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EFFECTS OF EXOGENOUS VITAMIN C ON ETHANOL TOXICITY IN RATS

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Abstract : The effect of a mega dose of ascorbic acid (200 mg/100 g body wt.) on alcohol–induced toxicity in rats was evaluated . In rats administered alcohol and ascorbic acid, malondialdehyde (MDA), hydroperoxide and conjugated dienes decreased in comparison with that given alcohol alone. The reduced activities of scavenging enzymes, eg. superoxide dismutase (SOD) and catalase, in ethanol-administered rats were also enhanced by the co-administration of ascorbic acid and ethanol. Co-administration of ethanol and ascorbic acid reduced phospholipids and MDA levels of the erythrocyte membrane in comparison with that of the ethanol fed rats. The reduction in the activities of glutamic oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), gamaglutamyl transpeptidase (GGT) and the decrease in triglycerides levels also clearly showed the protective action of ascorbic acid in reducing ethanol induced toxicity.

Key words : ascorbic acid, ethanol, antioxidant, lipid peroxidation, erythrocyte membrane

INTRODUCTION

Acute and chronic ingestion of ethanol causes a variety of changes in the liver (1, 2). Chronic ethanol consumption has been associated with increased lipid peroxidation manifested by an increase in the level of malondiadehyde (MDA) in the liver (3). Increased lipid peroxidation could occur as a consequence of induction of microsomal membrane free radical generation and also due to depletion of hepatocyte lipid peroxidation defences. In acute ethanol intoxication, liver microsomal metabolism of ethanol was accompanied by OH generation by the cytochrome p 450 system. Hydroxyl radicals are responsible for the conversion of ethanol to acetaldehyde (3). Vitamin C is a terminal water-soluble small antioxidant that protects lipids against peroxidation (4). Low leucocyte levels of vitamin C as a measure of tissue stores were found in patients with alcoholic cirrhosis (5). In vitro studies have shown that low-density lipoprotein oxidation can be prevented by naturally occurring antioxidants such as vitamin C, vitamin E, carotenes, etc (6). Chakraborthy *et al* (7) have shown that ascorbate protects guinea pig tissues against peroxides both *in vivo* and *in vitro*. So in this study

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we examined the role played by a mega dose of ascorbic acid (AA) in rats administered alcohol.

Experimental

Male albino rats (Sprague-dawley) having an average body of 120±10 g were used. They were fed with rat feed. (Lipton India*) (*Composition of rat feed: crude protein 24%, ether extract - 3.5%, crude fiber-12%, ash- 8%, calcium 1.2%, phosphorus-0.06%, nitrogen free extract-43%) Food and water were given *ad libitum*. The rats were divided into the following 4 groups of 6 rat each as follows.

Group I:	Control rats	----------
Group II:	Ethanol treated	(900 mg ethanol/100 g body weight/day)
Group III:	Ascorbic acid treated	(200 mg AA/100 g body weight/day)
Group IV:	Ascorbic acid+ethanol treated	(200 mg AA/100 g body weight + 900 mg ethanol/100 g body weight/day)

Control and ascorbic acid group animals were administered glucose solution equivalent to the caloric value of ethanol in group 2 and 4. Ascorbic acid and ethanol were given orally by gastric intubation daily for a period of 30 days. Ascorbic acid was freshly dissolved in distilled water and ethanol was diluted in the ratio 1:1(v/v). Ascorbic acid was purchased from M/s Sigma chemical Co. USA, and absolute ethanol from M/s E-Merck, Germany. After the end of the experimental period, the rats were deprived of food overnight, and killed by decapitaion. The Serum and various were removed to ice-cold containers for various biochemical estimations.

Biochemical analysis

The serum and the tissues were extracted according to the procedure of Folch *et al* (8). Tissues were stored in pre cooled containers (–4°C) for biochemical analysis. The tissues were homogenised and extracted with chloroform: methanol (2:1). The residue was washed with chloroform methanol at least 3 times. The filtrates were combined. To the filtrate, 0.7% KCl (20% of the total volume of the extract) was added and mixed. The aqueous upper phase was removed with a pasteur pipette and the lower layer was washed 3 times with 5 ml of chlorofrom: methanol: KCI (2: 48: 47) solution. The washed lower layer of chlorofrom was evaporated to dryness and the residue was redissolved in known volume of chlorofrom. Aliquots of the extracts were used for the estimation of various lipids.

Ascorbic acid in the serum and tissues were determined by the method of Roe and Kuether (9). Estimation of triglycerides was carried out as described by Menon and Kurup (10). Free fatty acids (FFA) were estimated by the method of Falhot *et al* (11). Malondialdehyde, hydroperoxide and diene conjugates were estimated according to John and Steven (12). Activities

of superoxide dismutase (SOD) and catalase were determined by the method of Kakker *et al* (13) and Miehly and Chance (14) respectively. Glutathione content was determined by the procedure of Patterson and Lazarrow (15). Glutamic oxaloacetic transaminase (GOT) and Glutamic pyruvic transaminase (GPT) were estimated by the method of Wooten (16) Gama glutamyl transpeptidase (GGT) activity in the serum was assayed by Szasz's method (17).

Erythrocyte membrane was separated according to the method of Kainimoto and Miura (18). Lipids were extracted from the erythrocyte membrane using chlorofrom and methanol in the ratio 2:1(v/v). Cholesterol was estimated by the method of Abell *et al* (19). Phosholipids in the erythrocyte were determinied by the method of Zilversmit and Davis (20).

Lipid peroxidation was measured by the formation of MDA (12). Protein was estimated by the method of Lowry *et al* (21).

Statistical analysis

Statistical analysis was carried out using one way analysis of variance (ANNOVA). Difference between treatments means were determined by the method of Snedecor and Cohran (22). Difference between treatments means were determined by the Bonferroni multiple comparison procedure. The results were expressed as the means ± SD of six values in each group, and statistical probability of $P<0.05$ was considered to be significant.

RESULTS

Ascorbate content

The ascorbate content was significantly increased in the serum of rats fed AA in comparison with control (Table I) But in rats administered ethanol, the ascorbate content was significantly higher in the serum in comparison with control. When a massive dose of AA and ethanol were administered together AA level did not alter significantly in comparison with the control. The ethanol induced higher levels of AA was brought down to normal levels in the serum when AA was administered along with alcohol.

TABLE I: Ascorbate content of blood in rats (mg/100 ml).

I.	Control	2.87±0.18
II.	Ethanol	3.73±0.24[a]
III.	Ascorbate	3.35±0.43
IV.	Ascorbate + Ethanol	2.38±0.28[b]

Values expressed as mean ± SD of 6 rats

[a]– $P < 0.05$ between control and other treated groups

[b]– $P < 0.05$ between ethanol and ethanol + ascorbic acid group

Triglycerides content

The concentration of triglycerides increased in the various tissues of group II and IV when compared with that of the control group (Table II) In group III the triglyceride level significantly decreased in all tissues studied, in comparison with the control group. But in the ethanol+ ascorbic acid treated group, the triglyceride level significantly decreased in all the tissues except liver in comparison with that for rats given alcohol alone.

TABLE II: Concentration of Triglycerides and Free Fatty acid (mg/100 g wet. tissue) in various rat tissues.

Groups	Liver		Kidney		Brain		Heart	
	TG	FFA	TG	FFA	TG	FFA	TG	FFA
Control	438.40±22.1	283.43±14.37	74.33±5.23	117.41±6.83	68.97±3.59	129.64±3.50	44.53±1.98	165.35±4.24
Ethanol	531.68±30.6[a]	341.77±17.22[a]	103.50±6.8[a]	226.46±10.05[a]	138.45±4.9[a]	144.13±4.66[a]	77.14±3.23[a]	217.53±5.88[a]
AA	327.57±18.37[a]	185.59±8.16[a]	69.72±3.18[a]	103.46±4.48	56.17±2.77[a]	133.37±3.06[a]	36.17±1.59[a]	143.43±3.85[a]
AA + Ethanol	488.29±24.89	303.83±15.1[b]	82.63±4.5[b]	124.40±7.33[b]	71.53±3.03[b]	142.56±4.32[a]	56.75±1.8[ab]	204.15±4.9[ab]

Values expressed as mean ± SD of 6 rats
[a]– P<0.05 between control and other treated groups
[b]– P<0.05 between ethanol and ethanol+ascorbic acid group

TABLE III: Concentration of Malondialdehyde, Hydroperoxides and Conjugated Diences (mM/100 g tissue) in various rat tissues.

	MDA				Hydroperoxides				Conjugated dienes			
	Liver	Kidney	Brain	Heart	Liver	Kidney	Brain	Heart	Liver	Kidney	Brain	Heart
Control	0.742±0.07	1.33±0.08	1.26±0.15	0.43±0.03	12.83±0.70	1.63±0.09	8.87±0.64	47.41±1.39	74.54±1.62	15.91±0.69	15.32±0.93	13.29±0.88
Ethanol	1.22±0.12[a]	1.75±0.09[a]	2.61±0.22[a]	1.07±0.07[a]	17.84±0.73[a]	2.25±0.13[a]	20.77±0.68[a]	82.28±1.7[a]	184.67±3.64[a]	26.21±0.92[a]	20.52±1.24[a]	15.92±0.69[a]
AA	0.627±0.10	1.03±0.02[a]	0.94±0.03	0.37±0.02	10.56±0.69[a]	0.85±0.04[a]	4.80±0.64[a]	43.26±1.33[a]	66.22±2.28[a]	13.97±1.04	13.27±0.97	10.42±0.69[a]
AA+Ethanol	1.03±0.09[a]	1.39±0.07[b]	1.32±0.09[b]	0.693±0.06[ab]	14.93±0.82[ab]	1.94±0.15[a]	8.91±0.70[b]	71.57±1.52[ab]	103.4±3.88[ab]	20.42±0.81[ab]	17.43±0.94[b]	13.97±0.73[b]

Values expressed as mean ± SD of 6 rats
[a]– P<0.05 between control and other treated groups
[b]– P<0.05 between ethanol and ethanol+ascorbic acid group

Free fatty acid content

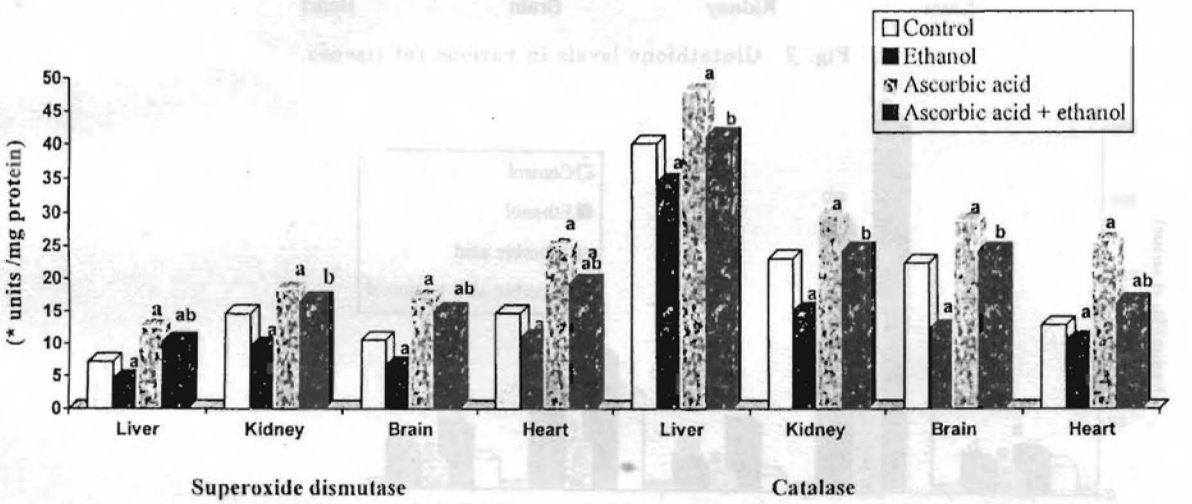
As shown in the Table II, concentration of FFA showed an increase in group II and IV when compared with that of group I. In group III the FFA decreased significantly in all the tissues except kidney in comparison with the control. In the rats fed ethanol + ascorbic acid there was a significant reduction in the FFA level in all the tissues except brain in comparison with those fed alcohol alone.

Lipid peroxidation products

The levels of MDA, hydroperoxides, and conjugated dienes were found to decrease in all the tissues of rats given ascorbic acid (Table–III). But a significant increase was noticed in the ethanol–treated group in comparison with the corresponding values for the control group. In the ascorbic acid + ethanol treated group, the lipid peroxides level was less than that of the alcohol–treated group.

Activities of SOD and catalase

The activities of SOD and catalase were found to increase in all the tissues studied in rats administered a mega dose of vitamin C and to decrease significantly in group II in comparison with control group (Fig. 1). In rats given ascorbic acid + ethanol, their activities increased in comparison with the activities of the ethanol–treated group.



SOD-*units -Enzyme concentration required to inhibit the chromogen production (OD at 560 nm) by 50% in 1 min.
Catalase-Values x10^{-3} units /mg protein (unit- Velocity constant /sec.)
Values expressed as mean ± SD of 6 rats
a - $p < 0.05$ between control and other treated groups
b - $p < 0.05$ between ethanol and ethanol + ascorbic acid group

Fig. 1 : Activity of Superoxide dismutase and Catalase in rats.

Concentration of glutathione

The concentration of glutathione significantly increased in all the tissues of groups II in comparison with the control (Fig. 2). But in the ascorbic acid+ethanol-treated group, the glutathione content decreased significantly in comparison with the ethanol treated group.

Activities of GOT, GPT and GGT

Activities of GOT, GPT and GGT in serum and liver significantly increased in rats given alcohol (Fig. 3). But co-administration of ascorbic acid and ethanol reduced their activities when compared with that of rats given alcohol alone.

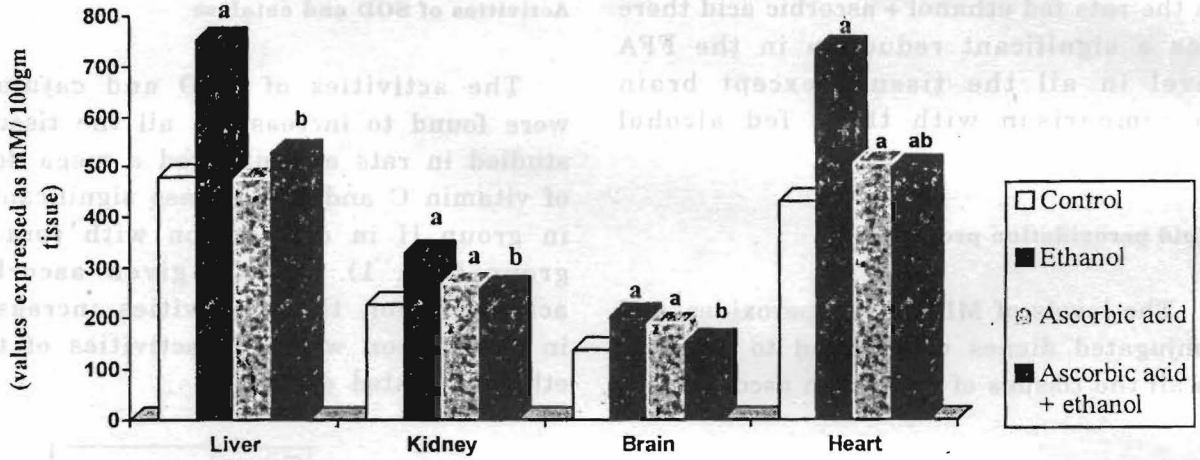
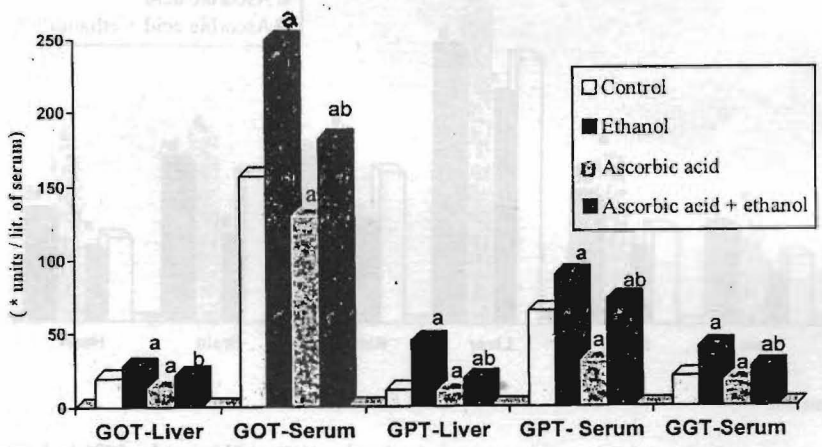


Fig. 2 : Glutathione levels in various rat tissues.



Values expressed as mean ± SD of 6 rats
a - $p < 0.05$ between control and other treated groups
b - $p < 0.05$ between ethanol and ethanol + ascorbic acid group

GGT-Units - The molar absorption coefficient of 4 nitro aniline at 405 nm is 9900/mol-1 cm-1 −1 cm −1
GOT- Units- Moles of OAA lib/min/mg protein.
GPT- Units- Moles of pyruvate lib/min/mg protein.

Fig. 3 : Activity of GOT, GPT and GGT in rats.

TABLE IV: Cholesterol, Phospholipids and MDA content of rat erythrocyte membrane.

Groups	Cholesterol (mg/dl)	Phospholipids (mg/dl)	MDA (μmole of MDA formed/mg protein)	Cholesterol: Phospholipids
I. Control	186.40±13.98	413.21±20.41	16.38±1.26	0.45±0.02
II. Ethanol	220.75±16.57[a]	412.17±24.92	21.53±1.85[a]	0.53[a]±0.03[a]
III. Ascorbic acid	178.40±11.25[a]	266.60±14.38[a]	12.33±0.097[a]	0.66±0.087[a]
IV. Ascorbic acid + Ethanol	211.71±14.60	377.08±17.17[ab]	18.13±1.32[b]	0.62±0.083[a]

Values expressed as mean ± SD of 6 rats
[a]– P<0.05 between control and other treated groups
[b]– P<0.05 between ethanol and ethanol + ascorbic acid groups

Erythrocyte membrane cholesterol, phospholipids and MDA

There was significant increase in the erythrocyte membrane cholesterol, and malondialdehyde in the alcohol fed rats when compared to the controls (Table-IV). In the ascorbic acid treated group the cholesterol, phospholipids and MDA levels were significantly reduced when compared with other groups. On co-administration of ascorbic acid + ethanol, phospholipid and MDA levels were significantly lower than that of the ethanol fed rats.

DISCUSSION

The desirable intake of ascorbic acid for the maintenance of optimal health is a subject of considerable controversy. It has been suggested by Pauling (23) that an adequate intake of ascorbic acid should be about 3 g per day under ordinary conditions and larger, upto 40 g per day, for a person under stress. Nandi et al. (24) reported large dose of ascorbic acid (0.5 mg to 250 mg in 0.75 ml water) had neither beneficial nor toxic effect on growth and maintenance of rats and guinea pigs fed a nutritionally balanced fortified wheat diet. Previous studies from our laboratory have shown that supplementation of 200 mg AA/100 g body weight of AA decreased the cholesterol level in rats (25, 26). And we have not observed any toxic effects at this dosage. Hence we selected this dose of ascorbic acid to study its effects on alcohol induced oxidative stress.

As reported in previous studies, supplementation of AA raised the serum level of AA in rats (27). But ethanol administration raised ascorbate level in the serum of rats.

The biochemical mechanism of ethanol induced-toxicity is poorly understood. One of the proposed mechanisms is the membrane damage due to the direct effect of lipid peroxidation. Consistent with this idea, increased levels of lipid peroxidation products, ie, MDA, hydroperoxides and diene conjugates were found in the alcoholic rats in the present study. But the administration of ascorbic acid along with alcohol offered some protection against lipid peroxidation. This supports Cadens (28) observations that vitamin C supplementation decrease endogenous oxidative damage in guinea pigs under

stressful condition. The FFA's which increased in the tissues of alcohol–administered rats serve as the substrate for the lipid peroxidation. Increase in the level of fatty acids may be closely associated with the breakdown of the membrane structure caused by the lipid peroxidation (29). Maintenance of normal cell function in the presence of O_2 largely depends upon the efficiency of the tissue defense mechanism against free radical–mediated oxidation system. Cells have developed several defense systems. The activities of antioxidant enzymes such as SOD and catalase were considerably increased in the ethanol + ascorbic acid group in comparison with those administered ethanol alone. However, in the ethanol group, the levels were considerably lower than those in the control group. This is in agreement with the studies of Asha *et al* (30), ethanol intoxication enhanced glutathione content, which was brought down to the normal level by the mega dose of ascorbic acid. This is in agreement with the result of Heter et al. (31), who observed that chronic ethanol feeding increased the glutathione content. The increase in glutathione content may be due to a feed back activation of glutathione synthesis.

According to Wayner *et al* (32) ascorbate contributes upto 24% of the total peroxy radical trapping antioxidant capacity in human plasma. Erythrocyte membrane was taken as a model to study the ethanol induced peroxidative damage, since it is more prone to lipid peroxidation because of its polyunsaturated lipid content. There was increased cholesterol, MDA content indicating alterations in cholesterol: phospholipids ratio and peroxidative damage to the membrane. Alterations in the cholesterol phospholipids ratio is the result of an adaptive mechanism to resist the fluidizing effect of ethanol. A number of studies in the experimental animals and human beings support our results (33). But a high dose of AA partly changed this picture, thus pointing out the beneficial effect of AA in reducing the alcohol induced toxicity. Only further *in vitro* studies will prove the actual mechanism of ascorbic acid's action.

There are a number of contradictory reports on the role of ascorbic acid in reducing lipid peroxidation. Barja *et al* (34) showed that 660 mg/kg body weight of ascorbic acid did not decrease any of the antioxidant defences in guinea pigs, such as SOD, catalase, glutathione peroxidase, and glutathione. But there are also reports showing that both low and very high levels of vitamin C decreased body weight, glutathione reductase, and the degree of unsaturation of fatty acid acids in membrane lipids. The diet supplying an amount of vitamin C 40 times higher than the maximum daily requirement has been shown to increase the global antioxidant capacity (34,35). *In vitro* studies have shown that a high concentration of ascorbate induced cytotoxicity and that a low concentration of it had a stimulatory effect on growth. This induction of toxicity is attributed to the generation of H_2O_2. In this *in vivo* study we have observed that administration of ascorbic acid at a dose of 200 mg/100g body weight enhanced the activity of catalase, the enzyme responsible for the removal of the peroxide radical.

Even though there have been s tudies showing the peroxidative damage caused by ethanol and the antioxidant capacity of ascorbic acid, experiments have not been undertaken to assess the effect of a mega dose of ascorbic acid on alcohol induced-toxicity. This study clearly shows that ascorbic acid protects against ethanol-induced toxicity by increasing the activities of scavenging enzymes, by reducing the lipid peroxidation products and by altering the lipid composition of the plasma membrane. The increase in the activities of the marker enzymes GOT, GPT and GGT in animals given ethanol and ascorbic acid in comparison with their levels in animal given alcohol alone also substantiates the protective action of ascorbic acid.

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