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Hepatoprotective Effects of Liv.52 and its Indirect Influence on the Regulation of Thyroid Hormones in Rat Liver Toxicity induced by Carbon Tetrachloride

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ABSTRACT

This study was undertaken to investigate the protective effect of Liv.52, a herbal formulation, on various serum and liver marker enzymes, lipid peroxidation and histological changes in the liver of male albino rats suffering long-term carbon tetrachloride (CCl_4) poisoning. It was observed that the activities of serum marker enzymes, hepatic enzymes and NADPH-dependent lipid peroxidation were significantly elevated after treatment with CCl_4. However, in rats given Liv.52 at the same time as CCl_4, the levels of all the marker enzymes were closer to oxidation after Liv.52 treatment. CCl_4 alone caused severe liver damage, but when Liv.52 was simultaneously given, there was much less effect on the structure of the organ. Circulating T_3 and T_4 concentrations were also determined after 6 weeks of simultaneous treatment with Liv.52 and CCl_4. Concentrations of T_3 were significantly lowered after CCl_4 treatment alone, but Liv.52 administration together with CCl_4 resulted in maintenance of T_3 activity within normal limits., thus suggesting indirect beneficial effects of Liv.52 on the regulation of thyroid hormone concentrations.

Key words: Liv.52 – Carbon tetrachloride – Hepatotoxicity – Tri-iodothyronine – Thyroxine

INTRODUCTION

The liver, because of its strategic anatomical location and its large capacity for metabolic conversions, is exposed to many kinds of xenobiotics and therapeutic agents. Moreover, the rapidly growing morbidity and mortality from liver disease is largely attributable to the increasing number of noxious medicinal agents and environmental pollutants[1]. Unfortunately, so far, in the modern era of medicine, there is no specific treatment to counter the menacing impact of these dreaded diseases. The therapeutic regimen followed in all these cases upto the present moment is by and large symptomatic and at best palliative, but it still confronts the practitioner with a formidable task. In view of this situation, attempts are perpetually being made to find some effective therapy for such conditions. However, during the last three decades, Liv.52, an indigenous herbal formulation of several well-known plant principles, prepared according to Ayurvedic concepts, is routinely being prescribed by the physicians and clinicians in India, as well as in many other countries, for various liver ailments, with highly promising results[2-4]. It has been subjected to extensive clinical and experimental evaluations and has been shown to be quite effective in restoring various biochemical and structural indices of hepatotoxicity[5-9] to within normal limits. Many workers have recorded their observations of different parameters and on the whole, the main conclusion has been that this preparation has a definite beneficial effect on the liver. Many of the active principles of this drug mixture are individually well known for their hepatoprotective potential, but since the mixture is prescribed and used as a whole, we were interested in investigating the efficacy of its combined effects, rather than in undertaking the characterization of its ingredients.

A typical and most frequently used experimental model to study liver damage involves the use of carbon tetrachloride (CCl_4)[10-12]. It is an established potent toxin that is metabolized by a microsomal drug-oxidizing system to a more toxic metabolite, the CCl_3 radical, which initiates peroxidative changes in polyunsaturated fatty acid constituents of various biomembranes[4,11]. An earlier report form our laboratory has also shown increased lipid peroxidation after CCl_4 poisoning[4], while increased activities of serum marker enzymes have been well documented[8,13].

It has also been suggested in a few reports that in certain cases liver injury can manifest itself through alterations in the hormonal status of the thyroid gland1[14,15]. Liver plays an important role in the metabolism of thyroid hormones, being involved in their conjugation, excretion and peripheral deiodination and in the synthesis of thyroxine binding globulin (TBG)[16,17]. Further observations have shown that although almost all patients with liver disease are clinically euthyroid, some abnormalities in the circulating hormone concentrations have been reported[16,17]. However, we have no information about the influence of liver toxicity on the function of the thyroid gland in experimental conditions.

In view of these observations, and bearing in mind the claims about the efficacy of the preparation in liver disease, we planned to ascertain in detail the hepatoprotective potential of Liv.52 in the long-term toxicity of liver. We also decided to investigate its indirect influence, if any, on the circulating concentrations of tri-iodothyronine (T_3) and thyroxine (T_4).

MATERIALS AND METHODS

Reagents

Carbon tetrachloride was purchased from Merck (India), Bombay. Liv.52 was a generous gift from The Himalaya Drug Co., Bombay, India, NADPH and BSA were procured from the Sigma Chemical Co. St. Louis, Mo. USA and NADH was obtained from Hi-Media Chemical Co., Bombay, India. All other reagents used were of analytical grade.

Animals

Mature male albino rats in the weight range of 150-180 g were procured from the Central Animal House, Punjab University, Chandigarh, India. The animals were fed with pelleted standard laboratory feed (supplied by Hindustan Lever, Bombay) and water ad libitum.

Experimental design

Animals were segregated into three treatment groups of ten rats each. Group I animals were injected with 0.2 ml of groundnut oil biweekly and served as controls. Animals in-groups 2 and 3 were injected subcutaneously (s.c.) with 0.2 ml of carbon tetrachloride mixed with 0.2 ml of groundnut oil, twice a week[18]. Animals in group 3 received in addition 0.2 ml of Liv.52 daily through intubation gavage technique. All treatments continued for a total period of 6 weeks, and then the animals were killed by exsanguination under light ether anesthesia. Livers were removed immediately and thoroughly rinsed in saline (NaCl, 9g/l w/v) and preserved for the determination of various enzyme activities and histoarchitectural studies.

Composition of Liv.52 syrup
Each 2.5 ml of Liv.52 syrup contains extracts of the following:

Capparis spinosa	17 mg
Cichorium intybus	17 mg
Solanum nigrum	8 mg
Cassia occidentalis	4 mg
Terminalia arjuna	8 mg
Achillea millefolium	4 mg
Tamarix gallica	4 mg

Body weight measurement and blood collection
To assess the general health of the animals during and after the various treatments, body weights of all the animals were recorded twice a week regularly for the whole of the treatment duration. Blood samples were drawn out of all the rats under light ether anesthesia by ocular vein puncture at the time of death. The serum was separated by centrifugation for determination of the activities of various serum marker enzymes and also for the estimation of T_3 and T_4 concentrations.

Biochemical estimations
Biochemical analyses of various serum and liver marker enzymes for the assessment of the extent of hepatotoxicity were carried out by standard methods. Alkaline phosphatase was estimated by the method of Wooton[19], alanine transaminase (ALT) and aspartate transaminase (AST) by the procedure of Reitman and Frankel[20], NADPH-dependent lipid peroxidation by the method of Pederson *et al.*[21] and proteins by the method of Lowry *et al.*[22].

Histological studies
As soon as the rats had been killed, small pieces of the apparently affected parts of the tissues were cut out and rinsed quickly and thoroughly two or three times in phosphate-buffered saline (PBS). The tissue pieces were then fixed in 10% buffered formaldehyde for 24 h, dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax (58-60ºC). Sections were cut at 5-6 µm, double stained with hematoxylin-eosin and examined under the light microscope.

Radioimmunoassay of T_3 and T_4
Serum concentrations of T_3 and T_4 were determined by radioimmunoassay from the kits procured from Bhabha Atomic Research Center, Trombay, Bombay, India. The sensitivities of the kits for the detection of T_3 and T_4 were 0.24 ng/ml and 0.5 µg/dl, respectively.

Statistical analysis
Data were expressed as the mean ± SD. All the studies requiring statistical evaluation included a minimum of six animals per group. The statistical significance of differences between the controls and the experimental groups was estimated by means of Student's '*t*'-test.

RESULTS

During the course of the present study, the body weights of control rats and those given Liv.52 + carbon tetrachloride rose steadily. However, animals given only CCl_4 showed fluctuating changes in

body weight throughout and no significant gain in net body weight was observed by the end of the study (Table 1).

The activities of the serum marker enzymes, AST, ALT and alkaline phosphatase increased significantly after CCl_4 treatment (Table 2). In rats treated with both Liv.52 and CCl_4 the activities of all these enzymes were significantly less than in rats treated with CCl_4 alone ($p<0.01$) and were close to control levels.

Similarly levels of all the hepatic enzymes, alkaline phosphatase, AST and ALT, were found to be elevated significantly after treatment with carbon tetrachloride (Table 3). Malonaldehyde products showed a considerable increase ($p<0.001$) as a result of CCl_4 damage, as shown by NADPH-dependent lipid peroxidation (Table 3). Simultaneous Liv.52 treatment prevented the rise in the activities of most of these enzymes, although some significant increase ($p<0.05$) was noticed in the activity of hepatic AST levels after 6 weeks of treatment. As regards the histoarchitecture of liver, the control animals revealed clear-cut hepatic lobules, separated by interlobular septa, traversed by portal veins, hepatic artery and bile ducts (hepatic triads). In the middle of each hepatic lobule is a central or intralobular vein with a few Kupffer cells around it. Within each lobule, plates of hepatic cells radiate from the central vein towards the periphery, while between the laminae, hepatic sinusoids prevail. Most of the cells are polyhedral and mononucleated

Table 1: Body weight of rats treated with carbon tetrachloride (CCl_4) or Liv.52 + CCl_4 (mean weight in grams ± SD)

Intervals	n	Control	CCl_4	CCl_4 + Liv.52
0 day	10	145.30 ± 20.30	169.63 ± 20.16	154.13 ± 9.13
1 week	10	143.30 ± 20.13	171.87 ± 15.30	156.42 ± 8.70
	10	155.91 ±22.30	171.50 ±17.50	158.00 ± 15.41
2 weeks	10	159.60 ± 21.13	163.25 ± 16.40	165.00 ± 7.39
	10	154.30 ± 18.57	160.37 ± 17.79	170.14 ± 14.15
3 weeks	10	158.80 ± 21.26	166.17 ± 22.13	171.00 ± 19.13
	9	162.32 ± 27.29	167.19 ± 13.16	176.16 ± 14.13
4 weeks	9	167.66 ± 21.13	162.81 ± 19.18	175.17 ± 16.13
	8	171.09 ± 31.09	162.11 ± 23.34	179.13 ± 34.18
5 weeks	8	169.18 ± 22.34	166.33 ±18.19	180.19 ± 10.19
	8	176.34 ± 18.19	168.14 ± 22.13	183.16 ± 12.26
6 weeks	8	179.88 ± 19.97	163.00 ± 31.09	180.19 ± 14.18
	7	186.88 ± 19.07	165.16 ± 19.16	184.16 ± 13.18

Table 2: Alterations in the activities of serum marker enzymes in rats after treatment with carbon tetrachloride (CCl_4) or CCl_4 + Liv.52 (Values are mean ± SD)

Group	n	Alkaline phosphatase[a]	AST[b]	ALT[b]
1 (Control)	6	13.71 ± 1.68	13.50 ± 2.94	10.50 ± 1.97
2 (CCl_4)	6	28.13 ± 3.79**	35.80 ± 6.90**	35.50 ± 7.14**
3 (CCl_4 + Liv.52)	6	15.24 ± 1.45	19.33 ± 4.38**	13.62 ± 2.29**

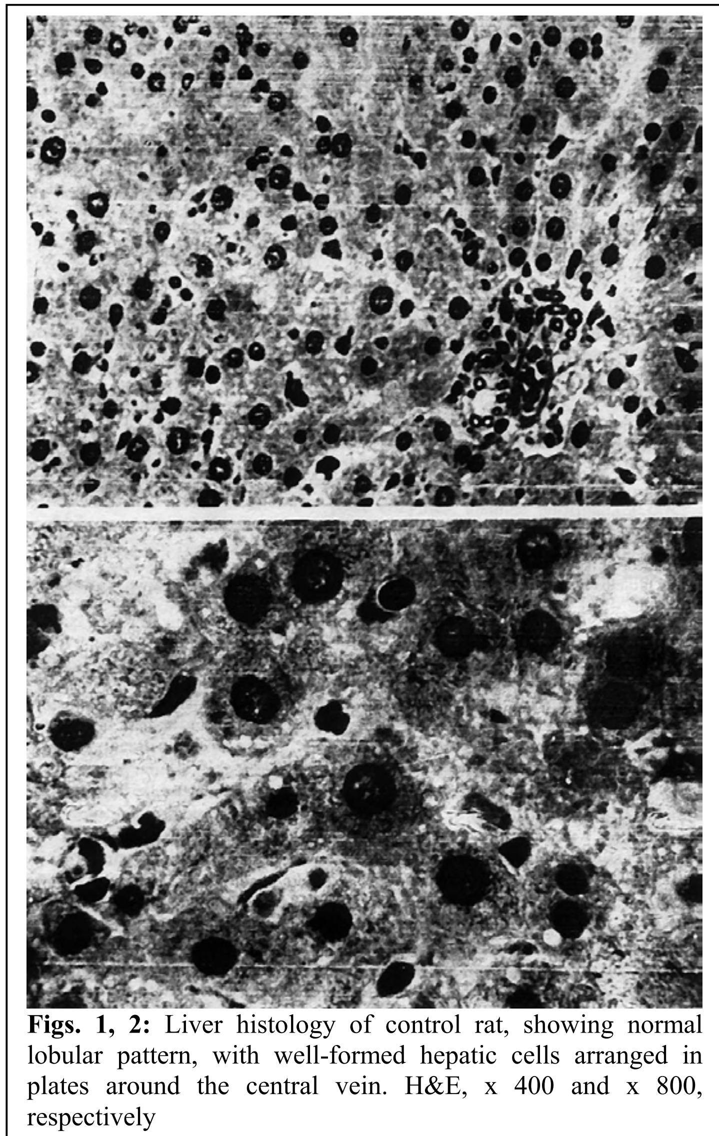
[a] Enzyme activity is expressed as King Armstrong units/100 ml serum
[b] Enzyme activity is expressed as IU/l of serum
*$p<0.01$; **$p<0.001$ (difference from controls/CCl_4-treated animals)

Table 3: Alterations in the activities of hepatic enzymes and NADPH-dependent lipid peroxidation in rats after treatment with carbon tetrachloride (CCl_4) or CCl_4 + Liv.52. Values are mean ± SD

Group	n	Alkaline phosphatase[a]	AST[b]	ALT[b]	NADPH-dependent lipid peroxidation[c]
1 (Control)	6	0.329 ± 0.060	2.54 ± 0.51	6.93 ± 0.86	0.203 ± 0.040
2 (CCl_4)	6	0.629 ± 0.072***	3.73 ±0.45**	10.31 ± 1.51***	0.729 ± 1.030***
3 (CCl_4 + Liv.52)	6	0.341 ± 0.051	2.89 ± 0.51	6.88 ± 0.53	0.258 ± 0.046*

[a] nmol pyruvate formed/min per milligram protein
[b] µg phenol formed/min per milligram protein
[c] nmol proteins in 15 min *$p<0.05$; **$p<0.01$; ***$p<0.001$ different from controls.

(Figs.1 and 2). Animals treated with CCl_4 showed drastic alterations in the internal structure of their livers. The main features were a disrupted pattern of hepatic cords, centrilobular vacuolization of hepatocytes and the incidence of necrosis, congestion and disruption of the central canal wall. A few areas were marked with severely swollen cells, “balloon cells”. There was a general collapse and condensation of liver architecture, accompanied by marked reduction of sinusoids and the conspicuous disappearance of interconnecting and branched sinusoids. Moreover, there were more Kupffer cells and more binucleated cells with variations in nuclear size (Figs.3 and 4).


Figs. 1, 2: Liver histology of control rat, showing normal lobular pattern, with well-formed hepatic cells arranged in plates around the central vein. H&E, x 400 and x 800, respectively

Rats treated with both CCl_4 and Liv.52 showed much less damage to liver structure. Hepatocytes were polyhedral, and hepatic cords were well defined. The hepatic cells in the middle zone were normal, although some vacuolization was visible in the peripheral cells. Necrosis, where present, was restricted to the centrilobular region, and only a few binucleated cells [with almost round nuceli) were seen. There were no “balloon cells”, while sinusoids were clearly visible (Figs.5 and 6).

Table 4 demonstrates that the animals poisoned with CCl_4 showed significantly depressed serum T_3 concentrations ($p<0.05$) but did not show any change in the scrum T_4 concentrations as compared to controls. However, when both CCl_4 and Liv.52 were administered, T_3 concentration remained within normal limits, while T_4 concentration was significantly depressed ($p<0.05$).

Table 4: Serum levels of thyroxine (T_4) and tri-iodothyronine (T_3) in male albino rats after treatment with carbon tetrachloride (CCl_4) or CCl_4 + Liv.52. Values are mean ± SD

Group	*n*	T_3 (ng/ml)	T_4 (µg/dl)
1 (Control)	6	0.422 ± 0.102	6.09 ± 1.56
2 (CCl_4)	6	0.138 ± 0.084*	4.98 ± 0.51
3 (CCl_4 + Liv.52)	6	0.345 ± 0.065	4.40 ±0.80*

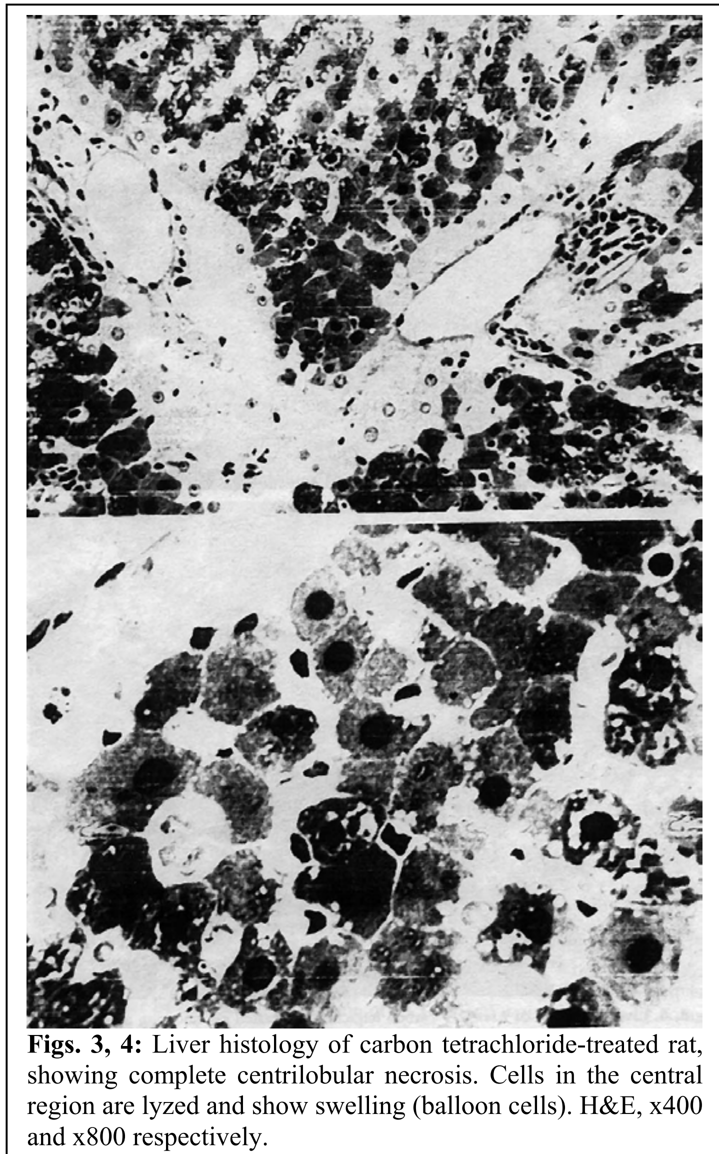
*$p<0.05$ (difference from control)

DISCUSSION

The present investigations revealed that the rats treated with CCl_4 did not gain in body weight throughout the study, while controls and those given Liv.52 with CCl_4 treatment put on weight steadily and showed appreciable net body weight gains at the end of the study. These observations could be explained if the animals given CCl_4 treatment alone responded by diminished food intake,

rather than suffering a primary toxic effect of CCl_4 itself. As we have not measured the quantity of food consumed by these animals, the above explanation remains speculative.

Alkaline phosphatase is an enzyme, which is loosely bound and is released into the blood stream when cell membranes are broken down[23]. The activity of alkaline phosphatase in liver as well as in serum was found to be significantly enhanced ($p<0.01$ to $p<0.001$) by CCl_4 treatment for 6 weeks. These results are in conformity with earlier findings[24,25] and could well be attributed to the toxic effects of CCl_4 on membranous lipids, CCl_4 is known to cause peroxidation of polyunsaturated lipids of biomembranes, resulting in the disruption of the membranous integrity and ultimately releasing the enzyme from the cell[26]. These observations are consistent with the observed increase in the extent of NADPH-dependent lipid peroxidation due to CCl_4 toxicity in group 2.



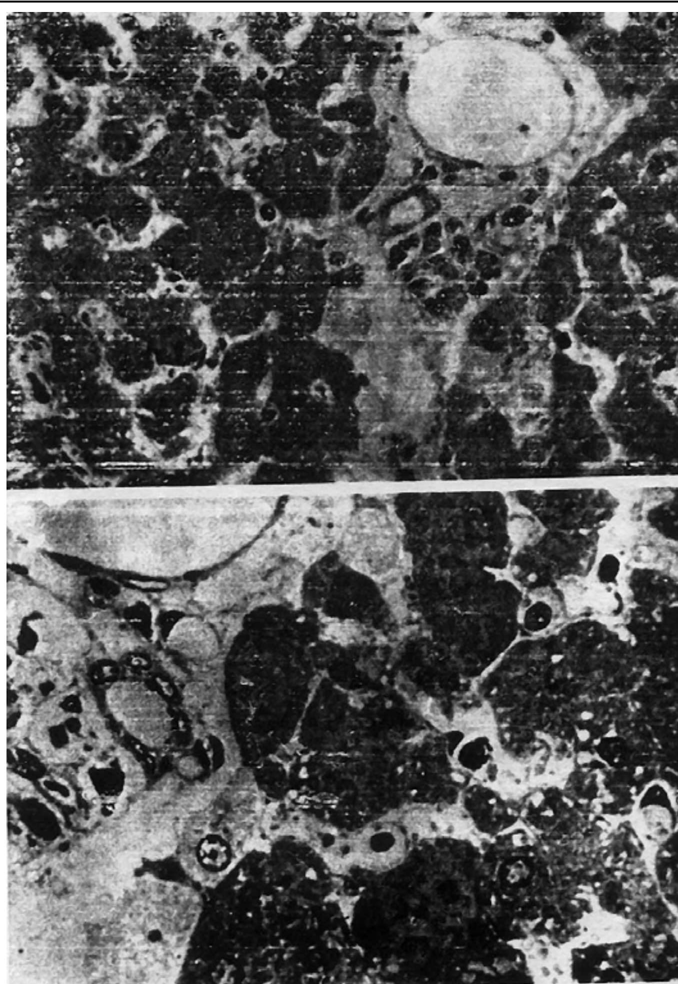
Figs. 3, 4: Liver histology of carbon tetrachloride-treated rat, showing complete centrilobular necrosis. Cells in the central region are lyzed and show swelling (balloon cells). H&E, x400 and x800 respectively.

Liv.52 treatment of CCl_4-poisoned rats resulted in almost normal levels of alkaline phosphatase in liver and serum at the end of the study. At the same time, NADPH-dependent lipid peroxidation also remained within the normal limits in Liv.52 treated animals. These protective effects in long-term CCl_4-poisoned rats suggests that Liv.52 can be attributed to its preventive action in the breakdown of the different membranous structures to varying extents, thus preventing the leakage of the enzyme into the blood stream. These observations are also consistent with the results of our histological studies, which show that Liv.52 is remarkably efficient in preserving the structural integrity of the liver.

The transaminases are amongst the important, specific liver enzymes that interconnect the metabolism of proteins and carbohydrates[27]. Similar results were observed regarding the activities of both the transaminases in serum and liver, which were seen to increase significantly ($p<0.001$) as a result of CCl_4 toxicity during 6 weeks. The increase in serum ALT level was more rapid than that of AST. Both of these transaminases are liver-specific enzymes[28] and are considered to be very sensitive and reliable indices in serum for measuring hepatoxic as well as the hepatoprotective or curative effects of various compounds[29,30]. Liv.52 treatment of CCl_4-poisoned animals furnished very dramatic and interesting results, since it restored the activity of hepatic AST and ALT almost

to within the normal limits. The activities of serum AST and ALT were significantly inhibited as compared to the levels in the CCl_4-treated group, but were not quite within the normal limits.

There are not many convincing reports on the efficacy of Liv.52 in maintaining normal serum and hepatic levels of transaminases in similar conditions, and the only reports available[7,31] are conflicting in nature. Further, another interesting aspect of the study was that the activity of AST in serum was appreciably less affected by treatment with Liv.52 than ALT. A similar trend was apparent in hepatic concentrations of these enzymes, implying a greater efficacy of Liv.52 in controlling the activity of ALT, since AST is known to be present in high concentrations in the heart, skeletal muscle and kidney apart from liver[28]. Thus, in our present study, normal hepatic but enormously increased serum AST levels could also be due to contributions of this enzyme from other organs after long-term CCl_4 toxicity, as CCl_4 is known to have damaging effects on various organs[28]. Though the mechanism of protection of Liv.52 is still obscure, the present results suggest that it probably helps in regulating protein metabolism, which in turn controls AST and ALT activities.



Figs. 5, 6: Liver histology of Liv.52 + carbon tetrachloride-treated rat, showing a few vacuolated cells in the central region, but otherwise most of the hepatocytes are normal in shape. H&E, x600 and x800 respectively.

Carbon-tetrachloride reaches maximum concentration in liver parenchyma within 2 hours of its administration. Thus, compositional and morphological changes in the cytoplasmic membrane systems of the hepatic cells may be expected to occur when the carbon tetrachloride concentration in liver is maximal[10]. The results obtained during the current investigations revealed that CCl_4 treatment for 6 weeks caused considerable necrosis of the parenchymal cells of liver, leading to acidophilic cytoplasm, which is finely granular or vacuolized and contains foci of hyaline necrosis. Few other cells had pyknotic or lysed nuclei. In the central zone, the effect of CCl_4 was much more severe, and the cells in this region were almost completely degenerated. The nuclei were in the stage of karyorrhexis or pyknosis and were even absent. This zone also showed an increased number of binucleated cells and more pronounced infiltration of Kupffer cells. The intermediary zone of the lobule was occupied by balloon cells, which had a characteristic round outline with a clear cytoplasm and a dark retracted nucleus. The peripheral zone was seen to be less affected and was intact to a large extent. All these observations by which we demonstrated the deleterious effects of CCl_4 are in line with various earlier reports[6,32]. The only positive difference between our

observations and the earlier findings relate to the severity of damage, and this could easily be attributed to the longer duration of the CCl_4 treatment that we used.

However, the animals given Liv.52 at the same time as CCl_4 showed promising results and responded by largely avoiding the usual deleterious effects of CCl_4. A few degenerated cells and some with pyknotic nuclei were observed, but there was definitely an overall improvement in the structure of the liver.

As regards serum concentrations of thyroid hormones, we observed that animals that were injected with carbon tetrachloride for 6 weeks (group 2) showed significantly depressed ($p<0.05$) T_3 concentrations but no significant decrease in T_4 levels. Our results are in line with the observations made by some earlier workers[14] on toxic conditions of liver induced by other agents. In fact, total and free thyroxin (T_4 and FT_4) serum concentrations have been reported as normal, increased or decreased in various liver diseases[17]. Similarly, Israel *et al.*[33] have observed a normal T_4 activity but depressed T_3 activity in serum of subjects suffering from alcohol toxicity. They considered that normal T_4 levels show that the thyroid gland function is normal in these patients, although a weak negative correlation between serum T_4 levels and the severity of liver disease was observed, which could be due to a reduction in thyroid hormone binding capacity of serum proteins. In some other studies, a similar decrease in T_3 levels was seen, and it was suggested that formation of T_3 is catalyzed by a particular enzyme, iodothyronine 5-deiodinase, located predominantly in microsomes of liver[15,34], and this decrease in T_3 could be due to the deficiency of this enzyme as such. At the same time, the existence of so-called low T_3 syndrome, i.e. low T_3 with normal total T_4 in the absence of clinical hypothyroidism, has been frequently reported in patients with chronic liver disease, as well as in many non-thyroid illnesses[16]. It has been shown to be dependent upon impaired liver conversion of T_4 toT_3[35].

However, the CCl_4-treated animals that were given Liv.52 (group 3) had normal levels of T_3 while a significantly lowered ($p<0.05$) T_4 activity was noticed. These observations suggest that Liv.52 treatment in hepatotoxic conditions has a positive effect on liver, aiding in the smooth conversion of T_4 to T_3, either by some direct mechanism or by indirectly increasing the possible deficiency of iodothyronine 5-deiodinase, and ultimately improves thyroid hormone status. Moreover, the observed decrease in activity of T_4 in Liv.52 treated animals has generated an interest in finding a plausible reason for it. This could possibly be due to its beneficial effect on liver, in which some kind of sensitization of T_4 degradation to either T_3 and some other metabolites might take place, leading to reduced concentration of T_4 and some other metabolites might take place, leading to reduced concentration of T_4. As TSH was not measured in the present investigation, it is not possible to conjecture its role, if any, in the hormonal changes observed.

Thus, in view of all these observations, the present study appears to be the only report so far emphasizing the efficacy of Liv.52 in maintaining the liver marker enzymes, lipid peroxidation and histology within the normal limits in carbon tetrachloride-induced hepatotoxic conditions. Moreover, the most interesting feature of the present study was Liv.52 helped in restoring the T_3 levels to within normal limits in carbon tetrachloride-induced hepatotoxic conditions, possibly by increasing the formulation of T_3 from T_4. In conclusion, these data, in conjunction with earlier

reports[15,17,33], suggest that Liv.52 treatment in toxic liver conditions not only improves the functioning of the liver, but at the same time may play a role in the regulation of thyroid hormones.

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