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Modulation of lecithin activity by vitamin-B complex to treat long term consumption of ethanol induced oxidative stress in liver

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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. Chronic alcohol abuse and its progression to ALD are associated with abnormal metabolism and low tissue or plasma levels, or both, of many micronutrients. Glutathione depletion is considered the most important sensitizing mechanism. In the present study efficacy of lecithin with vitamin-B complex to treat ethanol induced oxidative stress was compared with the effect of lecithin alone, tocopheryl acetate (vitamin E), as well as capacity of hepatic regeneration during abstention. Ethanol (1.6g / kg body weight/ day for 4 weeks) affects body weight in 16-18 week old male albino rats of Wistar strain weighing 200-220 g. Thiobarbituric acid reactive substance level, nitrite content, protein carbonyl group level, redox ratio (oxidized to reduced glutathione ratio), superoxide dismutase activity, and glutathione s-transferase activity significantly increased on ethanol exposure. Whereas reduced glutathione content, and activities of catalase, glutathione reductase and glutathione peroxidase significantly reduced due to ethanol exposure. These changes were reversed by different treatment. The results suggest that tocopheryl acetate (vitamin E) could partially reverse these changes and act as a potential therapeutic agent. However, lecithin with vitamin-B complex treatment is a promising therapeutic approach. Furthermore, preventive measures were more effective than curative treatment. Prevention of oxidative and nitrosative stress along with correction of nutritional deficiency is one of the proposed mechanisms for the therapeutic approach.

Keywords: Ethanol, Lecithin, Liver, Oxidative stress, Tocopherol, Vitamin-B Complex

The pendulum of opinion on whether malnutrition plays an important role in the pathogenesis of alcoholic liver disease (ALD) has swung in either directions[1]. Alcohol is a food that provides 7.1 kcal/g energy. It is most widely used drug in our society, with a potential for addiction and damage to multiple organs, particularly the liver[2]. Prolonged alcohol consumption has profound effects on metabolic pathways that influence the stages of fat accumulation, oxidant liver injury, and fibrosis. Chronic alcohol abuse and its progression to ALD are associated with abnormal metabolism and low tissue or plasma levels, or both, of many micronutrients[1]. Malnutrition is a frequent accompaniment of chronic alcohol abuse and is always present to varied degrees when chronic alcoholism progress to ALD[2].

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. This concept, albeit not novel, is becoming widely accepted by the field, and more research is directed toward identifying and characterizing the interfaces of the cross interactions to help understand individual predisposition to the disease. Glutathione depletion is considered the most important sensitizing mechanism. One of the contributing factors is decreased methionine metabolism[3].

The liver plays a central role in methionine metabolism, as half of the daily methionine is catabolized here. The first step in methionine metabolism is the formation of S-adenosylmethionine (SAM) catalyzed by methionine adenosyltransferase (MAT). Under normal conditions, most of the 6-8 g of SAM generated per day is used in transmethylation reaction. SAM is converted to homocysteine[4, 5].

There are three pathways that metabolize homocysteine (Fig. 1). One is the trans-sulfuration pathway, which converts homocysteine to cysteine. This is a unique pathway present only in the liver and lens[6] that condenses homocysteine with serine to form cystathionine in a reaction catalyzed by cystathionine

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β-synthase (CBS)[6,7]. Cleavage of cystathionine, catalyzed by another enzyme, γ-cystathionase, then releases free cysteine, the rate-limiting precursor for reduced glutathione (GSH) synthesis[6]. The other two pathways resynthesize methionine from homocysteine[4,7].

An alternate salvage pathway for homocysteine methylation occurs in the liver and kidney, where choline is precursor to betaine, the substrate for betaine homocysteine methyltransferase (BHMT). Endogenous betaine is maintained as a product of choline, which in turn is provided in the diet or as an endogenous product of phosphatidylcholine (PC), the major phospholipid

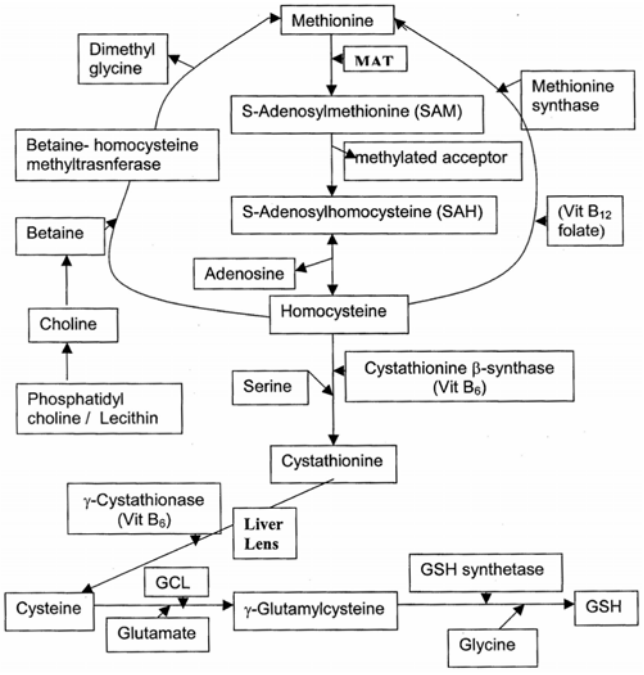

Fig. 1—Hepatic methionine metabolism. Methionine adenosyltransferase (MAT) catalyzes the synthesis of SAM for methionine and adenosine triphosphate; SAM is then converted to S-adenosylhomocysteine (SAH) as the methyl group is transferred. SAH hydrolase catalyzes the reversible hydrolysis of SAH to yield homocysteine and adenosine. Homocysteine in the liver can undergo three metabolic pathways, two of which regenerate methionine from homocysteine; the third is the transsulfuration pathway, which converts homocysteine to cysteine. In the transsulfuration pathway, cystathionine β-synthase catalyzes the formation of cystathionine. Both enzymes require normal levels of vitamin B_6 as cofactor. Cysteine is the rate limiting precursor for GSH synthesis as glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis, catalyzes the formation of γ-glutamylcysteine from cysteine and glutamate, and GSH synthetase catalyzes the formation of GSH from –glutamylcysteine and glycine. Methionine synthase regenerates methionine from homocysteine in a reaction that requires normal levels of folate and vitamin $B_{12,}$ and betaine homocysteine methyl transferase regenerates methionine from homocysteine that requires betaine.

constituent of cell membranes. Methionine that enters from the diet or is synthesized by the methionine synthase (MS) and BHMT reactions is converted to SAM by methionine adenosyl transferase (MAT)[6].

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[8]. SAM plays a central role in DNA nucleotide balance through its negative regulatory effect on methyl tetrahydrofolate reductase (MTHFR) and in promoting the production of antioxidant GSH[9]. It is believed that the protective effect of SAM is via increased GSH level, changing in DNA-methylation status and inhibiting TNF release by macrophages. But, controversy has surrounded whether SAM can be taken up by hepatocytes[7]. Therefore administration of phosphatidyl choline has been advocated for prevention and treatment of alcoholic liver disease[3].

Because 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. In the present study, efficacy of lecithin with vitamin-B complex to treat ethanol induced oxidative stress has been compared with the effect of lecithin alone. Tocopheryl acetate (vitamin E) is well-accepted nature's most effective antioxidant. Therefore, effectiveness of lecithin with vitamin B-complex has been further compared with vitamin E, as well as capacity of hepatic regeneration during abstention.

Materials and Methods

Chemicals—Ethanol was purchased from Bengal Chemicals, Kolkata. Fine chemicals were purchased from Sisco Research Laboratory (SRL), India; and Sigma Chemical Co., St. Louis, USA; and analytical grade chemicals from E.Merck or SRL.

Animal Selection—Male albino rats of Wistar strain (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 (25°± 2°C, 60-70% RH, and 12 hr light/dark cycle). All rats had free access of standard diet[10]; with modification containing (%) bengal gram, 31; gingelly oil cake, 30; wheat, 28; polished rice, 10; salt mixture, 0.5; vitamin-mineral mixture, 0.3; yeast with fish or liver oil, 0.2. Food and water were given *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 rats were divided into the following 9 groups of 6 each:

Group I: control rats — the rats were fed normal diet and water.

Group II: ethanol treated rats (1.6g ethanol/ kg body weight/ day for 4 weeks)

Group III: tocopheryl acetate + ethanol treated rats (1.6g ethanol and 80mg tocopheryl acetate/kg body weight/ day for 4 weeks)

Group IV: ethanol followed by tocopheryl acetate treated rats (1.6g ethanol/kg body wt/day for 4 wks, followed by 80mg tocopheryl acetate/kg body weight/day for 4 weeks)

Group V: lecithin + ethanol treated rats (1.6g ethanol and 500mg lecithin together/kg body weight/ day for 4 weeks)

Group VI: ethanol followed by lecithin treated rats (1.6g ethanol/kg body weight/ day for 4 weeks and followed by 500mg lecithin/kg body weight/day for 4 weeks)

Group VII: lecithin + vitamin B-complex + ethanol treated rats (1.6g ethanol and 500mg lecithin mixed with trace amount of vitamin-B complex together/kg body weight/day for 4 weeks)

Group VIII: ethanol followed by lecithin and vitamin B-complex treated rats (1.6g ethanol/kg body weight/ day for 4 weeks and followed by 500mg lecithin mixed with trace amount of vitamin-B complex/kg body weight/day for 4 weeks)

Group IX: ethanol treatment (1.6g ethanol/kg body weight/ day) for 4 weeks and followed by 4 weeks abstention.

Tocopheryl acetate was suspended in distilled water; and lecithin or vitamin-B complex was freshly dissolved in distilled water during treatment. Ethanol was diluted with distilled water to get desired concentration and fed orally by intragastric infusion technique.

Experimental procedure

At the end of the experimental period, the animals were sacrificed after over-night fast, by applying intraperitoneal thiopentone (thiosol/Na^+). The liver was dissected out and cleaned with ice-cold saline, blotted dry, and immediately transferred to the ice chamber. Various oxidative stress related parameters were estimated. Animals of group II were sacrificed at the end of 4 weeks of exposure to ethanol; and all other animals were sacrificed at the end of 8 weeks of treatment period as described above.

Methods

Liver was homogenized in 0.25 *M* sucrose solution, diluted with 0.9% saline, and these diluted samples were used for the estimation of tissue protein by the method of Lowry *et al*[11].

Determination of ascorbic acid—Weighed liver samples were homogenised in ice cold 6% TCA. Ascorbic acid content was estimated using thiourea reagent and 2,4-dinitrophenylhydrazine in cold H_2SO_4. The absorbance was recorded at 540 nm against blank. Concentration was calculated using standard ascorbic acid solution[12].

Determination of lipid peroxidation—Liver samples were homogenized in ice-cold 0.25 *M* tris buffer (*p*H 7.4); and 0.3 ml of this homogenate was mixed thoroughly with 2ml of TCA-TBA-HCl [trichloroacetic acid (TCA) 15% w/v, thiobarbituric acid (TBA) 0.375% w/v, and hydrochloric acid (HCl) 0.25 *N*]. The solutions were heated for 15 min in a boiling water bath, cooled, the flocculent precipitates were removed, and the absorbances were recorded at 535 nm. The extent of lipid peroxidation was calculated using molar extinction coefficient 1.56×10^5 $M^{-1}cm^{-1}$ for malondialdehyde[13].

Nitrite estimation—Nitrite was measured using Griess reagents[14]. Sulfanilamide (1%, 50 µl) in 2.5% ortho-phosphoric acid (Griess reagent 1) was added to the tissue homogenate, followed by N-(1-naphthyl)ethylenediamine (0.1%, 50 µl) in double distilled water (Griess reagent 2) was added, incubated in dark at room temperature for 10 min. The absorbance was measured at 540 nm. The concentration of nitrite was measured by using $NaNO_2$ as a standard.

Protein-carbonyl content—Proteins were precipitated with 20% trichloroacetic acid and centrifuged. The precipitate was resuspended in 2,4 dinitrophenylhydrazine (10 m*M*) and vortexed at 10 min intervals for 1 hr at room temperature. The pellets were washed thrice with ethanol/ethyl acetate to remove the free reagent before centrifugation. The

pellets were then resuspended in 0.6 ml of 6 *M* guanidine hydrochloride, incubated at 37°C for 15 min, and centrifuged at 5,000 *g* for 3 min. The absorbance of supernatant was measured spectrophotometrically at 366 nm for carbonyl content, and calculations were performed with an ε value of 22,000 $M^{-1}cm^{-1}$ (ref. 15).

Estimation of glutathione content—The liver (~100 mg tissue) samples were homogenized in ice-cold 0.1 *M* phosphate buffer (*p*H 7.4). For glutathione content, the homogenates were immediately mixed with sulfosalicylic acid, shaked well, centrifuged, and the supernatant was mixed with 5,5′-dithiobis(2-nitrobenzoic acid) (in 0.01 *M* phosphate buffer, *p*H 8) and absorbance was recorded at 412 nm[16]. For oxidized glutathione, 200 µl supernatant was added to 3.78 ml of water to which 40 µl of 2-vinylpyridine was mixed to mask the GSH and left at room temperature for 3 hr before estimation as described above[17]. Glutathione content was determined from standard curve obtained from pure GSH.

Determination of catalase (EC 1.11.1.6) activity—Catalase activity was determined based on the method as described by Beers and Sizer[18] with necessary modification. The tissues were homogenized in 0.05 *M* phosphate buffered saline (*p*H 7.0). The rate of decomposition of H_2O_2 (2µl, 30%) in 0.05 *M* phosphate buffer (1ml, *p*H 7.0) at 240 nm after addition of homogenized tissue was noted. The specific activity was calculated assuming molar extinction coefficient 40 $M^{-1}cm^{-1}$ for H_2O_2 at 240 nm[19].

Assay of glutathione reductase (GR; EC 1.6.4.2) activity—The tissues were homogenized using phosphate buffer (0.12 *M*, *p*H 7.2), and the tissue extracts were added to 15m*M* EDTA in phosphate buffer, and 9.6 m*M* NADPH. The reaction was initiated by adding oxidized glutathione (GSSG, 65.3 m*M*). Change in absorbance was monitored at 340 nm; and the specific activity of the enzyme was determined using extinction coefficient for NADPH of 6.22 $cm^{-1}/\mu mole$[20].

Assay of glutathione S-transferase (GST; EC 2.5.1.18) activity—The tissue was homogenized using 0.05 *M* phosphate buffer (*p*H 6.5). 1-chloro-2, 4-dinitrobenzene (CDNB) in phosphate buffer was mixed with reduced glutathione, and then tissue extract was added, and the change in absorbance was monitored at 340 nm/min, and calculated from extinction coefficient 9.6 $mM^{-1}cm^{-1}$(ref. 21).

Determination of glutathione peroxidase (GPx; EC 1.11.1.9) activity—Glutathione peroxidase activity was measured as described by Paglia and Valentine[22], which is based on the fact that oxidized glutathione produced by GPx is reduced at a constant rate by glutathione reductase with NADPH as a cofactor. The NADPH allows for the maintenance of predictable levels of reduced glutathione. The oxidative rate of NADPH was monitored spectrophotometrically at 340 nm during the reaction.

Determination of superoxide dismutase (EC 1.15.1.1) activity—The superoxide dismutase activity was measured by the inhibition of autooxidation of 0.2 m*M* pyrogallol (air equilibrated) in 50 m*M* Tris-HCl buffer (*p*H 8.2) containing 1 m*M* diethylenetriamine pentaacetic acid. The rate of autooxidation was monitored at 420 nm. The percentage inhibition of the rate of autooxidation of pyrogallol was initiated by addition of tissue homogenate[23].

Statistical analysis—Results are expressed as mean ± SD. All the statistical analyses were performed using one-way analysis of variance (ANOVA) with multiple comparison tests and Student's *t* test. Significance was based on a P value <0.05.

Results

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 and figures.

Preventive and curative effect of tocopheryl acetate, lecithin and lecithin with vitamin B-complex on percentage change in body weight of rats during the experiment in relation to its initial weight are presented in Fig. 2. When rats were simultaneously exposed with ethanol and tocopheryl acetate or lecithin with vitamin B-complex significantly increased in body weight was observed. After ethanol treatment for 4 weeks, and then follow-up treatment was given for another 4 weeks; again similar type of response was observed. The relative weights (g/ 100g body weight) of liver of different treatments are presented in Fig. 3, and no significant change was observed.

Levels of ascorbic acid, thiobarbituric acid reactive substances (TBARS) and nitrite content in liver homogenate are shown in Table 1. No significant change was observed in ascorbic acid content. The level of lipid peroxidation in liver of all the tested

groups was significantly higher than the control group. TBARS level was reduced significantly during curative and preventive treatment with tocopheryl acetate, lecithin and lecithin with vitamin-B complex; and even during abstention. The nitrite content of liver homogenates of different groups was found to be significantly higher than the control group. Same trend of response was observed in different treatments; but preventive treatment showed better result than curative treatment. Ascorbic acid level significantly reduced during ethanol treatment; but no significant change was observed due to different treatments.

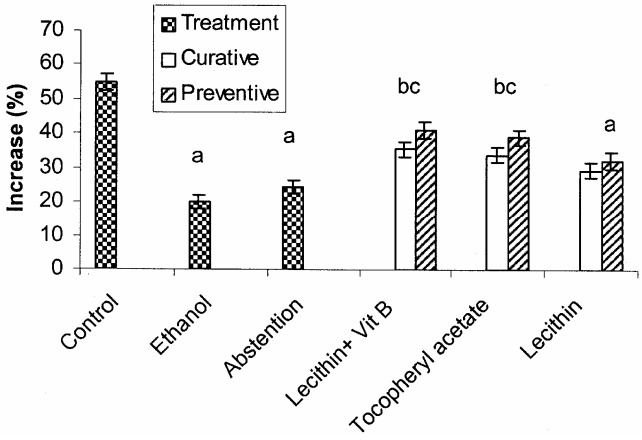

Fig. 2—Change in the body weight in different groups animals exposed to different treatments. Values are mean ± SD of 6 rats in each group. *P* values: [a] indicate *P*<0.05 compared to control group, [b]: <0.05 compared to ethanol treated group; [c]: <0.05 compared to abstention group.

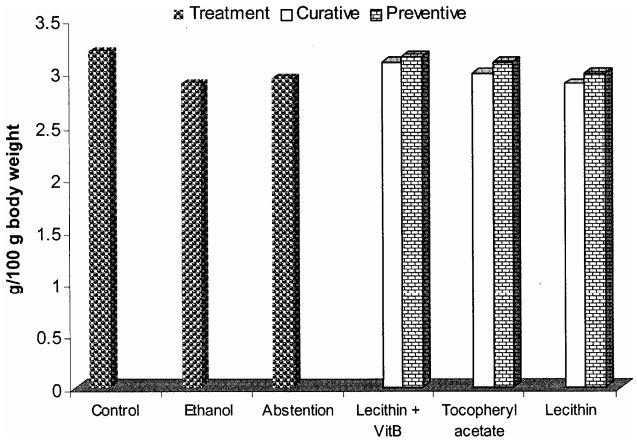

Fig. 3—Change in the liver weight in different groups animals exposed to different treatments. No significant change was observed in either group.

Table 1—Effect of tocopheryl acetate, lecithin and lecithin with vitamin-B complex on ascorbic acid content, thiobarbituric acid reactive substances (TBARS) and nitrite levels in liver homogenate

[Values are mean±SD of 6 rats in each group. Figures in the parentheses are % increase (+) or decrease (-) compared to experimental control group (Group II)]

Treatment	Ascorbic acid (mg/g tissue)		TBARS (μmole MDA formed/min/100mg tissue)		Nitrite (nmole/g wet tissue)	
Control	3.1 ± 0.17		0.638 ± 0.013		18.12± 1.26	
Ethanol	2.42± 0.38[a]		0.926 ± 0.023[a]		37.16± 1.97[a]	
Abstention	2.49± 0.25[b] (+2.9)		0.844 ± 0.038[ae] (-8.8)		32.74±0.80[ae] (-11.9)	
	Curative	Preventive	Curative	Preventive	Curative	Preventive
Lecithin + Vitamin B complex	2.87±0.30 (+18.5)	2.85±0.25 (+17.7)	0.728± 0.007[bdg] (-21.3)	0.716± 0.043[bdgqu] (-22.6)	26.62± 1.39[adgt] (-28.3)	25.43± 1.63[adgqs] (-30.5)
Tocopheryl acetate	2.72±0.12 (+12.4)	2.81±0.23 (+16.1)	0.735± 0.053[adg] (-20.6)	0.723± 0.039[bdgr] (-21.9)	25.52± 1.99[adgqs] (-31.3)	24.86± 1.93[adgps] (-33.1)
Lecithin	2.66±0.15 (+9.9)	2.68±0.17 (+10.7)	0.792± 0.014[ado] (-14.4)	0.782± 0.030[ad] (-15.5)	30.06± 1.88[adkm] (-19.1)	31.16± 2.85[adjm] (-16.1)
F Variance	4.389*		39.232*		54.527*	

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to normal healthy control (Group I); [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to ethanol treated group (Group II); [g]< 0.001, [h]< 0.01, [i]< 0.05 in drug treated groups when compared to abstention group (Group III); [j]< 0.001, [k]< 0.01, [l]< 0.05 in drug treated groups when compared to tocopheryl acetate curative treatment group (Group VI); [m]< 0.001, [n]< 0.01, [o]< 0.05 in drug treated groups when compared to tocopheryl acetate preventive treatment group (Group VII); [p]< 0.001, [q]< 0.01, [r]< 0.05 in drug treated groups when compared to lecithin curative treatment group (Group VIII); [s]< 0.001, [t]< 0.01, [u]< 0.05 in drug treated groups when compared to lecithin preventive treatment group (Group IX).

*Significant at *P*< 0.001

Effects of different treatments on protein carbonyl group, glutathione content and redox ratio in liver homogenate are presented in Table 2. The level of protein carbonyl group and redox ratio in liver of all the tested groups was significantly higher than the control group. However, protein carbonyl level was reduced significantly during curative and preventive treatment with different test compounds. The reduced glutathione (GSH) content of liver homogenates of different groups were found to be significantly lower than the control group. GSH level was significantly raised by different treatments when compared with ethanol fed rats. Here also preventive treatment showed better result than curative treatment. But oxidized glutathione (GSSG) and redox ratio (GSSG/GSH) decreased significantly by different treatments compared to ethanol treated group.

Glutathione reducatase (GR) and glutathione peroxidase (GPx) activities of liver of all the tested groups were found to be significantly lower in comparison to the enzyme activities of control group, and higher in comparison to ethanol treated group (Table 3). However, liver glutathione s-transferase (GST) activities of all the tested groups showed opposite trend of response.

The catalase activities of liver homogenates in treated groups were lower when compared with the control group (Table 4) and higher when compared with the ethanol treated group. On the other hand, the superoxide dismutase activities in liver of different groups were found to be higher than the activity observed in the control group. On the contrary, the liver superoxide dismutase activities of the treated groups were found to be lower than ethanol treated group.

Discussion

The intragastric ethanol infusion technique allowed maximal ethanol consumption and absolute control over ethanol-induced liver injury. In the present study, a dose of ethanol 1.6g/kg body weight/day for 4 weeks was used to induce maximum liver damage, which can

Table 2—Effect of tocopheryl acetate, lecithin and lecithin with vitamin-B complex on protein carbonyl group, reduced glutathione, oxidized glutathione levels and redox ratio in liver homogenate

[Values are mean±SD of 6 rats in each group. Figures in the parentheses are % increase (+) or decrease (-) compared to experimental control group (Group II)]

Treatment		Protein carbonyl group (nmol/mg protein)	GSH (nmole/ mg protein)	GSSG (mole/ mg protein)	Redox ratio (GSSG/GSH)
Control		2.08 ± 0.14	67.1±3.75	5.21± 0.27	0.078± 0.007
Ethanol		4.92 ± 0.21^{ad}	35.17±3.03^{a}	5.99± 0.25^{a}	0.172± 0.021^{a}
Abstention		4.16±0.31^{ad} (-15.4)	44.62±3.77^{ae} (+26.8)	5.62±0.20 (-6.5)	0.126±0.015^{a} (-26.7)
Lecithin + Vitamin B complex	Curative	2.98±0.22^{adgqu} (-38.4)	55.86±2.37^{adg} (+58.8)	5.36±0.22^{e} (-10.5)	0.096±0.008^{dg} (-44.2)
	Preventive	2.86±0.17^{adgpt} (-41.8)	58.95±4.12^{bdgqu} (+67.6)	5.34±0.19^{d} (-10.8)	0.090±0.006^{dg} (-47.6)
Tocopheryl acetate	Curative	2.92±0.2^{adgpt} (-40.6)	54.72±3.85^{adg} (+55.6)	5.31±0.24^{d} (-11.3)	0.097±0.004^{dg} (-43.6)
	Preventive	2.84±0.16^{adgps} (-42.2)	57.32±4.26^{adgr} (+62.9)	5.28±0.22^{d} (-11.8)	0.092±0.005^{dg} (-46.5)
Lecithin	Curative	3.56±0.20^{adgjm} (-27.6)	49.83±2.28^{ad} (+41.68)	5.41±0.24^{e} (-9.6)	0.108±0.006^{ad} (-37.2)
	Preventive	3.45± 0.22^{adgkm} (-29.8)	51.46±2.57^{adio} (+46.3)	5.44±0.17^{e} (-9.2)	0.105±0.005^{bdi} (-38.9)
F variance		92.62*	42.738*	6.463*	43.354*

P values: ^{a}< 0.001, ^{b}< 0.01, ^{c}< 0.05 compared to normal healthy control (Group I); ^{d}< 0.001, ^{e}< 0.01, ^{f}< 0.05 compared to ethanol treated group (Group II); ^{g}< 0.001, ^{h}< 0.01, ^{i}< 0.05 in drug treated groups when compared to abstention group (Group III); ^{j}< 0.001, ^{k}< 0.01, ^{l}< 0.05 in drug treated groups when compared to tocopheryl acetate curative treatment group (Group VI); ^{m}< 0.001, ^{n}< 0.01, ^{o}< 0.05 in drug treated groups when compared to tocopheryl acetate preventive treatment group (Group VII); ^{p}< 0.001, ^{q}< 0.01, ^{r}< 0.05 in drug treated groups when compared to lecithin curative treatment group (Group VIII); ^{s}< 0.001, ^{t}< 0.01, ^{u}< 0.05 in drug treated groups when compared to lecithin preventive treatment group (Group IX).

*Significant at *P*< 0.001

Table 3—Effect of tocopheryl acetate, lecithin and lecithin with vitamin-B complex on glutathione reductase, glutathione peroxidase, and glutathione s-transferase activity in liver homogenate

[Values are mean±SD of 6 rats in each group. Figures in the parentheses are % increase (+) or decrease (-) compared to experimental control group (Group II)]

Treatment	Glutathione reductase (nmole NADPH breakdown/ min/ mg protein)		Glutathione peroxidase (nmole NADPH breakdown/ min/ mg protein)		Glutathione s-transferase (μmole CDNB conjugate formed/ min/ mg protein)	
Control	56.38± 1.36		82.46±2.31		6.84±0.10	
Ethanol	26.77± 0.82[a]		52.94±1.57[a]		13.26±0.31[a]	
Abstention	34.23± 1.71[a] (+27.8)		59.63±3.84[af] (+12.6)		10.74±0.68[ad] (-19)	
	Curative	Preventive	Curative	Preventive	Curative	Preventive
Lecithin + Vitamin B complex	42.54± 2.87[adgq] (+58.9)	44.74± 2.42[adgpt] (+67.1)	72.12± 4.27[adgp] (+36.2)	74.32± 3.48[bdgpu] (+40.3)	7.81± 0.62[dgq] (-41.1)	7.63± 0.32[dgpu] (-42.4)
Tocopheryl acetate	42.16± 2.42[adgr] (+57.4)	44.36± 2.62[adgpt] (+65.7)	70.48± 3.69[adgq] (+33.1)	72.46± 2.16[adgp] (+36.8)	8.23± 0.51[bdg] (-37.9)	7.71± 0.42[dgqu] (-41.8)
Lecithin	37.24± 2.08[adlm] (+39.1)	38.91± 2.32[adin] (+45.3)	63.26± 2.31[adkm] (19.5)	67.43± 2.91[adh] (+27.3)	9.16± 0.62[adgn] (-30.9)	8.83± .81[adgo] (-33.4)
F Variance	85.579*		48.453*		83.33*	

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to normal healthy control (Group I); [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to ethanol treated group (Group II); [g]< 0.001, [h]< 0.01, [i]< 0.05 in drug treated groups when compared to abstention group (Group III); [j]< 0.001, [k]< 0.01, [l]< 0.05 in drug treated groups when compared to tocopheryl acetate curative treatment group (Group VI); [m]< 0.001, [n]< 0.01, [o]< 0.05 in drug treated groups when compared to tocopheryl acetate preventive treatment group (Group VII); [p]< 0.001, [q]< 0.01, [r]< 0.05 in drug treated groups when compared to lecithin curative treatment group (Group VIII); [s]< 0.001, [t]< 0.01, [u]< 0.05 in drug treated groups when compared to lecithin preventive treatment group (Group IX).

*Significant at *P*< 0.001

Table 4—Effect of tocopheryl acetate, lecithin and lecithin with vitamin-B complex on catalase and superoxide dismutase activity in liver homogenate

[Values are mean ±SD of 6 rats in each group. Figures in the parentheses are % increase (+) or decrease (-) compared to experimental control group (Group II)]

Treatment	Catalase (μmole H_2O_2 decomposed /min/ mg protein)		Superoxide dismutase (U/mg protein)	
Control	38.76± 0.56		6.33±0.15	
Ethanol	32.63±0.81[a]		9.07±0.21[a]	
Abstention	33.36±1.73[a] (+2.2)		8.62±0.0.66[a] (-4.9)	
	Curative	Preventive	Curative	Preventive
Lecithin + Vitamin B complex	37.06± 0.78[dgq] (+13.5)	37.96 ±1.61[dgpt] (+16.3)	7.26± 0.61[dh] (-19.9)	6.98±0.43[dgqu] (-23)
Tocopheryl acetate	36.22±1.40[cdir] (+11)	37.81±1.66[dgpt] (+15.8)	7.54±0.52[cei] (-16.8)	7.02±0.79[dgru] (-22.6)
Lecithin	33.73±1.19[alm] (+3.3)	34.63±0.96[an] (+6.1)	8.23±0.63[ao] (-9.2)	8.12±0.0.57[ao] (-10.4)
F variance	19.385*		15.702*	

P values: [a]< 0.001, [b]< 0.01, [c]< 0.05 compared to normal healthy control (Group I); [d]< 0.001, [e]< 0.01, [f]< 0.05 compared to ethanol treated group (Group II); [g]< 0.001, [h]< 0.01, [i]< 0.05 in drug treated groups when compared to abstention group (Group III); [j]< 0.001, [k]< 0.01, [l]< 0.05 in drug treated groups when compared to tocopheryl acetate curative treatment group (Group VI); [m]< 0.001, [n]< 0.01, [o]< 0.05 in drug treated groups when compared to tocopheryl acetate preventive treatment group (Group VII); [p]< 0.001, [q]< 0.01, [r]< 0.05 in drug treated groups when compared to lecithin curative treatment group (Group VIII); [s]< 0.001, [t]< 0.01, [u]< 0.05 in drug treated groups when compared to lecithin preventive treatment group (Group IX).

*Significant at *P*< 0.001

be reversed as observed by Das and Vasudevan[24]. Ethanol treated rats showed a lower increase in body weight due essentially to fat mass reduction. Reduced adipose tissue may be the foremost cause of lower body weight[24]. Because ethanol is metabolized rapidly in the liver with no net energy storage, its calories are considered "empty". Excessive use of alcohol results in changes in energy expenditure and dietary fat oxidation in the liver that may have an impact on body weight[25]. The substitution of alcohol for "normal" calories results in weight loss, whereas the effect of alcohol consumption on diminishing fat oxidation could contribute to weight gain in the setting of combining large amounts of alcohol with typical high-fat.

Animals with ALD exhibit impaired liver regeneration[26]. In the present study, the regenerative capacity was observed by administering ethanol (1.6g/ kg body weight/day) for 4 weeks, followed by abstention from alcohol for next 4 weeks. This group also acted as another experimental control group. Tocopheryl acetate, lecithin and lecithin with vitamin-B complex exhibited an ability to counteract the alcohol-induced changes in the body weight and biochemical parameters in preventive and curative aspects in varying degrees.

One of the proposed mechanisms of chronic ethanol induced-toxicity is the membrane damage due to the direct effect of lipid peroxidation products[24], i.e., TBARS, which was found to be increased in the ethanol treated rats in the present study. All these treatments reversed this level significantly. These suggest that these treatments offered some protection against lipid peroxidation. The tissue ascorbic acid concentration reflects oxidative stress in hepatocytes.

Exposure to reactive oxygen and nitrogen species (RONS) may cause lipid peroxidation in cell membranes, which in turn may generate species that damage cell proteins and promote their degradation[27]. Protein nitration has been suggested to be a final product of the highly reactive nitrogen oxide intermediates (e.g. peroxynitrite) formed in reactions between NO and oxygen-derived species such as superoxide. Nitrite is a stable metabolite of NO and can be used as an indicator of the overall formation of NO *in vivo*. Increased nitrite levels as a result of increased NOS activity have been observed in liver homogenate of animals when those were exposed to ethanol, indicating that these group of animals suffered from oxidative and nitrosative stress. Lecithin with vitamin-B complex and α-tocopherol were effective in liver, where it decreased the nitrite formation.

Glutathione is an important antioxidant; it protects cells against damage from free radicals and toxic endogenous and exogenous compounds either by reacting directly with these toxins or by facilitating the reduction of protein disulfide bridges[28]. Concentrations of total glutathione therefore give an indication of the intracellular oxidative state. One of the antioxidant properties of glutathione is mediated by the enzyme glutathione peroxidase. During the detoxification of lipid and other peroxides produced by free radical attack, glutathione peroxidase converts glutathione from a reduced state (GSH) to an oxidized one (GSSG)[29]. The NADPH-dependent enzyme glutathione reductase converts GSSG back to GSH, and so almost all intracellular glutathione is reduced. During an oxidative stress, there will be flux of glutathione to the oxidized form, and the ratio of reduced to oxidized glutathione may then be an indication of this stress[28]. GSH may also exist in another oxidized form, as mixed disulfides with protein thiols. Thiol-sulfide exchange reactions between GSSG and protein thiols are potentially important in biological samples, causing artifacts in measurement of glutathione.

Chronic ingestion of ethanol resulted in a significant decrease in glutathione peroxidase (GPx) activity in liver may be due to either free radical dependent inactivation of enzyme or depletion of its co-substrates i.e. GSH and NADPH in ethanol treated rats[24,30]. Glutathione s-transferase (GST) plays an essential role in liver by eliminating toxic compounds by conjugating them with glutathione. Increased glutathione s-transferase activity and decreased glutathione reductase activity, followed by thiol depletion, are important factors sustaining a pathogenic role for oxidative stress[24,31]. Supplementation of tocopheryl acetate, lecithin and lecithin with vitamin-B complex either as therapeutic or prophylactic treatments in ethanol induced liver damage could partly reverse these enzyme levels in the present study.

The presence of superoxide dismutase (SOD) in various compartments of human body enables SOD to dismutate superoxide radicals immediately[24]. In the present work, superoxide dismutase activities increased on ethanol exposure, and reduced significantly due to different treatments including abstention from alcohol. Catalase activity was

decreased in higher concentration of ethanol exposure may be due to loss of NADPH, or generation of superoxide, or increased activity of lipid peroxidation or combination of all[24]. Different treatment increased its activity in the present study.

Antioxidants represent a potential group of therapeutic agent for alcohol induced liver damage. They likely provide beneficial effects on hepatocytes via desensitization against oxidative stress while inhibiting priming mechanism for expression of proinflammatory and cytotoxic mediators[32]. From the present result it was observed that ethanol-induced stress can be partly prevented by tocopheryl acetate (vitamin E) supplementation. It is generally believed that α-tocopherol intercalates into the lipid bilayers of cell membranes and acts there directly to scavenge free radicals. Hydroxyl radical attack on tocopherol forms a stabilized phenolic radical that is reduced back to the phenol by ascorbate and NADH/NADPH reductase enzyme[33]. Vitamin E may largely modulate the expression of the toxicity by GSH depleting agents[34].

Supplementation of lecithin also showed a little better effect than abstention on reversing the effect of alcohol induced liver disease in the present study. Phosphatidyl choline (PC) is the product of SAM-dependent PEMT reaction, as well as a precursor of choline and betaine. Whereas PC is the principal phospholipids of cell membranes, phospholipid composition is disarranged in red cell and liver cell membranes from ethanol-fed rats[35]. Lieber *et al.*[36] have reported that choline supplementation failed to prevent alcohol-induced steatosis and fibrosis; while choline supplementation exerts moderate hepatotoxicity. Later Lieber *et al.*[37] concluded that some component of lecithin exerts a protective action against the fibrogenic effects of ethanol. However, supplementation of trace amount of vitamin-B complex with lecithin was very much effective in reversing the oxidative stress mediated effect.

Folate is water-soluble vitamin, required for DNA nucleotide synthesis and cell turnover, and as substrate for methionine synthase (MS), is essential as substrate for the transmethylation and reduction of homocysteine. The liver is the major site for folate storage and metabolism, both of which are altered in chronic alcoholism[38]. The intestinal absorption of thiamine is reduced in chronic alcoholics[39], owing in part to the inhibitory effect of ethanol on transport of thiamine across the enterocyte basolateral membrane[40].

Pyridoxine, or vitamin B6, is a coenzyme for many amino acid reactions and, after phosphorylation in the liver, circulates as a complex of pyridoxal phosphate (PLP) bound to albumin[41]. In addition, pyridoxine deficiency can contribute to homocysteine elevation in chronic alcoholics, because pyridoxine is a cofactor for two reactions that regulate the homocysteine transsulfuration pathway, cystathionine β-synthase (CBS) and γ-cystathionase[42]. Pyridoxine deficiency in alcoholics is related to ethanol metabolism in the liver, because production of acetaldehyde results in displacement of PLP from albumin, followed by urinary excretion of the unbound vitamin[43]. Therefore, vitamins are integrally involved in the metabolism of methionine and homocysteine. CBS requires vitamin B_6 as a cofactor, whereas MS requires normal levels of folate and vitamin B_{12}. The methionine metabolic cycle is present in all tissues and predominates in hepatocytes[44]. ALD affects many of these enzymatic steps. Hyperhomocysteinemia, a consequence of altered methionine metabolism, can be the result of genetic factors such as CBS deficiency, nutritional deficiencies in folate, vitamin B_6 or B_{12}, and impaired liver function[45].

SAM depletion causes reduced synthesis of reduced glutathione (GSH). The fall in hepatic GSH sensitizes the liver to oxidative injury. In these models, the methionine pool is partially conserved at the expense of lecithin as studied by change in body weight and biochemical parameters. Although folate and B_{12} deficiency contribute to the fall in MS activity, the enzyme is also postulated to be inactivated by possibly forming adducts with acetaldehyde or nitrosylated[46]. It is likely that the induction in BHMT was not sufficient to maintain methionine pool and thereby oxidative stress. From the present study, it can be concluded that tocopheryl acetate supplementation is a promising approach. Abstention from alcohol improved hepatic regeneration. However, lecithin with vitamin-B complex supplementation significantly reversed oxidative and nitrosative stress in alcohol induced liver damage. This protective combination is comparable with the benefit showed by the well-known potent antioxidant tocopheryl acetate (vitamin E). Moreover, preventive measures were better effective than curative treatment.

Conclusion

Correction of deficiencies or other strategies for nutritional support may be of benefit in the prevention

and treatment of ALD. The unraveling of the myriad connections between nutrients and alcohol and their metabolic and disease implications continues to challenge our critical thinking. Malnutrition is promoted by worsening liver function during development of ALD supports a vicious cycle whereby alcohol consumption begets specific nutritional deficiencies that promote mechanisms of liver injury that enhance the development of ALD and so forth. Therefore, prevention of oxidative and nitrosative stress along with correction of nutritional deficiency is the proposed mechanism for the therapeutic approach. Central to this concept is the likelihood that nutritional deficiencies will improve, liver injury will be arrested, and repair mechanisms will come to the forefront.

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