

Biochemical Changes in the Liver of Mice after Exposure to Different Doses of Diclofenac Sodium

Deepak Mohan, Sushma Sharma

Abstract—Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are a group of widely used drugs for the treatment of rheumatoid diseases and to relieve pain and inflammation due to their analgesic anti-pyretic and anti-inflammatory properties. The therapeutic and many of the toxic effects of NSAIDs result from reversible inhibition of enzymes in the cyclooxygenase (COX) group. In the present investigation the effect of the drug on the concentration of lipids, and on the activity of the enzymes i.e. acid and alkaline phosphatase, GOT, GPT and lipid peroxidase were studied. There was a significant enhancement in the activities of both acid and alkaline phosphatase after 21 days of treatment. Proportionate increase in the MDA contents was observed after different days of diclofenac treatment. Cellular damage in the liver resulted in decrease in the activity of both GOT (Glutamate oxaloacetate transaminase) and GPT (Glutamate pyruvate transaminase) in both low and high dose groups. Significant decrease in the liver contents was also observed in both dose groups.

Keywords—Anti-inflammatory, cyclooxygenase, glutamate oxaloacetate transaminase, malondialdehyde.

I. INTRODUCTION

THE diclofenac name is derived from its chemical name; 2-(2-6-dichloranilino) phenyl acetic acid [1]. The high levels of NSAIDs inhibit the activities of various enzymes, the ionic exchange rate and the processes depending on prostaglandins [2]. There is growing interest in the toxicity of diclofenac because of its clinical use and for the study of mechanism of hepatotoxicity, renal dysfunction, testicular toxicity and hypersensitivity reactions. This non-steroidal drug has been found to be a prime suspect in causing cell injury due to its ability to bind covalently to macromolecules in situations where intracellular levels of NADH, NADPH, GSH and other reducing agents are very low [3].

The drugs are converted into their simpler forms in liver, from where these can be easily eliminated from the body [4]. Drugs produce a wide variety of clinical and pathological hepatic injury. The elevation in the level of biochemical markers (e.g. alanine transferase, alkaline phosphatase) are often used to indicate liver damage [5].

The drug is found to generate protein adducts in the liver as well as hepatocytes via protein acylation by the drug glucuronide [6]. The possible mechanism of hepatotoxicity is the impairment of biochemical functions of hepatocytes by the

drug or its stable metabolites. Certain compounds can directly act as enzymes or ion transport inhibitors or compete with cellular metabolites in metabolic pathways; other hepatotoxins alter the energetic balance of cells by dramatically increasing the energy demand [7]. Depletion of ATP is, in fact, an early event in the course of drug induced cells by dramatically increasing the energy demand [7]. Depletion of ATP is, in fact, an early event in the course of drug induced toxicity.

Keeping the above information in mind, the present investigation has been carried out on liver of mice with the following aims and objectives: To study the effect of the drug on lipid contents, lipid oxides, and the activity of the enzymes acid phosphatase, alkaline phosphatase, GOT and GPT.

II. MATERIAL & METHOD

The protocol of the present investigation was approved by the Institutional Animals Ethics Committee (IAEC approval no. IAEC/Bio/5/2011-H.P.U.) Himachal Pradesh University, Shimla. Healthy, pathogen free Swiss albino mice of Balb C strain weighing 22-25 g were procured from the Central Research Institute (CRI) Kasauli, Himachal Pradesh. These were maintained in the animal house of the department of Biosciences, H.P. University, Shimla under suitable hygienic conditions with 16 hr day light and temperature of 24±2 °C. The animals were caged in polypropylene cages (six mice/cage) on soft chip bedding and provided with commercial feed (Hindustan Lever Ltd. New Delhi, India) and were given water *ad libitum*.

III. DRUG ADMINISTRATION

Diclofenac sodium was purchased from Sigma Aldrich Co., USA. A stock solution was made in distilled water. Dilutions were made according to the body weight of the mice and administered drug at the dose levels of 4 mg/kg body weight and 14 mg/kg body weight in the dose volume of 1 ml/100 g body weight. The control animals were given saline water only. Oral administration was selected as it is one of the proposed routes for toxicity and cannular feeding was preferred for accuracy.

IV. EXPERIMENTAL DESIGN

The animals were divided into three groups, as:

1. The mice of the first group served as normal (control) animals.
2. The mice of the second group were administered a daily dose of diclofenac sodium (4 mg/kg body weight) for 28 days.

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3. The mice of the third group were given a daily dose of diclofenac sodium (14 mg/kg body weight) for 28 days.

The body weight of the mice was recorded every week for 28 days. The animals were sacrificed at seven days, 14 days, 21 days, and 28 days of experiment by cervical dislocation. The liver was immediately excised, quickly blotted and weighed. Blood was collected by cutting the carotid artery. For serum collection, blood was kept as such for 30 minutes at room temperature and centrifuged at 6000 rpm for 20 minutes. Tissue homogenate (10%) was homogenized in 0.15 M Tris HCl buffer (pH 7.2). The homogenate was centrifuged at 3500 x g for 10 min at 4 °C and supernatant was collected. Protein concentration of the supernatant was calculated according to the method [8]. Spectrophotometric assays were performed on Hitachi VSU-double beam spectrophotometer. Acid dichromate method was employed for quantitative estimation of total lipids [9]. Levels of Malondialdehyde (index of lipid peroxidation) were estimated according to method [10] using thiobarbituric acid. The enzymes (GPT and GOT) were estimated by the method [11] and the quantitative estimation of acid and alkaline phosphatase activity was made by the method [12].

V. RESULTS

A. Lipids

1. Liver

Quantitative Estimation of total lipid content in the liver of normal mice exhibited values ranging between 22.29±0.204 to 23.40±0.161 mg/g fresh tissue weight (Table I and Fig. 1). There was no significant change in the lipid content of mice after seven days in the groups treated with doses of 4 mg/kg body weight and 14 mg/kg body weight. Diclofenac administration for 14 days led to a significant reduction in both groups as the lipid content decreased from 23.40±0.161 mg/g fresh tissue weight to 18.03±0.205 (p<0.001) in the low dose group and 18.67±0.216 mg/g fresh tissue weight (p<0.05) in the high dose group. Lipid biosynthesis continued to be inhibited on 21st day of treatment, as the concentration of lipids observed at this stage was 16.34±0.134 in 4 mg/kg and 15.19±0.228 mg/g fresh tissue weight in 14 mg/kg dose group (p<0.001).

TABLE I
TOTAL LIPIDS (MG/G FRESH TISSUE WEIGHT) IN THE LIVERS OF NORMAL AND DRUG TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	23.05±0.302	23.40±0.161	22.29±0.204	22.29±0.209
4 mg/kg b.w.	21.42±0.245	18.03±0.20**	16.34±0.13**	15.0±0.295**
14 mg/kg b.w.	22.08±0.168	18.67±0.216*	15.19±0.22**	14.04±0.20**

Values are mean ± sem; n=5 in each group [*p<0.05, **p<0.001]

On the 28th day of drug administration, the lipid content in the liver dropped from 22.29±0.209 mg/g tissue weight in normal mice to 15.2±0.295 in the 4 mg/kg dose group and 14.04±0.202 mg/g tissue weight in the high dose group (p<0.001).

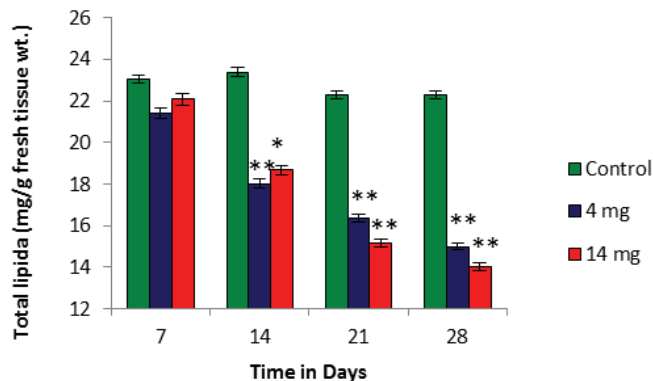


Fig. 1 Histogram showing total lipids (mg/g fresh tissue weight) in the liver of normal and drug treated mice from 7-28 day periods

2. Serum

Quantitative determination of serum total lipid content in normal mice revealed lipid levels ranging between 4.65±0.057 to 5.20±0.330 mg/ml (Table II and Fig. 2). Diclofenac treatment on the other hand resulted in a non-significant increase to 5.37±0.390 in the 4 mg/kg dose group and 5.59±0.450 mg/ml in the 14 mg/kg dose group against 4.86±0.178 in normal mice. A similar trend was noticed after 14 days, where the treated animals showed 6.19±0.608 in the 4 mg/kg dose group and 5.99±0.522 mg/ml in the 14 mg/kg dose group in comparison to 5.20±0.330 mg/ml in normal mice, though the changes up to this stage were non-significant. This increase was attenuated after 21 days when the lipid content decreased to 3.82±0.031 mg/ml in the 4 mg/kg dose group and 3.43±0.065 mg/ml in the 14 mg/kg dose group in comparison to 4.65±0.057 mg/ml in the normal control mice (p<0.05). The mice exposed to diclofenac at 28 days depicted similar results as the lipid level recorded was 3.53±0.115 mg/ml in the 4 mg/kg dose group and 3.65±0.106 in the 14 mg/kg dose group.

Quantitative estimation of total lipid content in the liver of normal mice exhibited values ranging between 22.29±0.204 to 23.40±0.161 mg/g fresh tissue weight. There was no significant change in the lipid content of mice after seven days in the groups treated with doses of 4 mg/kg body weight and 14 mg/kg body weight. Diclofenac administration for 14 days led to a significant reduction in both groups as the lipid content decreased from 23.40±0.161 mg/g fresh tissue weight to 18.03±0.205 (p<0.001) in the low dose group and 18.67±0.216 mg/g fresh tissue weight (p<0.05) in the high dose group. Lipid biosynthesis continued to be inhibited on the 21st day of treatment, as the concentration of lipids observed at this stage was 16.34±0.134 in 4 mg/kg and 15.19±0.228 mg/g fresh tissue weight in the 14 mg/kg dose group (p<0.001).

On the 28th day of drug administration, the lipid content in the liver dropped from 22.29±0.209 mg/g tissue weight in normal mice to 15.2±0.295 in the 4 mg/kg dose group and 14.04±0.202 mg/g tissue weight in the high dose group (p<0.001).

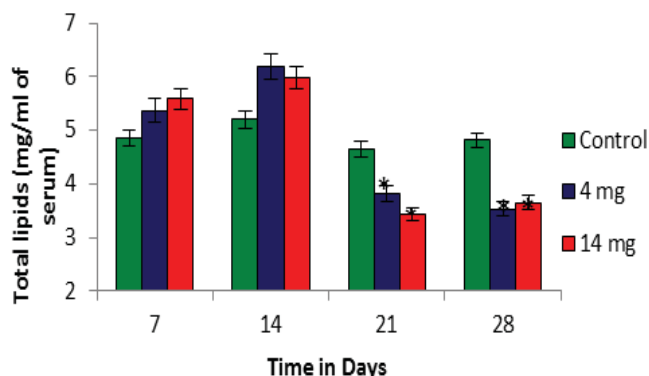


Fig. 2 Histogram showing total lipids (mg/ml of serum) in serum of normal and drug treated mice from 7-28 day periods

TABLE II
TOTAL LIPIDS (MG/ML OF SERUM) IN THE SERUM OF NORMAL AND DRUG TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	4.86±0.178	5.20±0.330	4.65±0.057	4.81±0.054
4 mg/kg b.w.	5.37±0.390	6.19±0.608	3.82±0.031*	3.53±0.115*
14 mg/kg b.w.	5.59±0.450	5.99±0.522	3.43±0.065*	3.65±0.106*

Values are means ± sem; n=5 in each group. [*p<0.05]

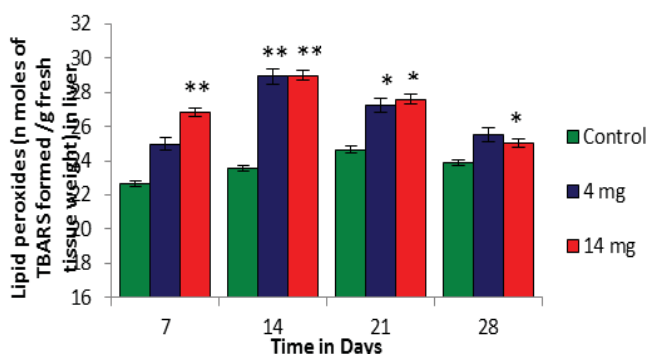


Fig. 3 Histogram showing the Lipid peroxides (n moles of TBARS formed/g fresh tissue weight) in the liver of normal and treated mice from 7-28 day periods

B. Lipid Peroxides

1. Liver

The results showed that administration of mice with diclofenac sodium (4 mg/kg body weight) for seven days and 28 days induced non-significant change on liver lipid peroxides (Table III and Fig. 3). Lipid peroxides in the liver were markedly increased as represented by a significant increase in the MDA level in mice treated with diclofenac sodium after 14 days and 21 days, compared to the control. Seven day exposure could only elevate the lipid peroxides in the high dose group where the values increased from 22.69±0.131 to 26.85±0.189 (n moles of TBARS formed/g fresh tissue weight) [p<0.001]. There was an elevation of MDA content from 23.58±0.107 to 28.95±0.138 n moles/g wet tissues weight after 14 days (p<0.001) and from 24.66±0.212 to 27.22±0.205 n moles/g wet tissues weight after 21 days of treatment (p<0.05). Mice treated with diclofenac at a dose of

14 mg/kg body weight demonstrated a significant increase from the control during all the stages of treatment. The MDA content increased from 22.69±0.131 to 26.85±0.189 n moles/g wet tissue weight and 23.58±0.107 to 28.95±0.219 n moles/g wet tissue weight after seven and 14 days, respectively (p<0.001). There was further increase from 24.66±0.212 to 27.59±0.256 n moles/g wet tissue weight after 21 days and 28 days, respectively, in the high dose group (p<0.05).

TABLE III
LIPID PEROXIDES (N MOLES OF TBARS FORMED/G FRESH TISSUE WEIGHT) IN THE LIVER OF NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	22.69±0.131	23.58±0.107	24.66±0.212	23.87±0.175
4 mg/kg b.w.	24.99±0.446	28.95±0.138**	27.22±0.205*	25.53±0.370
14 mg/kg b.w.	26.85±0.18**	28.99±0.219**	27.59±0.256*	25.05±0.348*

Values are mean ± SEM; n=5 in each group. (*p<0.05, **p<0.001)

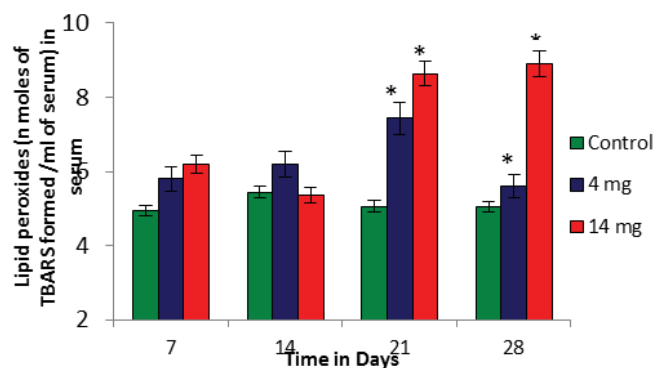


Fig. 4 Histogram showing the Lipid peroxides (n moles of TBARS formed/ml of serum) in the serum of normal and treated mice from seven-28 day periods

TABLE IV
LIPID PEROXIDES (N MOLES OF TBARS FORMED/ML OF SERUM) IN THE SERUM OF NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	4.95±0.104	5.44±0.204	5.06±0.105	5.05±0.144
4 mg/kg b.w.	5.80±0.168	6.19±0.402	7.43±0.656**	5.6±0.265*
14 mg/kg b.w.	6.20±0.091	5.36±0.404	8.63±0.400**	8.91±0.177*

Values are means ± SEM; n=5 in each group (*p<0.05, **p<0.001)

2. Serum

The lipid peroxidation products were not significantly altered in the serum of mice treated with diclofenac during first two stages i.e. 7th and 14th days in both dose groups (Table IV and Fig. 4). The extent of lipid peroxidation displayed MDA concentration between 4.95±0.104 to 5.44±0.204 n moles/l of serum in normal mice. There was a significant increase after 21 days and 28 days of treatment in mice treated with diclofenac at a dose of 14 mg/kg body weight. The elevated MDA concentration was recorded at 8.63±0.400 (p<0.001) after 21 days and at 8.91±0.177 n moles/l of serum (p<0.05) after 28 days.

C. Acid Phosphatase

1. Liver

Diclofenac treatment documented a different trend for acid phosphatase activity in the liver of mice (Table V and Fig. 5). The normal values in the control mice ranged from 0.413±0.001 to 0.428±0.002 μM Pi/mg of proteins. There was a significant decrease from 0.420±0.002 to 0.0389±0.003 μM Pi/mg of proteins in the low dose group after seven days (p<0.05). In the high dose group, the values also decreased considerably at 0.358±0.003 μM Pi/mg of proteins (p<0.001). After 14 days of diclofenac treatment, the specific activity of the enzyme showed insignificant change in both groups depicting the values at 0.396±0.003 and 0.417±0.002 μM Pi/mg of proteins for 4 mg and 14 mg/kg body weight, respectively.

TABLE V
ACID PHOSPHATASE ACTIVITY (μM PI/MG PROTEIN) IN THE LIVER OF
NORMAL AND TREATED MICE FROM SEVEN-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	0.420±0.002	0.427±0.002	0.428±0.003	0.413±0.001
4 mg/kg b.w.	0.389±0.003*	0.396±0.003	0.487±0.003*	0.339±0.002**
14 mg/kg b.w.	0.358±0.003**	0.417±0.002	0.508±0.003*	0.345±0.003**

Values are mean ± sem; n=5 in each group. (*p<0.05, **p<0.001)

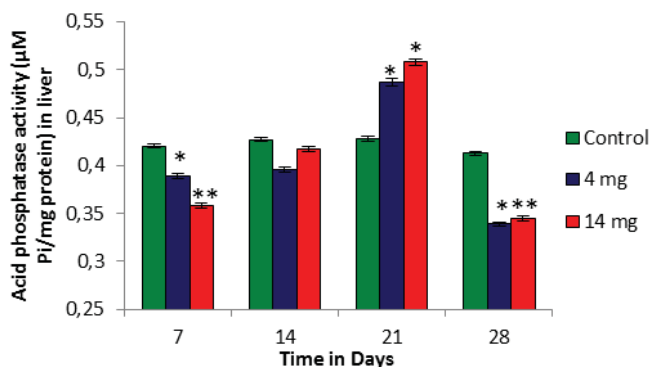


Fig. 5 Histogram showing acid phosphatase activity (μM Pi/mg protein) in the liver of normal and treated mice from 7-28 day periods

There was a significant enhancement in the activity after 21 and 28 days of diclofenac treatment. The specific activity depicted the values at 0.487±0.003 μM Pi/mg of proteins and 0.508±0.003 μM Pi/mg of proteins for the low and high dose groups, respectively, after 21 days of treatment (p<0.05) and after 28 days the values were at 0.339±0.002 μM Pi/mg of proteins for the low dose group and 0.345±0.003 μM Pi/mg proteins for the high dose group (p<0.001).

2. Serum

The activity of acid phosphatase in sera was estimated to determine the correlation with the release of enzymes after tissue damage due to diclofenac therapy during different stages of investigation. There were insignificant changes in the specific activity of acid phosphatase until the 14th day of drug administration as compared to the control (Table VI and Fig. 6). The activity in serum of control mice ranged from 0.181±

0.002 to 0.193±0.001 μM Pi/mg proteins. After the 7th and 14th day of treatment there was slight and insignificant increase in both dose groups. Continuous exposure of the animals to diclofenac for 21-28 days resulted in significant increase in the activity of mice sera. The values increased from 0.189±0.002 to 0.271±0.002 μM Pi/mg proteins in the low dose group and 0.251±0.003 μM Pi/mg proteins after 21 days (p<0.001). A parallel rise in the activity was noticed in the sera after 28 days of investigation as the values enhanced from 0.193±0.001 to 0.270±0.002 in the low dose group and 0.292±0.001 μM Pi/mg proteins in the high dose group (p<0.001).

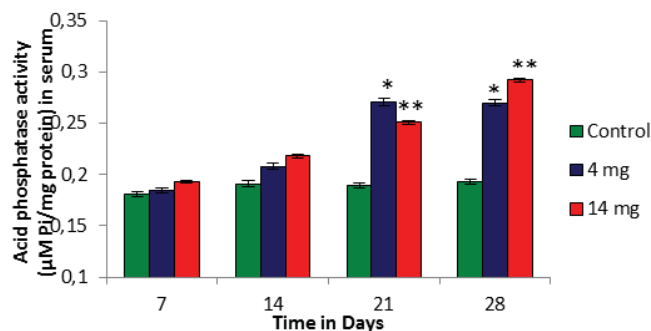


Fig. 6 Histogram showing the acid phosphatase activity (μM Pi/mg protein) in the serum of normal and treated mice from 7-28 day periods

TABLE VI
ACID PHOSPHATASE ACTIVITY (μM PI/MG PROTEIN) IN THE SERUM OF
NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	0.181±0.002	0.191±0.003	0.189±0.002	0.193±0.001
4 mg/kg b.w.	0.184±0.005	0.208±0.003	0.271±0.002**	0.270±0.002*
14 mg/kg b.w.	0.193±0.002	0.218±0.002	0.251±0.003**	0.292±0.001**

Values are mean ± sem; n=5 in each group. (*p<0.05, **p<0.001)

D. Alkaline Phosphatase

1. Liver

Alkaline phosphatase in the liver of normal mice demonstrated activity ranging from 0.568±0.002 to 0.577±0.001 μM Pi/mg proteins (Table VII and Fig. 7). Marked and significant reduction of enzyme activity occurred after seven days of diclofenac treatment as the liver exhibited activity of 0.526±0.002 μM Pi/mg proteins in the 4 mg/kg dose group and 0.538±0.002 μM Pi/mg proteins in the 14 mg/kg dose group (p<0.05). After 14 days and 21 days of treatment the activity showed sharp increase as compared to the normal, though the increase was not significant in the 4 mg/kg dose. The treated mice liver exhibited significantly increased values of 0.602±0.002 μM Pi/mg proteins in the 14 mg/kg dose group after 14 days (p<0.05). Similar trend continued until the 21st day where the increased activity recorded was 0.612±0.003 μM Pi/mg proteins in the 4 mg/kg dose group (p<0.05) and 0.621±0.002 μM Pi/mg proteins in the 14 mg/kg dose group (p<0.001).

The trend was reversed after the 28th day where a sharp decline in the activity was observed in both dose groups. The

activity recorded was 0.520 ± 0.002 $\mu\text{M Pi/mg proteins}$ in the 4 mg/kg dose group and 0.532 ± 0.002 $\mu\text{M Pi/mg proteins}$ in the 14 mg/kg dose group as compared to 0.575 ± 0.002 $\mu\text{M Pi/mg proteins}$ in normal mice ($p < 0.05$).

TABLE VII
ALKALINE PHOSPHATASE ACTIVITY ($\mu\text{M Pi/MG PROTEIN}$) IN THE LIVER OF NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	0.568 ± 0.002	0.569 ± 0.003	0.577 ± 0.001	0.575 ± 0.002
4 mg/kg b.w.	$0.526 \pm 0.002^*$	0.564 ± 0.005	$0.612 \pm 0.003^*$	$0.520 \pm 0.002^*$
14 mg/kg b.w.	$0.538 \pm 0.002^*$	$0.602 \pm 0.001^*$	$0.621 \pm 0.002^{**}$	$0.532 \pm 0.002^*$

Values are mean \pm sem; n=5 in each group. (* $p < 0.05$, ** $p < 0.001$)

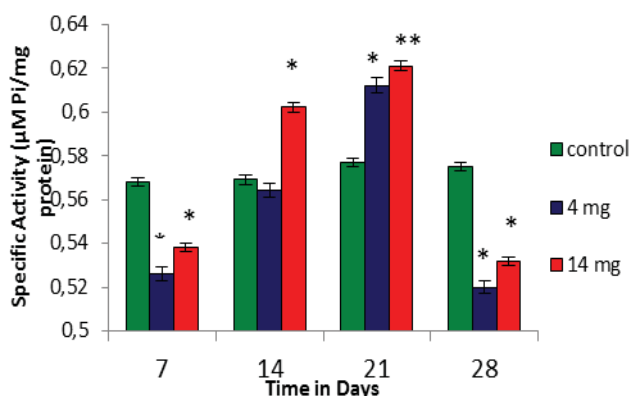


Fig. 7 Histogram showing the alkaline phosphatase activity ($\mu\text{M Pi/mg protein}$) in the liver of normal and treated mice from 7-28 day periods

2. Serum

Diclofenac treatment resulted in a significant increase in the specific activity of alkaline phosphatase in serum of mice, though the increase was evident after 21 days and 28 days of treatment (Table VIII and Fig. 8). Serum alkaline phosphatase of normal mice ranged from 0.250 ± 0.002 $\mu\text{M Pi/mg proteins}$ to 0.261 ± 0.002 $\mu\text{M Pi/mg proteins}$. Initial exposure of mice to diclofenac did not result in any significant change in the activity of the enzyme until the 21st day when there was a sharp increase in the activity. There was a significant increase in the specific activity of the enzyme from 0.251 ± 0.002 $\mu\text{M Pi/mg proteins}$ to 0.312 ± 0.002 $\mu\text{M Pi/mg proteins}$ in the 4 mg/kg dose group and 0.321 ± 0.002 $\mu\text{M Pi/mg proteins}$ in the 14 mg/kg dose group ($p < 0.001$). The enzymatic activity followed a similar trend after 28 days when an increase from 0.261 ± 0.002 $\mu\text{M Pi/mg proteins}$ to 0.329 ± 0.002 in the 4 mg/kg dose group and 0.333 ± 0.003 $\mu\text{M Pi/mg proteins}$ in the 14 mg/kg dose group was reported ($p < 0.001$).

TABLE VIII
ALKALINE PHOSPHATASE ACTIVITY ($\mu\text{M Pi/MG PROTEIN}$) IN THE SERUM OF NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	0.250 ± 0.002	0.256 ± 0.002	0.259 ± 0.002	0.261 ± 0.002
4 mg/kg b.w.	0.259 ± 0.001	0.267 ± 0.003	$0.312 \pm 0.002^{**}$	$0.329 \pm 0.002^{**}$
14 mg/kg b.w.	0.255 ± 0.003	$0.289 \pm 0.002^*$	$0.321 \pm 0.003^{**}$	$0.333 \pm 0.004^*$

Values are mean \pm SEM; n=5 in each group. (* $p < 0.05$, ** $p < 0.001$)

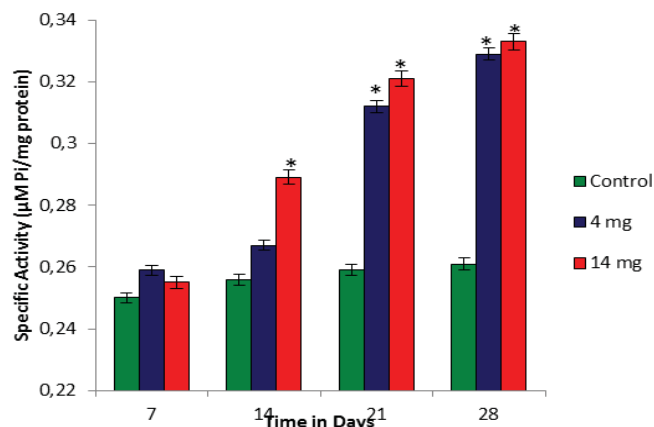


Fig. 8 Histogram showing the alkaline phosphatase activity ($\mu\text{M Pi/mg protein}$) in the serum of normal and treated mice from 7-28 day periods. Values are mean \pm SEM; n=5 in each group. (* $p < 0.05$, ** $p < 0.001$)

E. Glutamate Oxaloacetate Transaminase

1. Liver

There are very high levels of transaminases present in liver. Therefore, a quantitative estimation of GOT was done to confirm whether diclofenac was damaging the liver as well.

GOT enzyme in the liver of normal mice demonstrated very high activity ranging from 0.349 ± 0.002 to 0.361 ± 0.001 μ moles of sodium pyruvate formed/g protein/min at 37 °C (Table IX and Fig. 9). A marked and highly significant elevation of the enzyme level was observed with diclofenac treatment after seven days and 14 days. The activity noticed was 0.434 ± 0.001 and 0.486 ± 0.001 , which was 24.4% and 39.2% more than normal in the 4 mg/kg and the 14 mg/kg dose groups, respectively ($p < 0.001$). After 14 days of treatment the activity of the enzyme rose to 0.529 ± 0.006 in the 4 mg/kg dose group and 0.582 ± 0.003 in the 14 mg/kg dose group ($p < 0.001$). A reverse trend was observed after treating the mice with diclofenac for 21 days and 28 days. A significant decrease in the activity was noticed from 0.349 ± 0.001 in normal mice to 0.299 ± 0.002 ($p < 0.05$), depicting a change of 14.3% in the 14 mg/kg dose group after 21 days. This pattern continued until the 28th day when mice testes showed activities of 0.284 ± 0.002 ($p < 0.001$) and 0.294 ± 0.004 μ moles of sodium pyruvate formed/g protein/min at 37 °C ($p < 0.05$) in the 4 mg and 14 mg/kg dose groups, respectively.

TABLE IX
GOT ACTIVITY (μ MOLES OF SODIUM PYRUVATE FORMED/G PROTEIN/MIN AT 37°C) IN THE LIVER OF NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	0.350 ± 0.004	0.361 ± 0.001	0.349 ± 0.002	0.357 ± 0.001
4 mg/kg b.w.	$0.434 \pm 0.002^*$	$0.529 \pm 0.006^*$	0.309 ± 0.004	$0.284 \pm 0.002^{**}$
14 mg/kg b.w.	$0.486 \pm 0.002^{**}$	$0.582 \pm 0.003^{**}$	$0.299 \pm 0.003^*$	$0.294 \pm 0.004^*$

Values are mean \pm sem; n=5 in each group. (* $p < 0.05$, ** $p < 0.001$)

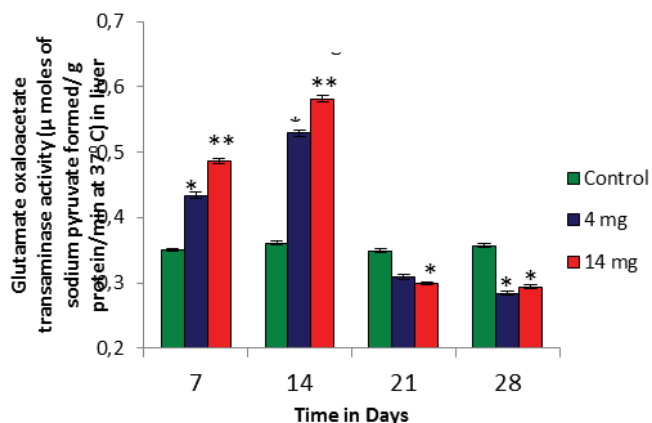


Fig. 9 Histogram showing the GOT activity (μ moles of sodium pyruvate formed/g protein/min at 37°C) in the liver of normal and treated mice from 7-28 day periods)

2. Serum

Presence of GOT in serum is an indicator of pathogenicity of various tissues in the body. The normal mice showed the activity in the range of 73.39 \pm 0.516 to 78.47 \pm 0.62 (μ moles of sodium pyruvate formed/ml of serum/min at 37 °C) (Table X and Fig. 10).

TABLE X

GOT ACTIVITY (μ MOLES OF SODIUM PYRUVATE FORMED/ML OF SERUM/MIN AT 37 °C) IN THE SERUM OF NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	74.84 \pm 0.335	78.47 \pm 0.627	73.39 \pm 0.516	74.48 \pm 0.292
4 mg/kg b.w.	73.67 \pm 0.330	76.59 \pm 0.290	82.63 \pm 0.088**	87.03 \pm 0.270**
14 mg/kg b.w.	73.16 \pm 0.753	79.39 \pm 1.38	83.03 \pm 0.392**	86.23 \pm 0.217**

Values are mean \pm SEM; n=5 in each group (*p<0.05, **p<0.001)

After 7 days of diclofenac treatment no significant change was recorded in the activity of the enzyme in the serum. The enzyme level was 73.67 \pm 0.330 and 73.16 \pm 0.753 (μ moles of sodium pyruvate formed/ml of serum/min at 37 °C) in the 4 mg/kg and 14 mg/kg dose groups and after 14 days the activities were at 76.59 \pm 0.290 and 79.39 \pm 1.39 (μ moles of sodium pyruvate formed/ml of serum/min at 37 °C) in both groups, as compared to 78.47 \pm 0.62 (μ moles of sodium pyruvate formed/ml of serum/min at 37 °C) in normal untreated mice.

Continuous exposure of mice to diclofenac for 21 days resulted in a significant increase in activity from 73.39 \pm 0.516 in normal mice to 82.63 \pm 0.088 in the 4 mg/kg group and 83.03 \pm 0.392 (μ moles of sodium pyruvate formed/ml of serum/min at 37 °C) in the 14 mg/kg dose group (p<0.001). This increasing trend continued for 28 days and an increase of 14.4% in the 4 mg/kg dose group and 13.6% in the 14 mg/kg dose group were noticed (p<0.001).

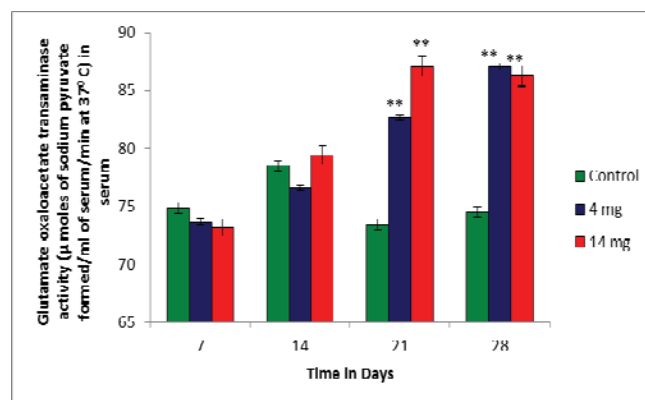


Fig. 10 Histogram showing the GOT activity (μ moles of sodium pyruvate formed/ml of serum/min at 37°C) in the serum of normal and treated mice from 7-28 day periods

F. Glutamate Pyruvate Transaminase

1. Liver

TABLE XI

GPT ACTIVITY (μ MOLES OF SODIUM PYRUVATE FORMED/G PROTEIN/MIN AT 37°C) IN THE LIVER OF NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	0.282 \pm 0.001	0.289 \pm 0.002	0.280 \pm 0.002	0.289 \pm 0.002
4 mg/kg b.w.	0.305 \pm 0.002	0.335 \pm 0.003*	0.210 \pm 0.004*	0.165 \pm 0.002**
14 mg/kg b.w.	0.326 \pm 0.005	0.350 \pm 0.004*	0.165 \pm 0.005**	0.168 \pm 0.003**

Values are means \pm SEM; n=5 in each group. (*p<0.05, **p<0.001)

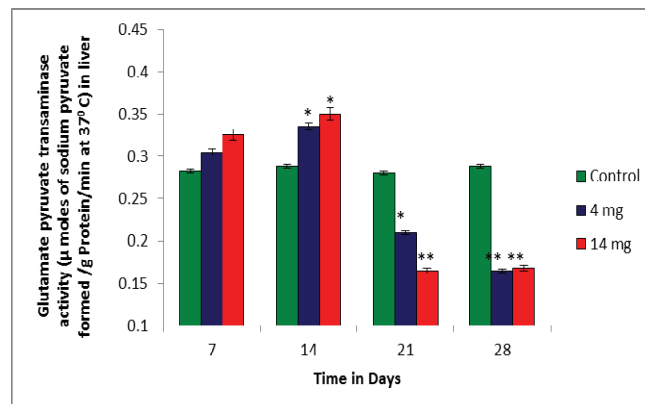


Fig. 11 Histogram showing the GPT activity (μ moles of sodium pyruvate formed/g Protein/min at 37°C) in the liver of normal and treated mice from 7-28 days period

Very high level of transaminases was found in the liver, so quantitative estimation of GPT may be an indicator of the damage incurred by the diclofenac to the organ.

The activity in normal mice ranged between 0.280 \pm 0.002 to 0.289 \pm 0.002 μ moles of sodium pyruvate formed/g protein/min at 37 °C (Table XI and Fig. 11). Diclofenac administration revealed insignificant increase in the activity of the enzyme in both the 4 mg/kg and 14 mg/kg dose groups after seven days. There were significant changes noticed after 14 days when there was increase in activity from 0.289 \pm 0.002 in the normal mice to 0.335 \pm 0.003 in the 4 mg/kg dose group

and 0.350 ± 0.004 μ moles of sodium pyruvate formed/g protein/min at 37°C in the 14 mg/kg dose group ($p < 0.05$). It was seen after 21 days that the enzyme activity decreased significantly from 0.280 ± 0.002 in normal mice to 0.210 ± 0.004 in the 4 mg/kg dose group ($p < 0.05$) and 0.165 ± 0.005 μ moles of sodium pyruvate formed/g protein/min at 37°C in the high dose group ($p < 0.001$). Continuous exposure of mice to diclofenac for 28 days resulted in significant decrease in enzyme activity, as it reduced to 0.165 ± 0.002 in the low dose group and 0.168 ± 0.003 μ moles of sodium pyruvate formed/g protein/min at 37°C in the high dose group, showing a decrease of 42.9% and 41.8% respectively ($p < 0.001$).

2. Serum

TABLE XII

GPT ACTIVITY (μ MOLES OF SODIUM PYRUVATE FORMED/ML OF SERUM/MIN AT 37°C) IN THE SERUM OF NORMAL AND TREATED MICE FROM 7-28 DAY PERIODS

Groups	Days			
	7	14	21	28
Control	45.45 ± 0.688	46.63 ± 0.214	46.74 ± 0.293	45.14 ± 0.442
4 mg/kg b.w.	44.46 ± 0.545	$53.34 \pm 0.382^*$	$61.04 \pm 0.367^{**}$	$53.64 \pm 0.639^*$
14 mg/kg b.w.	42.45 ± 0.728	$54.60 \pm 0.760^*$	$63.24 \pm 0.456^{**}$	$55.24 \pm 0.647^*$

Values are mean \pm sem; n=5 in each group. (* $p < 0.05$, ** $p < 0.001$)

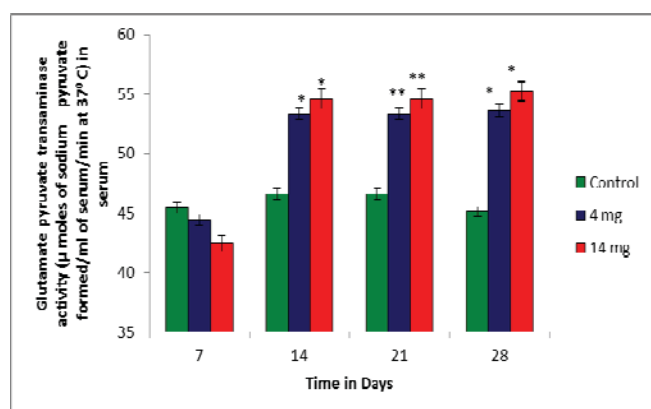


Fig. 12 Histogram showing the GPT activity (μ moles of sodium pyruvate formed/ml of serum/min at 37°C) in the serum of normal and treated mice from 7-28 days period)

The level of an enzyme in the serum acts as a biomarker showing the extent of damage in other tissues due to toxicity. The normal mice showed activity in the range of 45.14 ± 0.442 to 46.74 ± 0.293 μ moles of sodium pyruvate formed/g protein/min at 37°C (Table XII and Fig. 12). No significant change in the activity was recorded after seven days of diclofenac treatment. The serum enzyme level showed significant increase after 14 days of diclofenac treatment, as the activity recorded was 53.34 ± 0.382 and 54.60 ± 0.760 in the 4 mg/kg and 14 mg/kg dose groups, respectively ($p < 0.05$). Further treatment of mice to diclofenac for 21 days also showed significant rise in enzyme activity, as an increase of 23.4% in the low dose group and 26.04% in the high dose group was noticed. The enzyme level increased from 46.74 ± 0.293 in normal mice to 61.04 ± 0.367 μ moles of sodium pyruvate formed/ g protein/min at 37°C in the low dose group and

63.24 ± 0.456 μ moles of sodium pyruvate formed/g protein/min at 37°C in high dose group ($p < 0.001$). On the 28th day of treatment the enzyme levels were 53.64 ± 0.639 and 55.24 ± 0.647 μ moles of sodium pyruvate formed/g protein/min at 37°C , showing an increase of 15.8% and 18.2% in the 4 mg/kg and 14 mg/kg dose groups, respectively ($p < 0.05$).

VI. DISCUSSION

Investigations have shown that mitochondrial dysfunction is a major mechanism of drug induced liver injury, which involves the parent drug or a reactive metabolite generated through cytochromes P450 [13]. The initial increase in the lipid level in the low dose group after seven days may reflect that the drug failed to have any significant effect on the lipogenic pathways in the liver. Lipid synthesis was however inhibited thereafter until the 28th day. Significant decrease in the later stage of diclofenac treatment in the liver speculates the pharmaco-toxicological behaviour of diclofenac and further strengthens the relevance of lipid and free fatty acid role in synthesis of lipid mediators. Since, lipids form important constituents of the membranes, so various degenerative changes as a result of drug toxicity may also explain the continuous loss of lipids in the organs. In other studies, the lipolytic role played by diclofenac has accounted for the decline in lipid level in rats after diclofenac treatment [14].

Lipid peroxidation is known as a common mechanism of toxic manifestations of many drugs including most of the NSAIDs in the liver. Malondialdehyde (MDA) is a low molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. The significant increase in MDA concentration observed in the present study reflected cellular damage in the liver induced by diclofenac. These findings were significantly correlated with the increase of biochemical markers (ALT, AST and ALP activities) in serum suggesting liver failure. Moreover, the changes in MDA contents in both tissues were proportional to the diclofenac doses.

The significant increase in the activity of TBARS in the liver in the diclofenac treated groups (4 mg and 14 mg/kg body weight) indicated ongoing peroxidative stress and compromised antioxidant defense mechanism. The sharp increase in the MDA concentration until the 28th day of the experiment demonstrated the toxic effects of diclofenac on the tissues. Reference [15] reported an increase in liver transaminases, MDA, serum urea and creatinine after injecting diclofenac sodium intramuscularly. Similar results also showed hepatic and renal degenerative changes, necrosis, leukocyte infiltration and fibrosis [16]. Diclofenac sodium is metabolized in the liver through ring hydroxylation, catalyzed by cytochrome isoenzymes to the major oxidative metabolite, 4'-hydroxydiclofenac [17]. Diclofenac has been shown to increase MDA contents in liver and kidney tissue [18]. While another NSAID, flunixin decreased them in heart, kidney and spleen tissue [19].

The interlobular and intralobular fibrosis could be attributed to generation of reactive oxygen species. Our results are in

accordance to [20], who mentioned that oxidative stress is relevant to the formation of fibrosis in most chronic liver diseases associated with decline in antioxidant abilities.

Elevated serum lipid peroxidation in the 14 mg/kg dose group may be related to an imbalance in the antioxidant system and depression of antioxidant scavenger status that promoted production of MDA. Lipid peroxidation can be used as the hepatic oxidative stress parameters for measuring the damage that occurs in the membranes of tissues as a result of free radical generation [21]. Elevated lipid peroxide levels in the present studies may be as a consequence of generation of free radicals, which resulted in superoxide catalyzed oxidation process. A significant elevation of serum lipid peroxides after diclofenac treatment was noticed in rats [22]. Several other studies have been made to show serious liver damage due to effect of toxic doses of diclofenac [23].

Drug toxicity risks can be assessed by measuring the activities of marker or diagnostic enzymes in various tissues of the body, including the liver [24]. Some enzymes are considered as useful marker enzymes of liver cytolysis and damage to the plasma membrane of liver cells [25].

Alkaline phosphatase is one of these marker enzymes for the plasma membrane and endoplasmic reticulum in the liver. Since the enzyme is predominantly located in the plasma membrane of microvilli of bile canaliculi. It is often employed to assess the integrity of the plasma membrane [26]. The enzyme reaches the liver mainly from bone and excreted into bile, therefore, its elevation in serum occurs in hepatobiliary diseases [27]. The hepatotoxicity due to diclofenac has also been related to the consumption of cytosolic glutathione and protein thiols as well as pyridine nucleotide oxidation [28].

The initial decline in the specific activity of the enzyme in both the 4 mg/kg and 14 mg/kg body weight dose groups may be attributed to the inhibition of enzyme production due to the toxic effects of diclofenac and adaptive response towards the cytotoxic effects of the drug. The results were in accordance to the earlier findings where diclofenac is known to decrease the activity of alkaline phosphatase in the skeletal muscle of mice [29]. The significant increase in specific activity of the enzyme after diclofenac administration for 14 days and 21 days may be due to increased functional activity of the liver probably leading to de novo synthesis of the enzyme molecules. It is noteworthy that alkaline phosphatase plays different role in different tissues. The enzyme has been proposed to be a function related marker in the renal proximal epithelia [30]. However, significant decrease in alkaline phosphatase activity in rat hearts was documented to play a cardiovascular protective role by preventing calcification of the system [31]. Several histopathological changes due to diclofenac toxicity have also been shown to be associated with significant elevation of liver enzymes like alkaline phosphatase [32].

Since, phosphate monoesters are subjected to hydrolysis by the alkaline phosphatases (ALP) continuously in the cells, the hyper production of the enzyme could constitute a serious threat to the life of the cells that are dependent on a variety of phosphate esters for their vital processes [33]. Cytolysis as a

consequence of diclofenac toxicity may adversely affect the facilitation and transfer of metabolites across the cell membranes. Significant decrease in enzyme activity after 28 days of treatment is due to severe degenerative changes in the hepatic tissues resulting in the release of the enzyme into blood stream. Necrotic and other degenerative changes might have disturbed the integrity of the plasma membrane resulting in the leakage of the enzyme as suggested by concomitant increase in the serum ALP in the present study. Similar loss of alkaline phosphatase activity in liver has also been reported due to mercury toxicity as a consequence of changes in permeability of plasma membrane in addition to changes in the balance between synthesis and degradation of enzyme proteins [34]. Other results also demonstrated that decrease or inhibition of ACP and ALP activities are due to increased necrosis in the tissues like hepatocytes [35].

Serum alkaline phosphatase is produced by many tissues especially bone, liver, intestine, placenta and is excreted in the bile. Most type of liver injuries result in significant elevation of serum alkaline phosphatase. Bile acids induce alkaline phosphatase synthesis and exert a deterrent effect on the canalicular membrane, allowing leakage into the serum [36].

Alkaline phosphatases have also been categorized as cell activity biomarker [37], as well as hydrolytic enzyme functioning at alkaline pH optima by a number of workers in earlier studies [38]. The observed increase in the activities of alkaline phosphatase in the serum after 21 days of treatment is attributed to leakages of the enzyme from the degenerated tissues due to change in permeability characteristics. Elevated liver enzymes (ALT, AST and ALP) in serum were also earlier reported, when mice were treated with moderate and high dose of diclofenac indicating liver damage [39]. In other findings, the experimentally treated rats with diclofenac resulted in marked elevation of liver enzymes in the serum which reflects the severity of the liver injury [40]. The increase in ALP level after diclofenac administration has also been reported in rats [41]. Some other toxins like carbon tetra chloride are also known to cause significant elevation of serum enzymes [42].

Liver enzymes are cytoplasmic in nature and the hepatotoxic drugs alter the cell membrane function and because of cellular leakage, the hepatoprotective enzymes are released into circulation indicating liver toxicity. Increase in the alkaline phosphatase activity in the later stages suggested some deleterious effects caused due to the toxicity of the drug resulting in continuous release of the enzyme from various tissues into the blood stream. An increase in the activity of serum alkaline phosphatase after aspirin treatment was also reported earlier and this increase was linked to hepatotoxic effects caused by another non-steroidal anti-inflammatory drug, aspirin [43]. On contrary, a significant decrease in the activity of alkaline phosphatase was reported after treatment with aspirin in rats [44].

Because of the intracellular location of AST and ALT in the cytosol, toxicity affecting the liver with the subsequent breakdown in membrane architecture of the cells led to their spillage into plasma and subsequent increase in concentration.

The transaminase enzymes are AST (GOT) and ALT (GPT), the presence of elevated transaminases can be an indicator of liver damage. The significantly increased liver weight of diclofenac exposed animals in the present study was, however, found to be associated with concomitant increase of serum AST and ALT enzyme levels. It is worth mentioning that the elevated activity of serum AST and ALT may be due to loss of enzymes of liver tissue [45]. Liver function tests help in the diagnosis of abnormal condition of the liver and leakage of cellular enzymes into plasma indicates the sign of hepatic tissue damage [46]. Aspartate and alanine aminotransferases are normally localized within the cells of liver, heart, gill, kidney, muscles and other organs. These enzymes are of major importance in assessing and monitoring liver cytolysis [47] and their presence in serum may give information on organ dysfunction [48]. The general increase in the activity of AST in the liver following diclofenac administration for 7-14 days in the liver could be due to de novo synthesis of the enzyme molecule or an adaptation by the organs to the assault from the drug leading to the activity higher than the control. Similar increase in the activity of ALT was reported in fish kidney after Bisphenol-A treatment [49]. A rise in AST and ALT activity is a sensitive indicator of damage to the cytoplasmic and mitochondrial membrane due to liver toxicity. Biochemical symptoms of liver cytolysis are depicted by the pattern of AST and ALT activities. These aminotransferases being the important components of amino acid metabolism in the body help in retention of amino groups [50]. They also help in providing the necessary intermediates for gluconeogenesis. Alteration in their activities may have an adverse effect on the amino acid metabolism of the tissues and consequently the intermediates needed for gluconeogenesis.

Significant decrease in the activity of AST and ALT observed after 28 days may be a consequence of cellular damage arising from diclofenac administration resulting in leakage of the marker enzyme to the extracellular fluid and labialization of the membranes, further strengthening the hypothesis. These findings are supported by histopathological observations showing regressive and degenerative changes in the hepatocytes of the liver. Our results are compatible with previous findings, where administration of diclofenac sodium (9.5 mg/kg body weight) orally for 28 days induced significant increase in the levels of AST, ALT, and total bilirubin and significant decrease in total protein and albumin [51].

The elevated levels of serum enzymes are indicative of cellular leakages and loss of functional integrity of the cell membrane in liver. There was significant elevation of ALT and AST in the serum of diclofenac induced mice during the later stages of the investigation. Earlier studies reported that serum enzymes such as ALT and AST levels were elevated in NSAID induced hepatotoxicity [52]. The increase in serum levels of hepatic markers signifies structural and functional catastrophe of the hepatic system. The leaking of cellular enzymes into plasma caused by liver damage were due to disturbance in hepatocyte transport functions. Significant rise in AST and ALT levels observed in the serum of diclofenac treated mice for 28 days is in accordance to the earlier

observations, where a sharp rise in above biochemical parameters was reported in diclofenac treatment for the same number of days. Serum aminotransferase activities are considered as sensitive indicators of hepatic injury [53]. There was a mention of elevation of serum ALT in vultures following diclofenac administration [54].

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