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Full Length Research Paper

Evaluation of mechanism of hepatotoxicity of leflunomide using albino wistar rats

Ram Lal Lodhi[1], Siddhartha Maity[2], Pradeep Kumar[1], Shubhini A Saraf[1], Gaurav Kaithwas[1], Sudipta Saha[1]*

[1]Department of Pharmaceutical Sciences, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Rai Bareli Road, Lucknow 226025, India.
[2]Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India.

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Leflunomide (LEF) is used for the treatment of rheumatoid arthritis via inhibition of dihydroorotate dehydrogenase and tyrosine kinase enzymes. It is metabolised by cytochrome 2C9 enzyme to active form A771726 (melononitrilamide) which is responsible for both its pharmacological and toxicological responses. Food and Drug Administration (FDA) gave black box warning in 2010 due to acute liver failure in humans. While the exact mechanism of its toxicity remains unknown, it has been postulated that the formation of toxic reactive metabolites and elevation of serum transaminase level may be responsible for its toxicity. The purpose of this study is to investigate whether oxidative stress has any role in inducing hepatotoxicity of this drug. LEF was administered orally in 10 mg/kg body weight to albino wistar rats. Then, liver and blood were collected at 4, 8, 12 and 16 h intervals. Liver glutathione (GSH), oxidized lipid (malonaldehyde MDA), superoxide dismutase (SOD), catalase (CAT) and plasma alanine transaminase (ALT), aspartate transaminase (AST) levels were measured to determine the level of toxicity. GSH, CAT and SOD levels were found to be decreased with respect to control at all time points, whereas MDA level was significantly increased which signified liver cell injury after drug administration. The plasma ALT and AST levels were also increased at the same time, denoting liver tissue damage. Our result collectively indicated that oxidative stress might be responsible for LEF inducing hepatotoxicity.

Key words: Leflunomide, A771726 metabolite, hepatotoxicity, oxidative stress.

INTRODUCTION

Leflunomide (LEF, Figure 1) is used for the treatment of rheumatoid arthritis since 1998 and this action is mediated through inhibition of dihydroorotate dehydrogenase and tyrosine kinase enzymes (Alcorn et al., 2009; Fox 1998). On the other hand, this action is triggered through inhibition of T- and B-lymphocytes proliferation *in vitro* (Cutolo et al., 2009). Researcher proposed that LEF induces tumor necrosis factors-α and interleukin-1β factors during its pharmacological response (Déage et al., 1998). Cytochrome 2C9 is the key enzyme which metabolized LEF to its active form A771726 (melononitrilamide, major), 4-trifluoroaniline and other minor metabolites (Rozman, 2002). Food and Drug Administration (FDA) categorised this drug through black box warning in 2010 due to its acute liver failure in humans (FDA Drug Safety Communication, 2010). The

*Corresponding author. E-mail: sudiptapharm@gmail.com. Phone: +91-8090747008.

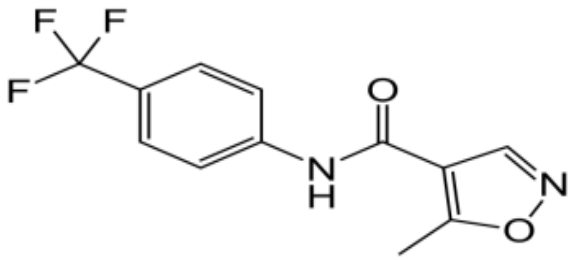

Figure 1 Chemical **s**tructure of LEF

exact nature of its acute liver failure inducing effect has not been completely established but several mechanisms have been proposed. One reason may be the formation of toxic reactive intermediates due to increasing activity of cytochrome 2C9 enzymes during metabolism (Yuyuan and Xuqing, 2010). Researcher also proposed that elevation of 10% serum transaminase level during LEF metabolism may be another reason of liver cell damage (Yuyuan and Xuqing, 2010; Gupta et al., 2011). At the same time, Shi et al. (2011) suggested that cytochorme P450 enzymes might be responsible for cytotoxicity in primary culture rat hepatocytes. Therefore, the question arose whether LEF produced hepatotoxicity via any other mechanisms in humans without formation of toxic reactive metabolites. To get the answer, we performed experiment where LEF was given orally to albino wistar rats in a single dose. Various biochemical parameters are measured at varying time intervals to evaluate the mechanism of LEF induced hepatotoxicity.

Glutathione (GSH), oxidised lipid (malonaldehyde, MDA), tissue catalase (CAT), superoxide dismutase (SOD) in liver and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma were measured to evaluate the mechanism of toxicity. It is demonstrated for the first time that LEF produced liver toxicity at normal dose in albino rats.

MATERIALS AND METHODS

LEF was kindly donated by IPCA Laboratories Ltd. Rampur, Dehradun. Disodium ethylenediaminetetra acetic acid (EDTA), sodium citrate, disodium hydrogen phosphate, trichloroacetic acid, potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from SD Fine Chemicals, Mumbai, India. Glacial meta phosphoric acid, 5,5-dithiotris-2-nitro benzoic acid, tris buffer, sodium carbonate, sodium chloride, sodium potassium tartarate were purchased from Loba Chemicals, New Delhi, India. All other chemicals were obtained from Himedia, Mumbai, India. All chemicals and solvents were of analytical grades and double distilled water was used throughout the experiment.

Animals and dose schedule

Albino Wistar rats of both sexes (weight 90 to 140 g) were purchased from Animal House, CARPS, Shobhit University, Meerut, India (Registration No. 1279/AC/09/CPCSEA, approval date 05.03.2012) and were kept in polypropylene cages under standard conditions of temperature (25 ± 1°C) with 12 h light and dark conditions, diet and water *ad libitum* for seven days. Animals were randomized and divided into the following two groups, control and test (six in each group). LEF was suspended in 1% sodium carboxy methyl cellulose given to test animals according to 10 mg/kg body weight. Blood samples were collected after 4, 8, 12 and 16 h of drug treatment from Retro orbital plexus and then 6 rats from each group were sacrified by the cervical dislocation at the above given time points. Livers were removed and kept in -20°C for further biochemical assays. Blood samples were taken and centrifuged at 2,500 g for 30 min. Plasma was separated, kept at -20°C and subjected to biochemical estimations.

Biochemical estimations

Plasma was subjected to AST, ALT analysis (Reitman and Frankel, 1957). Liver sample was further analysed for total GSH (Sedak and Kubdsay, 1968), CAT (Ellman, 1959), SOD (Claiborne, 1985) and MDA (Ohkawa et al., 1979) contents in tissues.

Plasma ALT

ALT was estimated according to method described by Reitman and Frankel (1957), with slight modifications and performed by using ALT assay kit obtained from Excel Diagonistic Pvt. Ltd., Hyderabad, India. Working solution was prepared by dissolving 4 ml of reagent 1 (mixture of L-alanine and α-ketoglutarate) and 1 ml of reagent 2 (mixture of nicotinamide adenine di neucleotide phosphate, NADP and lactate dehydrogenase, LDH) and kept at 2 to 8°C for future use. The whole assay was performed in 96-well plate and each plate contained 0.5 ml of working solution and 0.05 ml of plasma. This was incubated for 1 minute at 37°C and change in optical density (ΔA_{340}/min) was measured per minute for the next 3 min. Data was calculated by the following equations:

$$\Delta A_{340}/\text{min} = [A_{340}\ (\text{Time 2}) - A_{340}\ (\text{Time 1})] / [\text{Time 2 (min)} - \text{Time 1 (min)}]$$

$$\text{ALT Activity (unit/ml)} = \Delta A_{340}/\text{min} \times 1746 \times 10^{-3}$$

Plasma AST

AST was estimated according to method described by Reitman and Frankel (1957), with slight modifications and performed by using AST assay kit obtained from Excel Diagonistic Pvt. Ltd., Hyderabad, India. Working solution was prepared by dissolving 4 ml of reagent 1 (mixture of L-aspartate and α-ketoglutarate) and 1 ml of reagent 2 (mixture of NADP and malonate dehydrogenase, MDH) and kept at 2 to 8°C for future use. The whole assay was performed in 96-well plate and each plate contained 0.5 ml of working solution and 0.05 ml of plasma. This was incubated for 1 min at 37°C and change in optical density (ΔA_{340}/min) was measured per minute for the next 3 min. Data was calculated by the following equations:

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$$\text{ALT Activity (unit/ml)} = \Delta A_{340}/\text{min} \times 1746 \times 10^{-3}$$

Tissue GSH

Tissue GSH content was estimated by the method of Sedak and Kubdsay (1968), with slight modifications. 0.2 ml of 10% tissue homogenate suspension medium was taken in a tube and 1.8 ml of distilled water was added to it. 3.0 ml of precipitating solution (1.67 g of glacial meta phosphoric acid, 0.2 g disodium ethylenediaminetetra acetic acid and 30 g of sodium chloride in 100 ml distilled water) was added to the above mixture. The mixture was then allowed to stand for approximately 5 min and then filtered. 2.0 ml of the filtrate was added to 8.0 ml of the phosphate solution (0.3 M). 1 ml 0.4% 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was added to it, vortex-mixed and centrifuged at 13,000 rpm for 1 min. A blank solution was prepared in the above mentioned procedure where tissue sample was absent. The Optical density (OD) was measured at 412 nm. The data was calculated by the following procedure:

GSH (µM/mg of protein) = 310.4 × E_i × OD/mg of protein

Where O.D. at 412 nm and E_i is correction factor (0.542).

Tissue MDA

Tissue MDA assay was performed by the following procedure of Ohkawa et al. (1979), with slight modification. 1 ml of 10% tissue homogenate, 0.5 ml of 30 % trichloroacetic acid and 0.5 ml of 0.8% thiobarbituric acid were taken in a test tube and covered with aluminum foil and kept in a shaking water bath for 30 min at 80°C. After 30 min, tubes were taken out and kept in ice-cold water for 15 min. They were then centrifuged at 3,000 rpm for 15 min. The absorbance of supernatants was read at 540 nm at room temperature against appropriate blank. Blank consisted of 1.0 ml distilled water, 0.5 ml of 0.8% TBA solution, and 0.5 ml of 30% TCA solution. In a separate experiment, total protein content of the sample was estimated by bicinchotannic acid (BCA) assay kit using bovine serum albumin (BSA) as standard. The amount of MDA present in a sample was calculated according to the equation:

nM of MDA/mg of protein = (V × OD at 540 nM) / (0.56 × protein concentration)

Tissue CAT

Livers were homogenized in 50 mM stock solution of potassium phosphate buffer with a weight to volume ratio of 1:10. The homogenates were centrifuged at 10,000 rpm for 20 min. 50 µl of supernatant was added to a cuvette containing 2.95 ml of 19 mM/Liter solution of hydrogen peroxide (H_2O_2) prepared in potassium phosphate buffer. Disappearance of H_2O_2 was monitored at 1 min interval for 3 min at 240 nm. Catalase activity was calculated as:

nM of H_20_2/min/mg of protein = (ΔA/min × volume of assay) / (19 × volume of sample × mg of protein)

Tissue SOD

The supernatant was assayed for superoxide dismutase activity by following the inhibition of pyrogallol auto oxidation. 100 µl of cytosolic supernatant was added to tris HCl buffer, pH 8.5. The final volume of 3 ml was adjusted with the same buffer. At last, 25 µl of pyrogallol was added and changes in absorbance at 420 nm were recorded at one minute interval for 3 min. The increase in the absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD. One unit of SOD is described as the amount of enzyme required, causing 50% inhibition of pyrogallol auto oxidation per 3 ml of assay mixture and is given by the formula:

Unit of SOD/mg or protein = [100 × {(A - B) / (A × 50)}]/mg of protein

Where A = change in absorbance per minute in control and B = change in absorbance per minute in test sample.

Statistical analysis

Statistical analysis was carried out using Graph Pad Prism 5.0 (Graph Pad Software, San Diago, CA). All results were expressed as Mean ± standard deviation (SD). The data was analysed by one-way analysis of variance (ANOVA) followed by Bonferrori multiple comparison test, and statistically significant data was accepted when $p < 0.05$.

RESULTS

Tissue GSH

Tissue GSH level in the control group was found to be 21.70 ± 0.89 µM/mg of protein (Figure 2, Table 1). There was significant decrease in the GSH level in the LEF-treated rats beyond 4 h, which were lowest at 12 h post-treatment (16.43 ± 3.16 µM/mg of protein). Statistically significant differences were observed between control and 8, 12, 16 h treated groups (one-way ANOVA, $p < 0.05$).

Tissue MDA

Lipid peroxidation assay is another indicator for oxidative stress. Total MDA was measured in both control and LEF-treated rats at varying time points. According to the data presented in Figure 3, (Table 1), it could be observed that MDA concentration was ~ 0.08 nM/mg of protein in control group whereas it increased to ~0.21 nM/mg of protein at 16 h post LEF treatment. Significant increase in MDA concentration was observed at and above 4 h treatments. Statistically significant differences were observed between control and 8, 12, 16 h treated rats (one-way ANOVA, $p < 0.05$).

Tissue CAT

CAT enzyme is most abundant in the liver which is responsible for the catalytic decomposition of hydrogen peroxide (H_2O_2) to oxygen and water. The H_2O_2 level was measured in control and LEF-treated rats at varying time

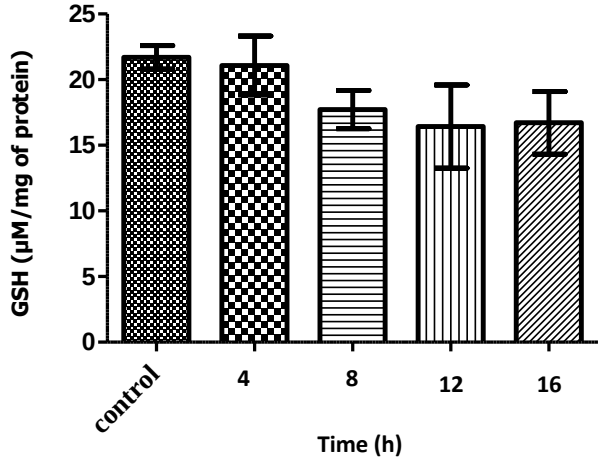

Figure 2. Effect of LEF on GSH level in liver with respect to control at varying time points in albino wistar rats (n = 6). GSH level were significantly reduced at and above 8 h treated rats with respect to control. Statistically significant differences were observed between control and 8, 12, 16 h treated groups (one-way ANOVA, $p < 0.05$).

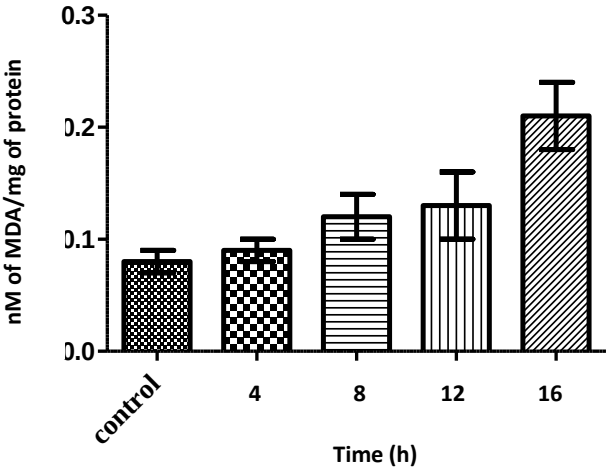

Figure 3. Effect of LEF on lipid oxidation (MDA) in liver with respect to control at varying time points in albino wistar rats (n = 6). MDA level was found to be increased at and above 8 h treated rats with respect to control. Statistically significant differences were observed between control and 8, 12, 16 h treated rats (one-way ANOVA, $p < 0.05$).

points. As could be observed from the data given in Figure 4, Table 1, there was increase in the concentration of the H_2O_2 from ~19 nM/min/mg of protein in control to ~126 nM/min/mg of protein at 16 h after LEF treatment. Although there was no change in H_2O_2 concentration at 4 h time point, its concentration was doubled in 8, 12 h, and four times higher in 16 h with respect to control. Increase in concentration of H_2O_2 depicted that there were less number of CAT enzyme available in the tissue to decompose the H_2O_2. Statistically significant differences were observed between control and 4, 8, 12, 16 h LEF treated rats (one-way ANOVA, $p < 0.05$).

Tissue SOD

SOD is a free radical scavenging enzyme which neutralizes superoxide free radical in normal physiological situations. From the data shown in Figure 5, Table 2, it is evident that there was significant reduction of SOD level with respect to control at and above 4 h. SOD level was 50% lower than control group at 16 h post-treated rats. Statistically significant differences were observed between control and 4, 8, 12, 16 h LEF treated rats (one way ANOVA, $p < 0.05$).

Plasma AST

AST is an important enzyme in human physiological system which helps in transamination of amino acids. Its level is increased during hepatic pathologic conditions. It was observed that there was slight increase of plasma AST level at 12 h albino rats after LEF administration (Figure 6, Table 2). Statistically significant difference was observed between control and 12 h treated rats (one-way ANOVA, $p < 0.05$).

Plasma ALT

ALT is also a transaminase enzyme like AST and its level is also increased during liver damage. As could be observed from the data given in Figure 7, Table 2, plasma ALT level was increased dramatically after 4 h of LEF treatment. The concentration of plasma ALT was almost double during 12 and 16 h after drug treatment (~60 unit/ml) in comparison to control (~25 unit/ml). Statistically significant difference was observed between control and 8, 12, 16 h treated rats (one-way ANOVA, $p < 0.05$).

DISCUSSION

More than 1,100 drugs are thought to be hepatotoxic in nature world-wide (Biour et al., 1998). Drug-induced liver injury is the most frequent cause for withdrawal of marketed drugs, despite rigorous preclinical and clinical testing (Bissell et al., 2001). The major causes of hepatotoxicity related withdrawal of drugs are elevation of liver enzymes levels and depletion of GSH during drug induced oxidative damage. One possible reason of hepatotoxicity is the formation of reactive intermediates during drug metabolism which ultimately binds with nucleophile GSH. These reactive intermediates are highly

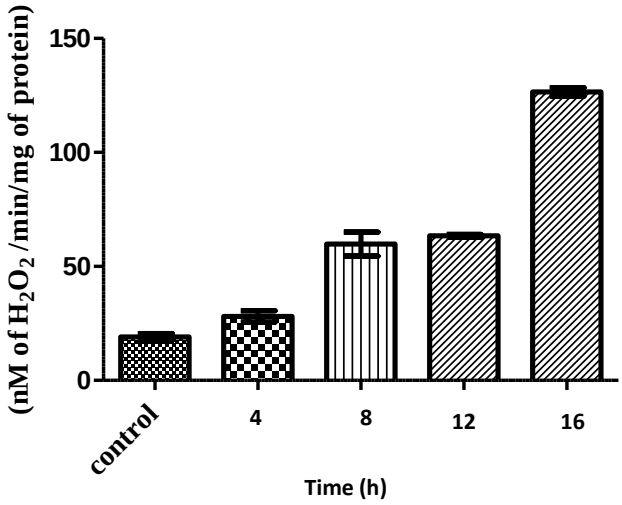

Figure 4. Tissue CAT was measured after LEF treatment at different time intervals with respect to control (n=6). Increase in concentration of H_2O_2 depicted that there were less amount of CAT available in the tissue to decompose the H_2O_2. Statistically significant differences were observed between control and 4, 8 12, 16 h LEF treated rats (one way-ANOVA, $p<0.05$).

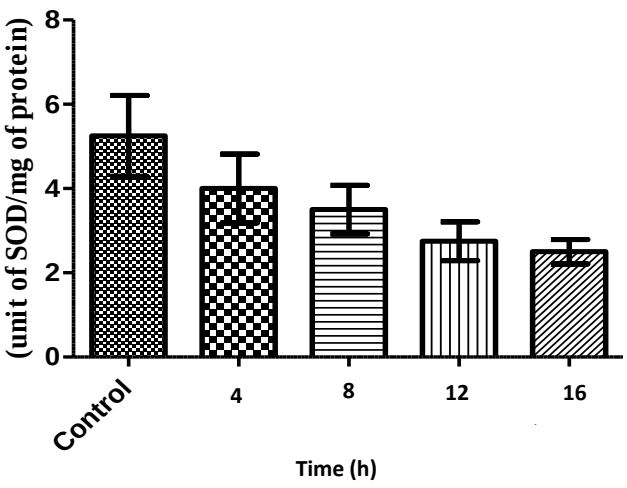

Figure 5. Tissue SOD was also measured at different time intervals with respect to control (n = 6). SOD level was decreased at all time points with respect to control. Statistically significant differences were observed between control and 4, 8 12, 16 h LEF treated rats (one way-ANOVA, $p < 0.05$).

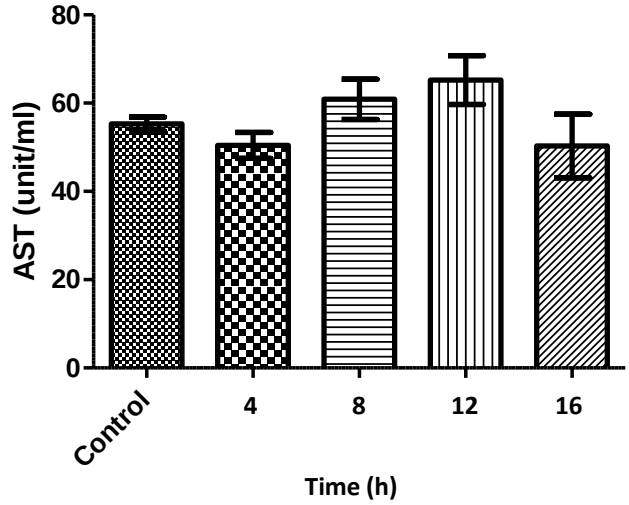

Figure 6. Plasma AST was measured at different time intervals with respect to control (n=6). No changes were observed on AST level after drug treatment. Statistically significant difference was observed between control and 12 h treated rats (one way-ANOVA, $p < 0.05$).

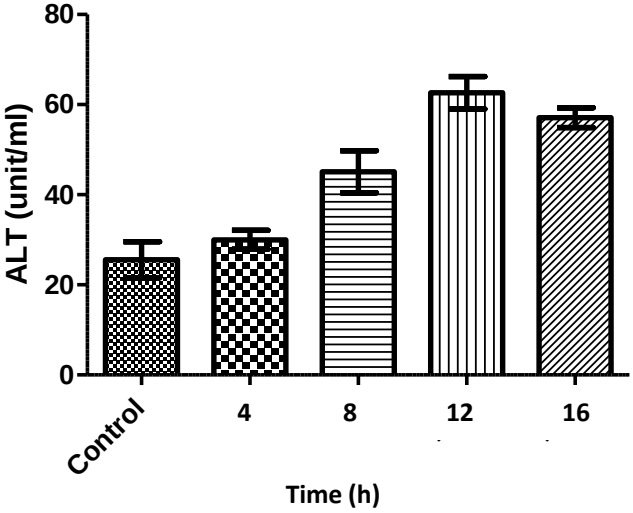

Figure 7. Plasma ALT was measured at different time intervals with respect to control (n=6). ALT levels were increased at and above 8 h with respect to control. Statistically significant difference was observed between control and 8, 12, 16 h treated rats (one way-ANOVA, $p < 0.05$).

toxic in nature which detoxify in presence of GSH. As a result, there is a reduction of GSH in liver which leads to liver injury. For example, five reactive intermediates have been identified for troglitazone in orally administered Sprague-Dawley rats which bound with GSH, and may lead to liver toxicity (Kassahun et al., 2001). This is the major cause for withdrawal of troglitazone from market in 2001 (Jaeschke, 2007).

On the other hand, few drugs also cause hepatotoxicity due to other mechanisms like changes in liver mitochondrial functions, alteration of expression of liver genes and oxidative stress (Russmann et al., 2009; Boverhof et al., 2005; Troudi et al., 2010). Oxidative stress induced hepatotoxicity is the major cause of drug withdrawal nowadays (Jaeschke et al., 2002). It is postulated that orally administered drugs may generate free radicals which cause reduction of GSH, CAT, SOD and elevation

Table 1. Effect of LEF on GSH, MDA, CAT, SOD and in liver at varying time points (n = 6).

Time (h)	GSH (µM/mg of protein)	MDA (nM of MDA/mg of protein)	Tissue CAT (nM of H_2O_2/min/mg of protein)	Tissue SOD (unit of SOD/mg of protein)
control	21.70±0.89	0.08±0.01	19.04±1.47	5.25±0.95
4	21.08±2.24	0.09±0.01	28.06±2.55	4.02±0.81
8	17.72±1.45	0.12±0.02	59.84±5.25	3.50±0.57
12	16.43±3.16	0.13±0.03	63.44±0.63	2.75±0.45
16	16.71±2.39	0.21±0.03	126.51±1.88	2.50±0.29

Table 2. Effect of LEF on ALT and AST levels in plasma at varying time points (n = 6).

Time (h)	AST (unit/ml)	ALT (unit/ml)
control	55.27±1.58	25.55±3.99
4	50.43±2.96	29.99±2.11
8	60.88±4.58	45.11±4.67
12	65.22±5.55	62.64±3.58
16	50.30±7.20	57.11±2.22

of MDA levels in liver. Elevation of plasma ALT and AST occurs during drug toxicity. Acetaminophen causes hepatotoxicity via oxidative stress mechanism in long term therapy (Reid et al., 2005).

Few recent surveys suggested that LEF has tendency to produce hepatotoxicity in human (Gupta et al., 2011). The final question arose how LEF produced hepatotoxicity in humans without formation of toxic reactive intermediates. To get the answer, we performed experiment where LEF was given orally to albino wistar rats in a single dose (10 mg/kg). One recent medical bulletin suggested that leflunomide is effective for organ transplantation at 10 mg/kg dose in rats (Drug Information Portal, Druglib.com). This information allowed us to perform the experiment at same dose to measure the toxicological responses. It is converted to major A771726 intermediate during first pass metabolism which is pharmacologically active than its parent drug (Rozman, 2002). A771726 is 99.3% plasma protein bound (albumin), has half-life ($t_{1/2}$) of 8 h and its concentration in blood is decreased after 12 h in rats (Silva et al., 1996). This data was very important to select time for collecting the liver and plasma from rats. Finally, we decided four time points (4, 8, 12 and 16 h) for collection after drug administration to get maximal effect. Various biochemical parameters were measured at varying time after drug treatment to evaluate the mechanism of LEF induced hepatotoxicity.

To evaluate, we performed GSH assay which explained that there was decrease in liver GSH level after drug treatment. Depletion of GSH by LEF has not been explained as the cause of the oxidative stress induced hepatotoxicity in rats. To get the satisfactory answer related to mechanism of toxicity, we measured MDA, CAT and SOD levels in liver.

Oxidation of lipids is an important parameter for measurement of oxidative stress in living system. Lipid is oxidized to MDA in presence of free radicals. From the result, it was observed that the MDA level was increased with time, which is an important indication of oxidative stress based hepatotoxicity of LEF. Antioxidant enzymes such as CAT are easily inactivated by peroxides or reactive oxygen species. It is most abundant in the liver, which is responsible for the catalytic decomposition of H_2O_2 to oxygen and water (Baudrimont et al., 1997; Reiter et al., 2000). The H_2O_2 level was measured and the values of control and LEF-treated groups were compared at varying time points. Increase in concentration of H_2O_2 in test rats depicted that there was less amount of CAT available in the tissue to scavenge the H_2O_2.

SOD is a free radical scavenging enzyme which neutralizes superoxide free radical in normal physiological situations (Karaman et al., 2006). SOD level was also decreased after LEF treatment. All these experiments suggested that LEF produced toxicity in liver via oxidative stress. To support this hypothesis, we further measured ALT and AST level in plasma. Both enzyme levels were also increased during pathophy- siological conditions and drug toxicity. Similar observation was found in our study where both these enzyme levels were increased after LEF treatment. It was also observed that both ALT and AST levels in plasma were higher at 12 h treated rats (Figures 6 and 7). These enzymes normally present within liver cells. If the liver is injured or damaged, the liver cells spill these enzymes into the blood, raising the enzyme levels in the blood. Increase in these enzyme levels in plasma might be due to LEF induced liver injury.

Conclusion

All biochemical assays suggested that LEF induced liver damage might be due to oxidative damage. LEF is converted to A771726 (major metabolite of LEF) via fast pass metabolism after oral administration in both rats and human. A771726 has higher plasma protein binding

capacity in rats after oral administration, and $t_{1/2}$ is 8 h (Baudrimont et al., 1997). From our findings, it is evident that LEF induced oxidative damage were higher at 12 and 16 h. It might be possible that LEF was converted to A771726 during oral administration which was responsible for liver damage at and above 12 h. The final conclusion of the study is that oxidative stress may occur during LEF hepatotoxicity. Prospective studies involving liver histopatological lesion is needed to confirm the toxic effect of LEF. There is no literature available for LEF induced oxidative stress related hepatotoxicity. This is the first report of LEF induced hepatotoxicity in a single dose after oral administration to albino wistar rats. Further studies can be performed with multiple dosing to evaluate the LEF induced hepatotoxicity as future scope of the work.

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