erythrocyte SOD activity initially may probably be an adaptive response towards

Table 5: Effect of ethanol on ascorbic acid in plasma, and nitrite, TGF-β_1 and VEGF-C levels in serum of rats for different time period
[Values are mean ± SD of 6 rats in each group]

	Ascorbic acid (mg/dl)	Nitrite (micromole)	VEGF-C (pg/ ml)	TGF-β_1 (pg/ ml)
Control	2.2 ± 0.14	18.1 ± 1.26	22.9 ± 3.55	15.8 ± 3.32
4 weeks	1.9 ± 0.18[c]	37.1 ± 1.97[a]	25.8 ± 3.31	18.6 ± 2.16
12 weeks	1.7 ± 0.14[a]	67.6 ± 4.27[ae]	26.6 ± 3.72	26.3 ± 3.5[ae]
24 weeks	1.7 ± 0.07[a]	104.9 ± 15.3[adg]	28.8 ± 7.36	37.8 ± 4.91[adg]
36 weeks	1.6 ± 0.14[af]	139.7 ± 24.77[adg]	29 ± 5.58	41.5 ± 2.88[adg]
F value	17.209	110.158	1.52	65.646
Significance	<0.001	<0.001	0.227	<0.001

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to control group and; [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to 4 weeks ethanol treated group; [g]< 0.001, [h]< 0.01, [i]< 0.05 compared to 12 weeks ethanol treated group; [j]< 0.001, [k]< 0.01, [l]< 0.05 compared to 24 weeks ethanol treated group.

oxidative stress (27). But, long term ethanol exposure diminished its activity. Decreased catalase activity due to ethanol exposure may be due to loss of NADPH, or generation of superoxide, or increased activity of lipid peroxidation or combination of all (28).

Ethanol-induced toxicity is protected by ascorbic acid and its level decreased with duration of ethanol exposure. It has often been viewed that low levels of nitric oxide (NO·) are cytoprotective, whereas higher levels lead to cytotoxicity. Cytotoxicity of NO· may also be related to the genesis of oxidative intermediates such as peroxynitrite, a product of NO· and superoxide (29).

Reactive oxygen species such as super oxide anion ($O_2{\cdot}^-$) and hydrogen peroxide (H_2O_2) are involved in the signaling pathways mediating any stress and growth responses, including angiogenesis (30). In endothelial cells, H_2O_2 stimulates cell migration, proliferation (31) and was found to induce VEGF mRNA (32). Nitric oxide (NO·) is an upstream and downstream regulator of VEGF mediated angiogenesis (33). VEGF participates in the regulation of normal (physiological or therapeutic) and pathological angiogenesis (VEGF-A, VEGF-B) and lymphangiogenesis (VEGF-C, VEGF-D) (34). No significant change in VEGF-C level in this study indicated that long-term ethanol exposure is not associated with lymphangiogenesis. However, chronic ethanol consumption associated increased TGF-b_1 level is believed to be involved in hepatic fibrosis (35) in this study.

This study revealed ethanol (1.6 g/ kg body weight/ day) ingestion perturbs the antioxidant system and caused deleterious effects on animals in a time dependent manner. These are associated with hepatic fibrosis and elevate pro-angiogenic factors, but not associated with lymphangiogenesis.

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