

# LYSOSOMAL ENZYME ACTIVITY DURING DEVELOPMENT OF CARBON TETRACHLORIDE INDUCED CIRRHOSIS IN RATS

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**Abstract :** The present study was undertaken to determine whether there is any alteration in the activities of lysosomal enzymes in the liver and sera of rats during the course of carbon tetrachloride (CCl<sub>4</sub>) induced cirrhosis in rats. Cirrhosis was induced by the chronic administration of carbon tetrachloride plus phenobarbitone. N-acetyl glucosaminidase,  $\beta$ -glucuronidase and acid phosphatase were assayed spectrophotometrically in the liver homogenates and in the sera at different stages of liver injury i.e., necrosis, fibrosis, and cirrhosis. Significant increase in the “basal” activities of N acetyl glucosaminidase,  $\beta$ -glucuronidase, and acid phosphatase were observed in the livers of rats during the course of development of cirrhosis. As the liver injury progressed from necrosis to cirrhosis, the ‘free’ activities of these three enzymes also increased. The ‘total’ activities of the enzymes studied were either decreased or remained unaltered. The increased ‘free’ activities of the lysosomal enzymes in the liver of CCl<sub>4</sub> treated rats may contribute to cellular autophagy and tissue catabolism, which may subsequently lead to cirrhosis.

**Key words :** carbon tetrachloride  
 $\beta$ -glucuronidase

N-acetyl glucosaminidase  
acid phosphatase

## INTRODUCTION

Ingestion of carbon tetrachloride (CCl<sub>4</sub>) produces liver cell necrosis in humans (1) as well as in experimental animals (2). Long term administration of the toxin by the experimental animals produces liver cirrhosis which is similar to human alcoholic cirrhosis both histologically (3) and systematically (4).

Cirrhosis is characterized by increased

collagen accumulation in the liver, secondary to cell death (5). Several mechanisms have been proposed for liver cell necrosis and collagen accumulation. One of the extensively investigated mechanisms is lipid peroxidation (6, 7). Persistent necrosis of the liver precedes the development of cirrhosis in both humans as well as animals (8, 9), and lysosomes play an important role in cell death and tissue damage (10). The lysosomes release

hydrolytic enzymes that cause auto digestion of the cellular contents. Also, lysosomal enzymes play an important role in the metabolism of glycosaminoglycans (11), which are closely associated with collagen metabolism (12, 13). Therefore, it is hypothesised that lysosomal enzymes may be altered in the liver during the course of development of cirrhosis. A few studies have been reported earlier on alteration of lysosomal enzymes in the liver in cirrhosis and controversial results have been obtained (14, 15). Alterations in the activities of the lysosomal enzymes in the liver and sera of rats in different stages of liver injury leading to cirrhosis i.e., necrosis, fibrosis have not been reported yet, to the best of my knowledge.

Therefore, in the present study the "free" (basal) and "total" (maximal) activities of three lysosomal enzymes namely  $\beta$ -glucuronidase, N-acetyl glucosaminidase and acid phosphatase were measured in the liver  $\text{CCl}_4$  treated rats in different stages of liver injury i.e., necrosis, fibrosis and cirrhosis.  $\beta$ -Glucuronidase and N-acetyl glucosaminidase were chosen because these two enzymes play an important role in the metabolism of glycosaminoglycans, which are closely associated with collagen molecules. Acid phosphatase does not play a role in glycosaminoglycan metabolism and therefore it was assayed for comparison. The activity in the presence of Triton X100 denotes "maximal" or "total" activity and the activity in the absence of Triton X100 denotes "basal" or "free" activity. The activities of these enzymes were assayed in the sera also.

## METHODS

### Animals

Adult male Wistar rats weighing 150–200 g at the start of the study were used in the experiments. The rats were allowed free access to commercial rat feed pellets (supplied by Lipton India Limited) and tap water. They were housed in galvanised iron cages in a thermostatically controlled room ( $28 \pm 2^\circ\text{C}$ ) and maintained in 12 hr natural dark/light cycle.

### Chemicals

p-nitrophenyl  $\beta$ -glucuronide, p-nitrophenyl-N-acetyl glucosaminide, p-nitrophenol, and glycine were purchased from Sigma (St. Louis, Mo. USA). Carbon tetrachloride was obtained from S.D. Fine Chem. Ltd., India. All other chemicals were of the analytical grade.

### Animal treatment

#### *Induction of cirrhosis:*

Cirrhosis was induced in the rats by chronic exposure to carbon tetrachloride ( $\text{CCl}_4$ ) vapours twice a week for 12 consecutive weeks as described by Mc Lean et al (16) with modifications described previously (17). Tap water containing phenobarbitone at the concentration of 500 mg/L was the only source of drinking water for the rats 10 days prior to the first dose of carbon tetrachloride vapours and throughout the treatment period.

Phenobarbitone was co-administered with carbon tetrachloride because it has been shown earlier that phenobarbitone hastens the development of cirrhosis (16).

*Control rats:* Eighteen rats were kept under the same condition as the experimental rats, but were administered phenobarbitone alone.

Twenty rats were exposed to carbon tetrachloride vapours twice a week. After exposure to  $\text{CCl}_4$  for 2 weeks, six rats were sacrificed 3 days after the last dose of  $\text{CCl}_4$ . Similarly, six rats each were sacrificed after 6 weeks and 12 weeks of treatment with  $\text{CCl}_4$  (3 days after the last dose of  $\text{CCl}_4$ ). Two rats died after 4 weeks of treatment with  $\text{CCl}_4$ . Similarly, in the control group, six rats each were sacrificed at the same time as the  $\text{CCl}_4$  treated rats.

### **Histology**

Slices of liver tissue were fixed in 10% buffered formalin, processed and stained with Haematoxylin-Eosin, Foot's reticulin and Van Geison stains for histological assessment.

### **Biochemical analysis**

The rats in the experimental group as well as those in the control group were fasted overnight and blood was drawn by heart puncture under light ether anaesthesia. The livers were excised, weighed and used for biochemical analysis and histological assessment. Sera were

separated from the clotted blood and used for the assay of N-acetyl glucosaminidase,  $\beta$ -glucuronidase and acid phosphatase.

### *Preparation of the homogenate :*

Liver homogenates were prepared as described by Kyaw et al (18). Briefly, portions of the liver (approximately 250 mg) was homogenised in 10 ml of ice-cold 0.25 M sucrose-1 mM  $\text{Na}_2\text{EDTA}$  in a Potter-Elvehjem homogeniser. The homogenate was divided into two equal parts, marked 'maximal' and 'basal'. To the 'maximal', Triton X100 was added to give a final concentration of 0.1% and to the 'basal' equivalent amount of distilled water was added. They were incubated at  $37^\circ\text{C}$  for one hour with gentle shaking now and then. The homogenates were centrifuged at  $4^\circ\text{C}$  at  $11,000 \times g$  for 30 minutes to remove unlysed particles. The supernatant was used for the assay of N-acetyl glucosaminidase (NAG),  $\beta$ -glucuronidase ( $\beta$  glc), acid phosphatase and protein.

### *Assay of N acetyl glucosaminidase, $\beta$ -Glucuronidase and acid phosphatase*

NAG and  $\beta$  glc were assayed in the liver homogenates and serum spectrophotometrically using p-nitrophenyl glycosides as substrates (19). Acid phosphatase was assayed in the homogenate and serum using p nitrophenylphosphate as substrate (20). Protein content in the liver homogenate supernatant was determined according to the method of Lowry et al (21) using bovine serum albumin as standard. One milliunit of enzyme activity is defined as the nmoles of substrate hydrolysed per minute at  $37^\circ\text{C}$ .

Enzyme activities are expressed as mU/mg protein in the liver supernatants and as U/L in sera.

### Statistical analysis

All values are expressed as mean  $\pm$  S.D. The results were compared using Student's t test. Differences were considered significant if the P value  $<0.05$ .

## RESULTS

**Histology:** The livers of phenobarbitone treated rats showed normal histology. After 2 weeks of treatment with carbon

tetrachloride, the rat livers showed perivenular necrosis. After 6 weeks of treatment with  $CCl_4$ , the rat livers showed fibrosis and after 12 weeks the rat livers showed cirrhosis.

**Biochemical results:** The biochemical results are shown in Table I. In general, the "free" activities of N-acetyl glucosaminidase, acid phosphatase as well as  $\beta$  glucuronidase progressively increased as liver injury progressed from necrosis to cirrhosis via fibrosis.

With regard to  $\beta$  glucuronidase, a 69% increase in free activity was observed in the

TABLE I: Hepatic and seral activities of Lysosomal enzymes during the development of carbon tetrachloride induced liver cirrhosis.

| Enzyme activity                         | Time after treatment with $CCl_4$ |                     |                   |                    |                   |                     |
|-----------------------------------------|-----------------------------------|---------------------|-------------------|--------------------|-------------------|---------------------|
|                                         | 2 weeks                           |                     | 6 weeks           |                    | 12 weeks          |                     |
|                                         | Control                           | $CCl_4$             | Control           | $CCl_4$            | Control           | $CCl_4$             |
| <b><math>\beta</math>-Glucuronidase</b> |                                   |                     |                   |                    |                   |                     |
| Liver free activity (mU/mg protein)     | 2.79 $\pm$ 0.70                   | 4.72 $\pm$ 1.04***  | 2.57 $\pm$ 0.38   | 4.99 $\pm$ 0.83*** | 2.44 $\pm$ 0.74   | 5.73 $\pm$ 0.75***  |
| Liver total activity (mU/mg protein)    | 9.94 $\pm$ 1.13                   | 5.80 $\pm$ 0.58***  | 11.70 $\pm$ 3.61  | 7.13 $\pm$ 0.69*   | 10.35 $\pm$ 1.59  | 11.40 $\pm$ 2.75    |
| Serum Enzyme Activity (U/L)             | 0.82 $\pm$ 0.09                   | 1.45 $\pm$ 0.08***  | 0.67 $\pm$ 0.10   | 1.28 $\pm$ 0.28*** | 0.89 $\pm$ 0.29   | 3.41 $\pm$ 0.44***  |
| <b>N-acetyl glucosaminidase</b>         |                                   |                     |                   |                    |                   |                     |
| Liver free activity (mU/mg protein)     | 3.89 $\pm$ 0.42                   | 3.52 $\pm$ 0.25     | 4.15 $\pm$ 0.79   | 6.77 $\pm$ 1.30**  | 4.06 $\pm$ 0.61   | 8.96 $\pm$ 3.01***  |
| Liver total activity (mU/mg protein)    | 4.83 $\pm$ 0.46                   | 4.23 $\pm$ 0.28     | 6.78 $\pm$ 1.06   | 7.71 $\pm$ 2.6     | 9.49 $\pm$ 2.43   | 11.28 $\pm$ 4.77    |
| Serum enzyme Activity (U/L)             | 9.39 $\pm$ 1.47                   | 12.35 $\pm$ 1.56**  | 10.96 $\pm$ 2.77  | 16.04 $\pm$ 1.00** | 10.32 $\pm$ 1.72  | 20.53 $\pm$ 4.34*** |
| <b>Acid phosphatase</b>                 |                                   |                     |                   |                    |                   |                     |
| Liver free activity (mU/mg protein)     | 30.85 $\pm$ 3.93                  | 28.21 $\pm$ 3.47    | 28.60 $\pm$ 2.90  | 35.75 $\pm$ 6.53*  | 29.89 $\pm$ 3.97  | 62.52 $\pm$ 30.0**  |
| Liver total activity (mU/mg protein)    | 73.45 $\pm$ 10.24                 | 31.27 $\pm$ 4.61*** | 61.88 $\pm$ 13.08 | 55.48 $\pm$ 7.22   | 69.91 $\pm$ 15.41 | 84.38 $\pm$ 39.30   |
| Serum enzyme Activity (U/L)             | 35.47 $\pm$ 3.47                  | 39.03 $\pm$ 2.45    | 39.37 $\pm$ 4.82  | 28.78 $\pm$ 7.12*  | 43.42 $\pm$ 3.87  | 40.00 $\pm$ 7.19    |

Data represent mean value  $\pm$  SD, number of rats = 6 in each group.  
\*\*\*P $<0.001$ , \*\*P $<0.01$ , \*P $<0.05$  vs respective control.

CCl<sub>4</sub> treated rats at 2 weeks, when the rat livers showed perivenular necrosis. Six weeks after treatment with CCl<sub>4</sub>, when the rats developed fibrosis, the free activity increased by 94% as compared with the control. At 12 weeks (when the rat livers developed cirrhosis), the free activity increased to 135% as compared with the control. With regard to the total activity of the enzyme, a 42% decrease in the activity was observed at the necrosis stage as compared with the control. In the fibrotic stage, a 39% decrease in total activity was observed. No significant change in the total activity was observed in cirrhosis. In the serum, a 77% increase in activity was observed 2 weeks after treatment with CCl<sub>4</sub>, the activity increased to 91% at 6 weeks and further to 283% at 12 weeks as compared with the control.

With respect to N acetyl glucosaminidase significant increase (63%) in the free activity of NAG was observed in the fibrotic stage. In cirrhosis, a 121% increase in the free activity was observed as compared with the control. No significant difference in the total activity was observed between the experimental rats and control rats throughout the treatment period. It may be noted that the activity of NAG in the control rats progressively increased throughout the experimental period. In plasma, a 30% increase in NAG was observed in necrosis stage and fibrosis stage. The activity increased further (99%) in cirrhosis.

No significant difference in the free activity of acid phosphatase was observed between the experimental rats and control rats in the perivenular necrosis stage. In the fibrotic stage, a 25% increase in free

activity was observed as compared with the control. The free activity increased further (109%) in the cirrhotic stage as compared with the control. With regard to the total activity, a 57% decrease was observed in the CCl<sub>4</sub> treated rats as compared to the controls at the perivenular necrosis stage. No significant difference in total activity was observed after this stage of liver injury. In the serum, no significant change in the activity was observed except for a slight decrease in the activity at 6 weeks after treatment with CCl<sub>4</sub>.

A decrease in total/free activity of all 3 lysosomal enzymes studied was observed throughout the treatment period with CCl<sub>4</sub>.

## DISCUSSION

It may be noted that the 'free' activities all the lysosomal enzymes studied was increased in the liver irrespective of whether they are involved in glycosaminoglycan metabolism or not. This suggests that the increase in the activities of the lysosomal enzymes may be due to generalised damage to the lysosomes and may not be related to glycosaminoglycan metabolism.

In normal liver, lysosomal enzymes generally are latent i.e., they are contained in the lysosomes. Therefore, the free activity of the lysosomal enzymes in the liver is very low. In the present study, a significant increase in the free activity of all the lysosomal enzymes studied was observed in the liver throughout the treatment period with CCl<sub>4</sub>, irrespective of the pathological state of the liver namely necrosis, fibrosis, or cirrhosis. In fact, as the liver injury

progressed from necrosis to cirrhosis via fibrosis, the free activities of the lysosomal enzymes also increased progressively. The increase in the 'free' activity of the lysosomal enzymes suggests the release of the lysosomal enzymes into the cytosol. Increase in the free activity of other lysosomal enzymes such as acid ribonuclease and cathepsin have been reported earlier by other workers in CCl<sub>4</sub>-induced liver injury.

The total/free ratio of the lysosomal enzymes was decreased throughout the treatment period with CCl<sub>4</sub>. The ratio of total/free activity of lysosomal enzymes has been interpreted as an expression indicating the stability of lysosomal membrane (22). A decreased value suggests decreased stability and/or an increased vulnerability of lysosomal membrane (fragility) resulting in leakage of the lysosomal enzymes and consequently leading to cell injury (23). The increased lysosomal membrane fragility could be due to (1) low energy levels in the parenchymal cells of damaged liver because of reduced ATP generation by the damaged mitochondria (2) enhanced lipid peroxidation

of the lysosomal membrane by CCl<sub>4</sub> or both (3). It has been reported earlier that in CCl<sub>4</sub> induced liver injury, mitochondrial function is impaired with the most frequent change being impaired function of the respiratory chain and ATP metabolism (24). A study by Younes et al (25) supports the view of lipid peroxidation. With respect to the enzyme N-acetyl glucosaminidase, the 'total' activity progressively increased in the control rats also. There is no clear explanation for this.

The increase in serum level of lysosomal enzymes may be as a result of increased fragility of liver lysosomal membrane allowing more enzymes to be leaked into the serum. It is also possible that increase in production and release of lysosomal enzymes from macrophages could also contribute to the increased serum lysosomal enzyme levels, as suggested by Hultberg et al (14).

In conclusion, it may be stated that the increased free activities of the lysosomal enzymes in the liver may contribute to cell death and tissue damage which are probable consequences leading to cirrhosis.

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