

Antioxidant and Hepatoprotective Activity of Ascorbic Acid Against Diclofenac Induced Hepatotoxicity in Rats

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In this study, the effect of diclofenac sodium induced liver damage in rats was investigated, morphologically and biochemically. Damaging effects of reactive oxygen species (ROS) on living systems are well documented. They include oxidative attack on vital cell constituents. Administration of diclofenac sodium (150 mg/kg/d) for 28 d produced severe liver injury, as demonstrated by dramatic elevation of serum hepatospecific markers like serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (ALP), serum acid phosphatase (SAP), blood urea nitrogen (BUN), serum total cholesterol and bilirubin levels and typical histopathological changes, hepatocyte necrosis or apoptosis, haemorrhage, fatty degeneration, *etc.* In addition, diclofenac sodium administration caused oxidative stress in rats, as evidenced by increased ROS production and malondialdehyde (MDA) concentrations in the liver of rats, along with a remarkable reduction in hepatic superoxide dismutase (SOD), catalase (CAT) activity and reduced glutathione (GSH) content. However, simultaneous treatment with ascorbic acid as a food supplement (200 mg/kg/d) significantly attenuated diclofenac sodium induced hepatotoxicity. The results showed that serum SGOT, SGPT, ALP, SAP, BUN, cholesterol, bilirubin levels and hepatic MDA content as well as ROS production were reduced dramatically and hepatic SOD, CAT activity and GSH content were restored remarkably by ascorbic acid supplementation, as compared to the diclofenac sodium treated rats. Moreover, the histopathological damage of liver and the number of apoptotic hepatocytes were also significantly ameliorated by ascorbic acid treatment. It is, therefore suggested that ascorbic acid can provide a definite protective effect against acute hepatic injury caused by diclofenac sodium in rats, which may mainly be associated with its antioxidative effect.

Key Words: Diclofenac, Ascorbic acid, Hepatotoxicity, Antioxidant.

INTRODUCTION

Non-steroidal antiinflammatory drugs (NSAIDs) are widely used for the treatment of pain and inflammation. NSAIDs produce their therapeutic effect by inhibiting the cyclooxygenase (COX) enzymes, which are involved

in the biosynthesis of prostaglandins (PGs)^{1,2}. Conventional NSAIDs inhibit both COX-1 and COX-2 at therapeutic doses³.

Diclofenac sodium has a antipyretic, analgesic and antiinflammatory effects, is an inhibitor of cyclooxygenase enzyme and is decreased in leukocyte intracellular free arachidonate level⁴. Diclofenac sodium is a common non-steroidal antiinflammatory agent (NSAIDs)⁵ that is effective in the treatment of painful inflammatory diseases and has been used to treat more than 75 million people worldwide⁶. Diclofenac belongs to a chemical subgroup of NSAIDs that are an arylakanoic group of phenylacetic acid⁷ and advocated for use in painful and inflammatory rheumatic and non-rheumatic conditions. The exact mechanism is not known but it is probably related to the decrease in the fatty acid entering the cell or released from the cell⁴. Diclofenac is an oral non-steroidal antiinflammatory drugs. A group of metabolic products called free radicals can damage liver cells and promote inflammation, impairing vital functions such as energy production. The body's natural defenses against free radicals (*e.g.* antioxidants) are inhibited by diclofenac sodium consumption, leading to increased liver damage.

Ascorbic acid (vitamin C) may protect lipids and lipoproteins in cellular membranes against oxidative damage caused by toxic free radicals at early stage. The antioxidant function of ascorbic acid is related to its reversible oxidation and reduction characteristics. Thus, ascorbic acid may partially prevent certain types of hepatocellular damage^{8,9}.

The aim of this study was to investigate the possible hepatoprotective effects of ascorbic acid on the serum hepatospecific markers and free radical damage of liver caused by diclofenac in rats.

EXPERIMENTAL

Diclofenac sodium was obtained from Dey's Medical Stores Mfg. Ltd., Kolkata, India, ascorbic acid obtained from sigma UK. Serum levels of glutamate pyruvate transaminase (SGPT), glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), acid phosphatase (SAP), cholesterol and bilirubin (total and direct), were determined by spectrophotometric methods. These kits were obtained from Ranbaxy diagnostic laboratory Ltd., HPSIDC, Baddi, India. TBA, TCA and HCl were obtained from Merck, Germany. Chemicals for SOD, GSH and catalase were obtained from Sigma, UK. All other reagents used for the experiments were of analytical grade.

Studies were carried out using male Wistar albino rats weighing 115 to 120 ± 5 g. They were obtained from the animal house, Indian Institute of Chemical Biology (IICB), Kolkata, India. The animals were grouped and housed in cages with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C) with dark and light cycle (12/12 h). They were allowed free access to standard dry

pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The rats were acclimatized to laboratory condition for 10 d before commencement of experiment. All procedures described were reviewed and approved by the Institutional Animal Ethics Committee (IAEC).

Experimental design: The rats were divided into the following groups each containing 6 rats ($n = 6$). **Group I:** Control rats which were fed normal diet and water. **Group II:** Diclofenac sodium treated rats: Diclofenac sodium 150mg/kg body weight/day for 4 weeks. **Group III:** Diclofenac sodium treated rats: 150 mg Diclofenac sodium/kg body weight/day + 200 mg ascorbic acid/kg body weight/day as a food supplement for 4 weeks.

Treatment

Group I: Rats were given normal saline 2 mL/100g body weight per oral dose for 28 d. The rats were fasted overnight, anesthetized with an intramuscular injection of ketamine hydrochloride (30 mg kg⁻¹) and were sacrificed by cervical dislocation. The hearts were exposed and 1-2 mL of blood samples were taken through a hypodermic syringe and transferred into universal bottles. Portions of the liver were also excised. A portion was fixed in 10 % formalin and another portion was blended as tissue homogenate. Biochemical and histopathological analyses were carried out on the blood and liver samples.

Group II: Hepatotoxicity was induced by the administration of diclofenac sodium 150 mg/kg/d for 28 d. On the 29th day, they were sacrificed and treated as described earlier.

Group III: In addition to the treatment described above, rats were given 200 mg/kg ascorbic acid orally for four week as food supplements. On the 29 th days, they were sacrificed and treated as described above.

Biochemical analysis: Serum levels of glutamate pyruvate transaminase (SGPT) and glutamate oxaloacetate transaminase (SGOT)¹⁰, bilirubin¹¹, (total and direct); serum alkaline phosphatase (ALP), serum acid phosphatase (SAP)¹², blood urea nitrogen (BUN)¹¹ and serum total cholesterol¹³ were determined by using standard analytical kit.

Estimation of superoxide dismutase (SOD): SOD activity was determined by the modified method of NADH-phenazinemetosulphatenitrobluetetrazolium formazon inhibition reaction spectrophotometrically¹⁴ at 560 nm. A single unit of enzyme was expressed as 50 % inhibition of nitroblue tetrazolium (NBT) reduction/min/mg protein.

Estimation of catalase (CAT): Catalase (CAT) was assayed calorimetrically¹⁵ using dichromate-acetic acid reagent (5 % potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). The intensity was measured at 620 nm and the amount of hydrogen peroxide hydrolyzed was calculated for the catalase activity.

Estimation of reduced glutathione (GSH): Reduced glutathione (GSH) was determined according to reported method¹⁶. 1 mL of supernatant (0.5 mL plasma/0.5 mL liver homogenate precipitated by 2 mL of 5 % TCA) was taken and 0.5 mL of Ellman's reagent (0.0198 % DTNB in 1 % sodium citrate) and 3 mL of phosphate buffer (pH 8.0) were added. The colour developed was read at 412 nm.

Estimation of lipid peroxidation: Lipid peroxidation in plasma and liver was estimated colorimetrically by measuring malondialdehyde (MDA)¹⁷. In brief, 0.1 mL of plasma was treated with 2 mL of (1:1:1 ratio) TBA-TCA-HCl reagent (TBA 0.37 %: 0.25 N HCl: 15 % TCA) and placed in water bath for 15 min, cooled and centrifuged and then clear supernatant was measured at 535 nm against reference blank.

Estimation of total protein: Protein estimation was performed as per method of Lawry *et al.*¹⁸.

Histopathological analysis: Portions of the liver were then fixed in buffered form formalin, processed through graded alcohol and xylene and embedded in paraffin wax following the standard micro-technique¹⁹. Sections of 5-6 μ were made at multiple levels and stained routinely with hematoxylin and eosin (H&E). Mounted slides were examined and photographed under a light microscope.

Statistical analysis: The results are expressed as means \pm standard deviation (SD) and values were calculated for each group and a one-way analysis of variance (ANOVA) was done for each quantitative parameter to determine the significance of inter-group differences²⁰.

RESULTS AND DISCUSSION

Biochemical analysis: The activities of serum hepatospecific markers like serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum alkaline phosphatase (ALP), serum acid phosphatase (SAP), blood urea nitrogen (BUN) and bilirubin were significantly increased ($p < 0.05$, $p < 0.001$, $p < 0.001$, $p < 0.05$ and $p < 0.05$, respectively) by diclofenac sodium, but significantly decreased ($p < 0.05$ in all parameters) with the administration of ascorbic acid (Table-1).

TABLE-1
EFFECT OF ASCORBIC ACID ON HEPATIC MARKER ENZYMES SGOT, SGPT, ALP, BILIRUBIN AND BUN ON CONTROL AND DICLOFENAC SODIUM TREATED RATS

Groups	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Bilirubin (μ mol/l)		BUN (mg/dl)
				Direct	Total	
Control	36.97 \pm 1.31	53.92 \pm 3.16	73.43 \pm 2.42	0.09 \pm 0.02	0.84 \pm 0.02	18.40 \pm 0.98
DS	91.10 \pm 1.33 [#]	108.87 \pm 2.46 [*]	152.33 \pm 3.42 [*]	0.74 \pm 0.03 [#]	6.26 \pm 0.28 [#]	30.95 \pm 1.81 [#]
DS-AA	51.01 \pm 1.76 [#]	70.19 \pm 1.51	95.88 \pm 3.97 [*]	0.27 \pm 0.09 [#]	1.00 \pm 0.09 [#]	22.58 \pm 1.29 [#]

DS = Diclofenac sodium; DS-AA = Diclofenac sodium & ascorbic acid.

Data are expressed as means \pm SE; Statistical significance with respect to the control group:

[#] $p < 0.05$ * $p < 0.001$.

The activities of serum hepatospecific markers like cholesterol, total protein and acid phosphatase were significantly altered by diclofenac sodium, but significantly corrected with the uses of ascorbic acid (Table-2).

TABLE-2
EFFECT OF ASCORBIC ACID ON HEPATIC MARKER ENZYMES
CHOLESTEROL, TOTAL PROTEIN AND ACID PHOSPHATASE ON
CONTROL AND DICLOFENAC SODIUM TREATED RATS

Groups	Cholesterol (mg/dl)	Protein (mg/dl)	Acid phosphatase (SAP) (IU/L)
Control	61.57±1.30	7.12±0.52	52.12±4.07
Diclofenac sodium	39.60±1.20 [#]	4.22±0.77 [#]	86.16±1.82 [#]
Diclofenac sodium & ascorbic acid	59.90±1.22 [#]	6.29±0.42 [#]	57.27±2.59 [*]

Data are expressed as mean ± SD, n = 6 per group; Statistical significance with respect to the control group: [#]p < 0.05, ^{*}p < 0.001.

There was a significant effect of diclofenac sodium on serum lipid peroxidase levels of group II rats ($p < 0.01$) when compared with control group. This effect was still significant in the ascorbic acid treated rats (Fig. 1). A significant decrease ($p < 0.05$) in the activities of enzymic antioxidants such as superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) of the liver was noted in diclofenac sodium induced rats when compared with the normal rats. Upon administration of ascorbic acid the activities of SOD, GSH and CAT were significantly ($p < 0.05$) reversed to near normal (Figs. 2-4). Results showed that administration of ascorbic acid significantly enhanced the hepatic level of glutathione dependent enzymes and superoxide dismutase and catalase activity suggesting that the hepatopro-

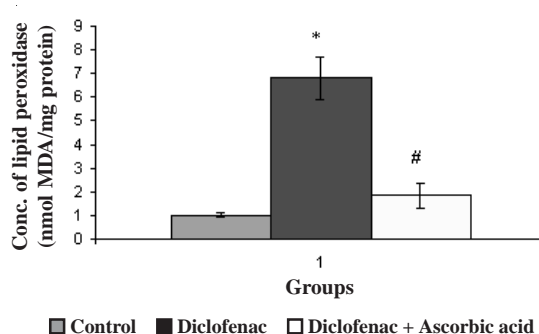


Fig. 1. Effect of ascorbic acid on activity of hepatic antioxidant enzymes lipid peroxidase in diclofenac sodium treated rat liver. Each bar represents the mean ± SD, n = 6. ^{*}p < 0.001 diclofenac sodium treatment alone (150 mg/kg/d) compared with control. [#]p < 0.001 activity of antioxidant enzyme lipid peroxide after simultaneously ascorbic acid treatment compared with diclofenac sodium

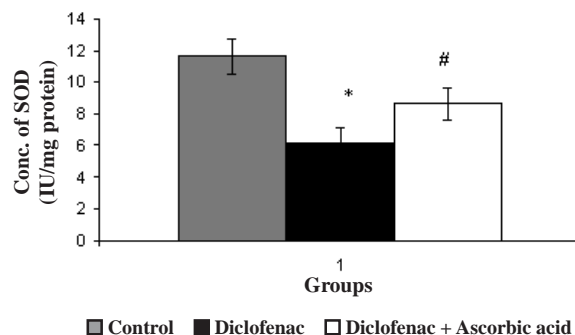


Fig. 2. Effect of ascorbic acid on activity of hepatic antioxidant enzymes superoxide dismutase (SOD) in Diclofenac sodium treated rat liver. Each bar represents the mean \pm SD, $n = 6$. * $p < 0.001$ Diclofenac sodium treatment alone (150 mg/kg/d) compared with control. # $p < 0.05$, activity of antioxidant enzyme (SOD) after simultaneously Ascorbic acid treatment compared with Diclofenac sodium

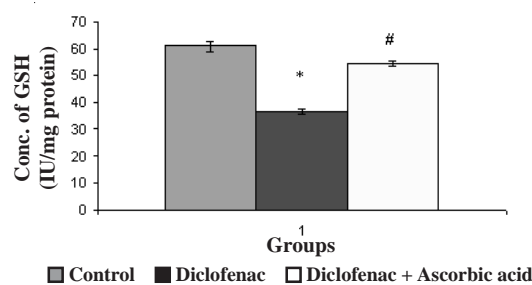


Fig. 3. Effect of ascorbic acid on activity of hepatic antioxidant enzymes reduced glutathione (GSH) in diclofenac sodium treated rat liver. Each bar represents the mean \pm SD, $n = 6$. * $p < 0.001$ diclofenac sodium treatment alone (150 mg/kg/d) compared with control. # $p < 0.05$, activity of antioxidant enzyme (GSH) after simultaneously ascorbic acid treatment compared with diclofenac sodium

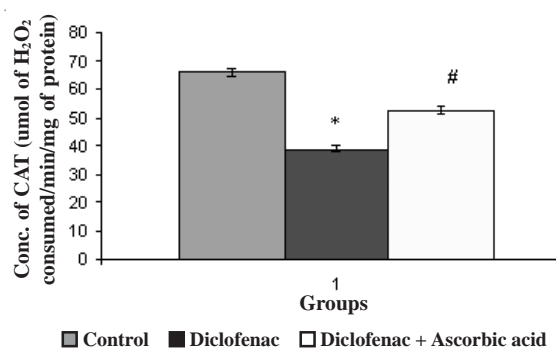


Fig. 4. Effect of ascorbic acid on activity of hepatic antioxidant enzymes catalase (CAT) in diclofenac sodium treated rat liver. Each bar represents the mean \pm SD, $n = 6$. * $p < 0.001$ Diclofenac sodium treatment alone (150 mg/kg/d) compared with control. # $p < 0.05$, activity of antioxidant enzyme (CAT) after simultaneously ascorbic acid treatment compared with diclofenac sodium

tective effect of the extract on diclofenac sodium induced hepatotoxicity may be due to its antioxidant activity. It was observed that the weight of animals fed with high dose diclofenac sodium reduced gradually and after simultaneous ascorbic acid supplementation there was no such significant alteration been noticed (Fig. 5). Results showed that appetite is close to normal in case of administration of ascorbic acid (Fig. 6).

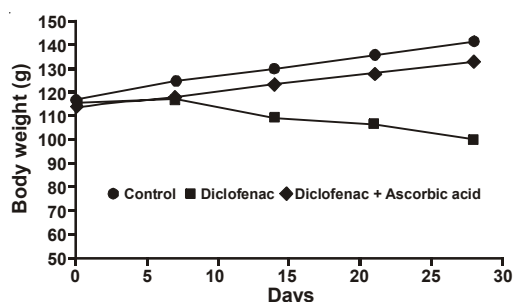


Fig. 5. Effect of ascorbic acid on body weigh in diclofenac sodium treated rat. Diclofenac sodium treatment alone (150 mg/kg/d) compared with control. Changes of body weight after simultaneously ascorbic acid treatment compared with diclofenac sodium

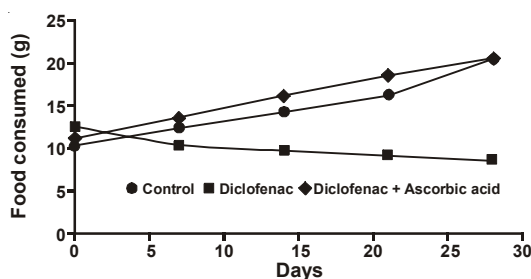


Fig. 6. Effect of ascorbic acid on daily food consumption in diclofenac sodium treated rat. Diclofenac sodium treatment alone (150 mg/kg/d) compared with control. Condition of appetite after simultaneously Ascorbic acid treatment compared with diclofenac sodium

Histopathological analysis

Group I (control): The normal lobular architectural pattern of the liver section is as shown in (Fig. 7). The lobulation is modest as a result of the low content of interstitial tissues and can be determined only with reference to the central vein. Sinusoids at the periphery or the lobule are fused into a reticulum. The hepatocytes are arranged in a series of branching and anatomizing perforated laminae to form a labyrinth, between which were sinusoidal spaces. The cytoplasm of the hepatocytes was clearly eosinophilic with prominent nuclei (Fig. 8).

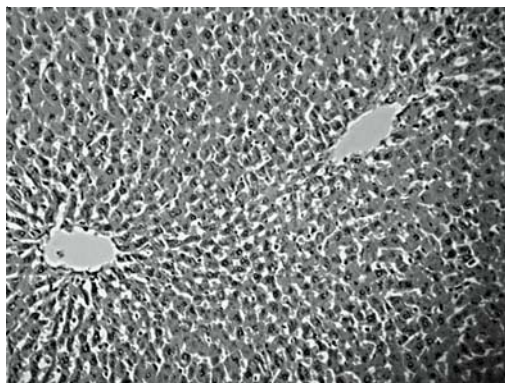


Fig. 7. Control rat: LM section of liver showing the normal lobular histological picture (H & E $\times 100$)

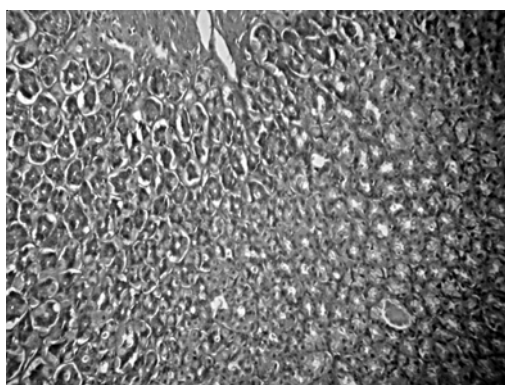


Fig. 8. Group-I. Control rat: LM section of liver showing a normal histological picture. Normal hepatocytes with brought out nuclei (n), cytoplasm (c) and a well distinct hepatic laminae (H & E $\times 400$)

Group II (diclofenac sodium only): The normal lobular architectural pattern of the liver section can not be discerned, though several central veins are seen (Fig. 9). It was observed that the hepatocytes were swollen and the sinusoidal spaces occluded. The cytoplasm of the hepatocytes appeared cloudy poorly stained (hydropic degeneration) and with necrotic signs (Fig. 10).

Group III (diclofenac sodium + ascorbic acid): The photomicrograph of the liver section of the vitamins treated group, (Figs. 11 and 12) showed a histological picture that closely approximates that of the control group (Fig. 7). The plate-like arrangements of the hepatocytes were seen, the sinusoidal spaces were also visible, but not as prominent as in the control group. The cytoplasm of the hepatocyte were clearly eosinophilic as in the control group.

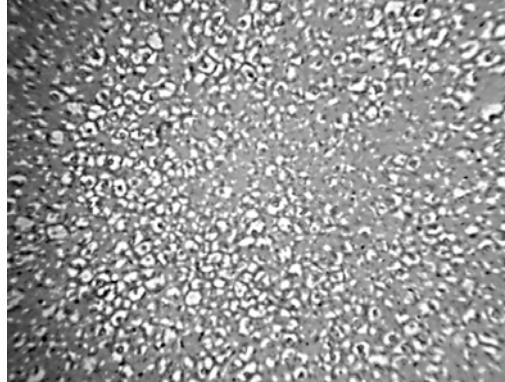


Fig. 9. Group-II. Diclofenac sodium treated rat: LM Section showing non-zonal macrovesicular lipid accumulation (H & E \times 100)

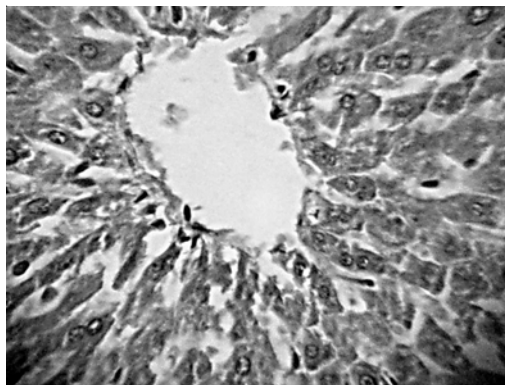


Fig. 10. Group-II: Diclofenac sodium treated features of fatty liver. Most of the hepatocytes are distended with large lipid vacuole with peripherally displaced nuclei. Large fat cysts are seen in places due to coalescence and rupture of fat containing hepatocytes (H & E \times 400)

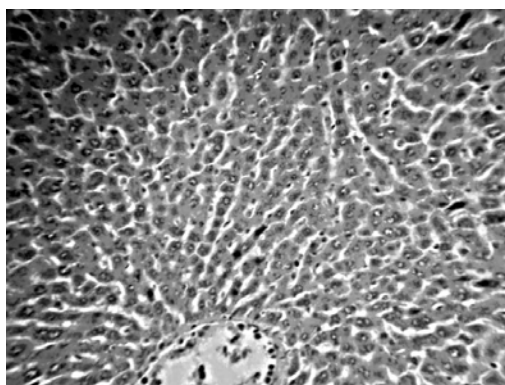


Fig. 11. Diclofenac + vitamin C treated rat: LM section of liver (H & E \times 100)

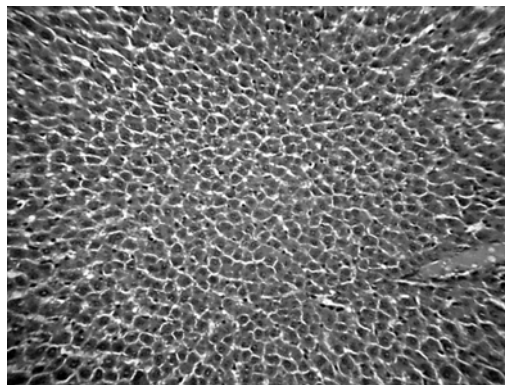


Fig. 12. Diclofenac + vitamin C treated rat LM Section showing mild closure of sinusoidal spaces and less conspicuous hepatic laminae (H & E \times 400)

Estimating the activities of serum marker enzymes, like SGOT, SGPT, ALP and SAP, can make assessment of liver function. When liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released in to the blood stream. Their estimation in the serum is a useful quantitative marker of the extent and type of hepatocellular damage²¹. The enhanced activities of these serum marker enzymes observed in diclofenac sodium treated rats in present study correspond to the extensive liver damage induced by diclofenac sodium. The tendency of these enzymes to return towards a near normal level in group III rats is a clear manifestation of anti-hepatotoxic effect of ascorbic acid. Presence of significantly high concentration of total cholesterol in the serum of group II animals and its recovery towards near normal values in ascorbic acid supplemented rats unearthing the hepatoprotective effect ascorbic acid against diclofenac sodium induced hepatotoxicity.

In present studies, the ascorbic acid co-administered rats showed significantly decreased levels of these lipids peroxidation markers as compared with diclofenac sodium treated rats. The inhibition of lipid peroxidation by ascorbic acid, therefore, may be one of the mechanisms by which ascorbic acid exerts its protection against diclofenac sodium mediated tissue injury.

Free radical scavenging enzymes, such as SOD and CAT, are the first line of defense against oxidative injury. SOD is ubiquitous cellular enzyme that dismutates superoxide radical to H_2O_2 and oxygen and is one of the chief cellular defense mechanisms. The H_2O_2 formed by SOD and other processes is scavenged by catalase that catalyzed the dismutation of H_2O_2 into water and molecular oxygen. Thus, the antioxidant enzyme catalase is responsible for detoxification of H_2O_2 . The catalase enzyme may also be released into the extracellular environment in which it has the potential to function as a potent antioxidant and thereby regulated cell survival²². In

light of these considerations, it seems plausible that extracellular catalase might function as an important autocrine antioxidant and survival factor²³.

The second line of defense consists of the non-enzymic scavengers reduced glutathione. GSH is an important naturally occurring antioxidant as it prevents the hydrogen of sulfhydryl group to be abstracted instead of methylene hydrogen of unsaturated lipids. GSH is a naturally occurring antioxidant important in the antioxidant defense of the body. It has been reported that determination of GSH, can serve as a key to know the amount of antioxidant reserve in the blood and probably in the organism and also, contribute in evaluating the possibilities available for the recuperation of alcoholic patients²⁴.

As shown in Figs. 1-4, diclofenac sodium treatment decreased SOD, catalase, reduced glutathione and increased lipid peroxidation. Pretreatment with ascorbic acid (200 mg/d) as a food supplement improved the SOD, catalase, glutathione and peroxidase levels significantly and reduced lipid peroxidation. The present study focussed that ascorbic acid possesses antioxidant activity, in treatment of NSAIDs induced hepatic cell injury.

Liver histology of diclofenac sodium administered rats showed pathomorphologic alterations (Figs. 9 and 10). These changes were predominant in the centrilobular region, having reduced oxygen perfusion. Hepatic damage may be partially attributed to cytochrome P450-dependent enzyme activity in liver that tends to be present in greatest concentration near the central vein and lower near the peripheral sites. Treatment with ascorbic acid reduced the histological changes (Figs. 11 and 12) produced by diclofenac sodium and significantly lessened the diclofenac sodium-induced liver changes, which correlates with the biochemical findings.

Conclusion

In conclusion, diclofenac sodium could increase the liver enzyme levels and affects some hepatospecific biochemical parameters. Increase in these parameters may occur due to peroxidation reactions, arising in diclofenac sodium biotransformation during drug administration and these reactions may inflict oxidative injury to cellular components. In the light of these results, ascorbic acid (vitamin C) may play a role in the prevention of hepatic cellular injury produced by non steroidal antiinflammatory drugs. However, there is a need for more detailed studies in order to assess the possible relationships between antioxidants and diclofenac sodium hepatotoxicity.

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