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Hepatoprotective effects of Liv-52 on ethanol induced liver damage in rats

Rajat Sandhir* & K D Gill**

Department of Biochemistry, Postgraduate Institute of Medical Education & Research, Chandigarh 160 012, India

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The mechanism of protective effects of Liv-52, a multiherbal hepatoprotective drug, on ethanol induced hepatic damage has been investigated. The results indicate that Liv-52 treatment prevents ethanol induced increase in the activity of the enzyme γ–glutamyl transpeptidase. Concomitantly there was also a decrease in ethanol accentuated lipid peroxidation in liver following Liv-52 treatment. The activity of antioxidant enzymes; superoxide dismutase, glutathione peroxidase and the levels of glutathione were decreased following ethanol ingestion. Liv-52 treatment was found to have protective effects on the activity of superoxide dismutase and the levels of glutathione. The results obtained from the study indicate hepatoprotective nature of Liv-52 which might be attributed to its ability to inhibit lipid peroxidation.

Ethanol is currently recognized as the most prevalent known cause of abnormal human development. Alcohol abuse and alcoholism represents one of the major health, social and economic issues facing the world. Liver is among the organs most susceptible to the toxic effects of ethanol[1]. It is now generally accepted that alcohol can induce *in vivo* changes in membrane lipid composition and fluidity[2], which may eventually effect cellular functions. Among the mechanisms responsible for the effects of alcohol, lipid peroxidation appears to be a likely candidate, since this process can account for alterations in membrane phospholipid composition observed after ethanol intoxication[3,4]. Aykac *et al.*[5] have observed an increase in hepatic lipid peroxidation following chronic ethanol ingestion.

Traditional medicines are effective in certain disorders and are based on experience in the use of plant products in amelioration of common diseases. Liv-52, an ayurvedic multiherbal formulation is widely used in various hepatic disorders[6-8]. Liv-52 has recently been reported to have protective effects in carbon tetrachloride[9,10], paracetamol[11] and ethanol[12] toxicity. However, very less scientific data regarding the identification and effectiveness of these herbs is available. Therefore, this study has been designed with an aim to understand the mechanism by which Liv-52 may exert its hepatoprotective effects following ethanol exposure.

*Present Address:
Department of Biochemistry, Dr. Ram Manohar Lohia Avadh University, Faizabad 224 001, India.
**Correspondent author
Tel 0172 747585-92 Ext. 516
Fax 0172 744401
Email medinst@pgi.chid.nic.in

Materials and Methods

Ethanol was obtained from E. Merck, Munich, Germany and Liv-52 was a kind gift from Himalaya Drug Co., Bangalore, India. Every 2.5 ml of Liv-52 syrup contains an extract of the following : *Capparis spinosa* (17mg); *Cichorium intybus* (17mg); *Solanum nigrum* (8mg); *Cassia occidentalis* (4mg); *Terminalis arjuna* (8mg); *Achillea millefolium* (4mg); and *Tamaria gallica* (4 mg). Glutathione, NADPH, DTNB, thiobarbituric acid, BSA, Tris were obtained from Sigma Chemical Co., USA. All other chemicals were obtained from local sources and were of analytical grade.

Animals and treatment—Male albino rats (Wistar strain) of 8-10 weeks of age weighing between 100 and 120g were used for the study. The animals were housed in polypropylene cages, fed on pellet diet (Hindustan Lever Ltd., India) and water *ad libitum*. Animals were divided into three groups of 6 animals each. Group I received normal saline, intragastrically. Group II received ethanol 3g/kg body wt, intragastrically, for a period of 4 weeks. Group III received ethanol (3g/kg body wt) and Liv-52 (1.0 ml/kg body wt) for 4 weeks intragastrically.

At the end of treatment, animals were anaesthetized with ether and sacrificed by decapitation. Blood was drawn from the supraorbital sinus, and serum separated for γ–glutamyl transpeptidase assay. Livers

were removed, washed with ice cold saline (0.15 *M*) and a 10% (w/v) homogenate prepared in 0.1 *M* Tris HCl, *p*H 7.4 for lipid peroxidation and glutathione estimation. The postnuclear fraction for catalase was obtained by centrifugation of homogenate at 1000 *g* for 20 min at 4°C and for other enzyme assays, the post nuclear fraction was centrifuged at 12,000 *g* for 60 min at 4°C.

Lipid peroxidation—The quantitative measurement of lipid peroxidation was performed according to the method of Wills[13]. The amount of malondialdehyde (MDA) formed was quantitated by reaction with thiobarbituric acid and used as an index of lipid peroxidation. The results were expressed as nmol MDA/mg protein using molar extinction co-efficient of the chromophore ($1.56 \times 10^{-5}\ M^{-1}\ cm^{-1}$)

Enzyme assays—The activity of antioxidant enzymes, viz. superoxide dismutase, catalase and glutathione peroxidase was assayed in livers of experimental animals and the activity of γ–glutamyl transpeptidase was assayed in serum of rats.

Superoxide dismutase was assayed according to the method of Martin *et al.*[14], wherein the autooxidation of hematoxylin to hematin is inhibited by the enzyme. The results were expressed as units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50%.

Catalase was assayed by the method of Luck[15] wherein breakdown of H_2O_2 by the enzyme is measured at 240nm. Enzyme activity was calculated using the millimolar extinction coefficient of H_2O_2 (0.07) and the results were expressed as μmol H_2O_2 decomposed/min/mg protein.

Glutathione peroxidase was assayed by the method of Lawrence and Burk[16], wherein oxidation of NADPH by H_2O_2 was followed at 340nm. Enzyme activity was calculated using the molar extinction coefficient of NADPH (6.22×10^{-6}) and the results expressed as nmol NADPH oxidized/min/mg protein.

γ–glutamyl transpeptidase activity was ascertained in serum by the method of Szasz[17], wherein the transfer the γ–glutamyl group of γ–glutamyl-4-nitroanilide to glycyl-glycine is measured. The results were expressed as IU/L.

The control activities of various enzymes studied are in accordance to those previously reported[18].

Glutathione estimation—Glutathione (GSH) was estimated in the samples by the method of Ellman[19] and the results were expressed as μmol GSH/mg protein.

Protein estimation—Protein in the samples was quantitated by the method of Lowry *et al.*[20] using bovine serum albumin as standard.

Statistical analysis was carried out using the Student's t-test. Values having $P<0.05$ were considered significant.

Results

The activity of γ–glutamyl transpeptidase was used as an index of ethanol induced hepatic damage. It was observed that ethanol exposure (3g/kg body wt., intragastrically) for 4 weeks resulted in a 2-fold increase of γ–glutamyl transpeptidase activity, whereas in the animals given Liv-52 along with ethanol, the activity of γ-glutamyl transpeptidase was completely restored, indicating the *in vivo* protective effects of Liv-52 against ethanol induced damage (Fig. 1).

In an attempt to understand the mechanism by which Liv-52 prevents hepatic damage caused by ethanol,detailed investigations were carried out relating to lipid peroxidation and antioxidant enzymes. The results in Fig. 2 indicate that ethanol *in vitro* (10 μg/ml) enhanced the amount of malondialdehyde formed, confirming that ethanol induced hepatotoxic effects are mediated through enhanced generation of free radicals. However, the effect of exogenously added Liv-52 on ethanol induced lipid peroxidation could not be studied, since the colour of Liv-52 extract interfered with the assay of lipid peroxidation. Therefore the *in vivo* effect of Liv-52 on ethanol induced lipid peroxidation was studied. The data in Fig. 3 indicate that ethanol could accentuate lipid peroxidation, a mediator of tissue damage, even after *in vivo* exposure. Whereas, when Liv-52 was given along with ethanol, the levels of lipid peroxidation were

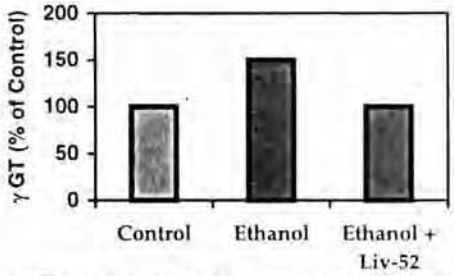

Fig. 1—Ethanol induced increase in γ–glutamyl transpeptidase activity in rat serum. Values are presented in percentages relative to control from the data obtained from studies on 6 animals in each group. a : $P<0.001$ as compared to control group, b : $P<0.001$ as compared to ethanol treated group.

restored to that observed in control, indicating protective efficacy of Liv-52 against hepatotoxicity of ethanol.

The activity of antioxidant enzymes, superoxide dismutase and glutathione peroxidase was significantly inhibited in liver, following ethanol exposure, whereas the activity of catalase increased markedly following ethanol exposure. The levels of reduced glutathione were observed to decrease in liver of ethanol exposed animals. Liv-52 treatment on the other hand was able to restore the activity of superoxide dismutase and the levels of glutathione in ethanol treated animals (Table 1). No significant effect was observed on the activity of catalase and glutathione peroxidase.

Discussion

The results obtained indicate that ethanol induced hepatotoxic damage in terms of the increase in γ–glutamyl transpeptidase activity, a known marker of ethanol induced hepatic damage[21]. The increase in γ–glutamyl transpeptidase activity was prevented by Liv-52 treatment, thereby confirming the efficacy of Liv-52 in counteracting the ethanol induced liver damage. Liv-52 treatment also restored the levels of ethanol induced lipid peroxidation to that in control liver. An increase in lipid peroxidation has already been reported after both acute and chronic exposure[22,23]. The effect of ethanol has been suggested to be a result of the enhanced generation of oxyfree radicals during its oxidation in liver[24]. The peroxidation of membrane lipids, results in loss of membrane structure and integrity resulting in elevated levels of γ–glutamyl transpeptidase, a membrane bound enzyme in serum. Goel and Dhiman[25] have reported protective effect of Liv-52 on carbon tetrachloride induced NADPH dependent lipid peroxidation and hepatic functions. These authors have further reported efficacy of Liv-52 in preserving the structural integrity of liver.

Our study demonstrates that ethanol exposure in-

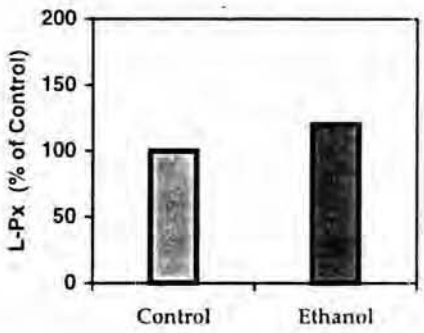

Fig. 2.—Effect of *in vitro* ethanol on lipid peroxidation in rat liver. Values are presented in percentages relative to control from the data obtained from five sets of observations. a : $P<0.001$ as compared to control group.

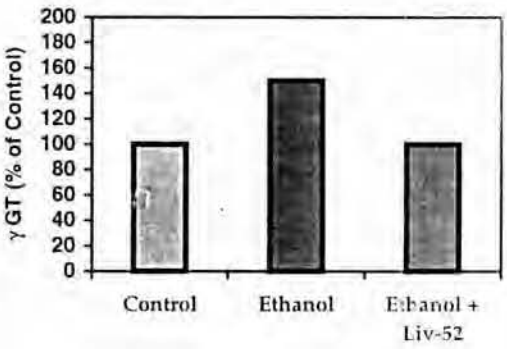

Fig. 3—*In vivo* effect of Liv-52 treatment on ethanol induced lipid peroxidation in rat liver. Values are presented in percentages relative to control from the data obtained from studies on 6 animals in each group. a : $P<0.001$ as compared to control group, b : $P< 0.001$ as compared to ethanol treated group.

Table 1—Ethanol induced alterations in antioxidant enzymes and glutathione levels in rat liver
[Values are mean ± SD of 6 animals/group]

	Superoxide dismutase (Units/mg protein)	Catalase (μ mol H_2O_2 decomposed/ min/mg protein)	Glutathione peroxidase (μ mol NADPH oxidised/ min/mg protein)	Glutathione (μ mol GSH/ mg protein)
Control group	16.47 ± 1.47	166.57 ± 7.93	297.85 ± 13.44	45.11 ± 2.13
Ethanol treated group	10.05 ± 0.83 [a]	209.84 ± 8.97 [a]	200.34 ± 10.43 [a]	32.73 ± 1.62 [a]
Ethanol + Liv-52 treated group	15.43 ± 1.29 [b]	201.58 ± 10.09 [NS]	213.34 ± 6.25 [NS]	43.13 ± 1.54 [b]

P values: [a] <0.001 compared to control group; [b] <0.001 compared to ethanol treated group; [NS] Not significant

peroxide dismutase, glutathione peroxidase and levels of glutathione in liver, whereas, Liv-52 treatment restored the activity of superoxide dismutase and the levels of glutathione to nearly those observed in control livers. Superoxide dismutase is an enzyme responsible for dismutation of highly reactive and potentially toxic superoxide radicals (O_2^-) to H_2O_2. A reduced activity of this enzyme may reduce its cellular efficacy to detoxify these potentially toxic oxyradicals which will lead to an increase in the levels of lipid peroxidation[26]. Glutathione is an important naturally occurring antioxidant as it prevents the hydrogen of the sulfhydryl group to be abstracted instead of methylene hydrogen of unsaturated lipids[27]. Therefore, the levels of glutathione are of critical importance in tissue injury caused by toxic substances. The binding of acetaldehyde, a metabolite of ethanol with glutathione may contribute to reduction in the levels of glutathione[29]. The ability of Liv-52 to protect the liver from ethanol induced damage might be attributed to its direct antiperoxidative effect or may be due to its ability to restore the activity of antioxidants, superoxide dismutase and glutathione. The enzyme superoxide dismutase and glutathione constitute the first line of defense against free radical induced damage and a restoration of the superoxide dismutase activity and glutathione levels by Liv-52 may account for its protective effects. The decrease in the activity of antioxidant enzymes superoxide dismutase, glutathione peroxidase and glutathione are speculated to be due to the damaging effects of free radicals produced following ethanol exposure or alternatively could be due to a direct effect of acetaldehyde, formed from oxidation of ethanol, on these enzymes[30,31]. A decrease in the activity of certain metabolic enzymes induced by free radicals generated on the oxidation of ethanol has been reported following ethanol exposure[32]. The antioxidant effect and resultant hepatoprotective ability of Liv-52 may be attributed to flavinoids, α and β–carotenes, vitamin A and C present in the multiherbal preparation[33,34], which explains its ability to reduce the levels of lipid peroxidation and restore the antioxidant status. Chauhan *et al.*[35], have demonstrated that Liv-52 enhances acetaldehyde elimination and also prevents binding of acetaldehyde to cellular proteins and thereby exerts its protective effects. The activity of glutathione peroxidase, an enzyme which reduces the levels of peroxides in the cell and thus protects the cell from peroxidative damage was also inhibited on ethanol exposure. On the contrary, coexposure of ethanol and Liv-52 failed to restore the activity to that observed in the control animals. The reduced activity of glutathione peroxidase might not contribute towards peroxidative damage following ethanol exposure, since the critical antioxidants superoxide dismutase and glutathione, which are the first lines of defense, offer protection against free radicals and thus maintain low levels of lipid peroxides. However, the increase in the activity of catalase, an important antioxidant enzyme responsible for detoxification of H_2O_2 dependent ethanol oxidation[36], may be a adaptive mechanism in response to ethanol ingestion. Increased catalase activity after ethanol exposure is considered to be harmful as it results in the formation of acetaldehyde, a very reactive compound.

The results of the present study thus demonstrate that Liv-52 protects liver from ethanol induced damage by preventing the peroxidation of membrane lipids. Further studies are, however, needed to isolate the specific components responsible for the antioxidant action of this multiherbal drug and to establish its mechanism of action.

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