Amelioration of Paracetamol-induced Hepatotoxicity in Mice with Ferulic acid

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Abstract— Paracetamol (PAR) is a widely used analgesic used in therapeutic purposes but its use is limited by hepatotoxicity. The present work concerns on the ability of ferulic acic (FA) to offer protection against acute hepatotoxicity induced by paracetamol (150 mg/kg) in Swiss albino mice. Oral administration of FA (100 mg/kg *p.o* and 200 mg/kg *p.o*) offered a significant dose dependent protection against paracetamol induced hepatotoxicity as assessed in terms of biochemical and histopathological parameters. The paracetamol induced elevated levels of serum marker enzymes such as serum glutamate pyruvate transaminase (GPT), serum glutamate oxaloacetate transaminase (GOT), alkaline phosphatase (ALP), and bilirubin in peripheral blood serum and deformed hepatic tissue architecture along with increased levels of lipid peroxides (LPO) and reduction of superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and glutathione peroxidase (GPx) in liver tissue. Post administration of the FA after paracetamol insult restored the levels of these parameters to control (untreated) levels. Thus the present study revealed that FA offered protection against hepatotoxicity induced by paracetamol.

Keywords- Hepatotoxicity, Paracetamol, antioxidant, lipid peroxidation

1 INTRODUCTION

Liver is a very important organ in maintaining homeostasis of the body. It involved in almost every biochemical pathways connected to growth, to fight against disease, nutrient supply, energy provision and reproduction. It plays a pivotal role in regulating metabolism of endogenous as well as xenobiotic substances, secretion and storage. Liver has got the capacity to detoxicate toxic substances and manufacture useful chemical principles. Liver disease is a worldwide health problem. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders [1]. Most of the hepatotoxic chemicals harm liver cells mainly by producing reactive species which form covalent bond with the lipids of the tissue. Due to excessive exposure to hazardous chemicals, sometimes the free radicals generated are so high that they overpower the natural defencive system leading to hepatic damage and cause jaundice, cirrhosis and fatty liver [2]. Production of the reactive species manifest in tissue thiol depletion, lipid peroxidation, plasma membrane damage etc., leading into severe hepatic injury [3].

Acetaminophen or paracetamol (PAR) is one of the most widely used analgesics with few side effects when taken in therapeutic doses [4] and hepatotoxicity is a common consequence of acetaminophen overdose [5],[6]. A number of reports indicate that overdose of paracetamol can produce centrizonal hemorrhagic hepatic necrosis in humans and experimental animals [1]. Acetaminophen toxicity is caused by the reaction metabolite N-acetyl-p-benzoquinoneimine (NAPQI), which is partly metabolized by cytochrome P-450 [7]. This species causes severe oxidative damage and glutathione depletion leading to liver necrosis. An overdose of acetaminophen, causes elevated levels of toxic NAPQI metabolite, which can extensively deplete hepatocellular GSH and covalently modify cellular proteins resulting in hepatocyte death. It is generally recognized that at higher doses the drug induces lipid peroxidation and oxidative stress, each contributing to hepatocellular damage and also produces hepatic necrosis. Introduction of

cytochrome or depletion of hepatic glutathione is a prerequisite for acetaminophen -induced hepatotoxicity [8], [9].

Liver dysfunction is a clinically significant problem, for people of all the reported cases of acute liver failure [10]. Therefore very often hepatoprotective drugs are recommended to ameliorate liver problems. Herbal drugs are often used widely because of their effectiveness, fewer side effects and relatively low cost [11]. It has been reported that about 160 phyto constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotenoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes from 101 plants have hepatoprotective activity [12], [13].

Phenolics are widely distributed in the plant kingdom and are integral part of human diet [14]. As a phenolic compound, Ferulic acid (FA or 4-Hydroxy-3-Methoxycinnamic Acid) (**Figure. 1**) play a major role in the body's defense against carcinogenesis by inhibiting the formation of N-nitroso compounds [15]. Moreover, FA is a strong scavenger of free radicals and it has been approved in certain countries as food additive to prevent lipid peroxidation [16]. FA is shown to preserve physiological integrity of the cells exposed to various stress. This can be attributed to the effective antioxidant property of FA. Usually phenolic compounds acts by scavenging free radicals and quenching the lipid peroxidative side chain. Phenolic compounds can act as free radical scavengers by virtue of their hydrogen donating ability and forming aryloxyl radicals [17]. Thus the present chapter concerns, prevention of paracetamol induced hepatotoxicity in mice by Ferulic acid.

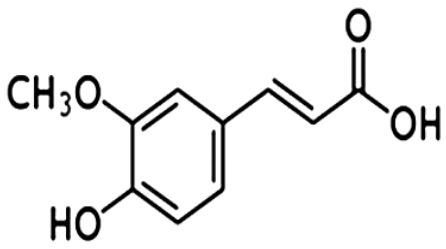

Figure. 1 Ferulic acid (FA)

2 MATERIALS AND METHODS

2.1 Animals

Female Swiss albino mice of 8-10 week old weighing 26-28g, selected from inbred group was used for hepatototoxicity studies and were kept under standard conditions of tempera-ture (25 ± 5℃) and humidity in the Centre's Animal House Facility. The animals were given standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) consti-tuted by the Animal Welfare Division of Government of India.

2.2 Chemicals

Ferulic acid (FA), Nitroblue tetrazolium (NBT), riboflavin, reduced glutathione (GSH), 5-5' dithiobis-2- nitro benzoic acid (DTNB) were purchased from Sigma Chemical Company Inc., St. Louis, MO, USA. Paracetamol was obtained from Variety Pharmaceuticals (P) Ltd., Shornur, Kerala, India. EDTA was from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. H2O2 was from Merck India Ltd., Mumbai, India. Thiobarbituric acid (TBA) was purchased from Hi-media Laboratories, Mumbai, India. All other chemicals and reagents used in this study were of analytical grade procured from reputed Indian manufacturers.

2.3 Administration of Drugs

The animals were randomly divided into 5 groups of five each and treated as follows.

Group I - Untreated Control

Group II - Paracetamol control, administered with paracetamol (150 mg/kg) as single dose after 18 h starvation.

Group III - Silymarin (75 mg/kg body weight) one hour after paracetamol administration

Group IV- Ferulic acid 100 mg/kg b.w **(**in 0.1 ml distilled water *p.o*) one hour after paracetamol administration

Group V- Ferulic acid 200 mg/kg b.w **(**in 0.1 ml distilled water

p.o) one hour after paracetamol administration

Group I administered with distilled water alone was kept as untreated control. Group II was given acetaminophen (150 mg/kg body weight) as single dose orally after 18 h starvation and kept as control. Group III served as reference control, received Silymarin (75mg/kg body weight), a clinically used hepatoprotective drug, one hour after paracetamol administration. Ferulic acid was administered to Group IV and V animals, one hour after paracetamol (150 mg/kg) administration. The test drugs and paracetamol were administered orally to all animals. After 24 hours of paracetamol feeding, the animals were sacrificed and blood was collected by direct cardiac puncture under light ether anesthesia and serum was separated for the biochemical estimations. The liver was removed, washed with ice-cold PBS, weighed and morphological changes were observed. Then liver homogenates (10% w/v) was analyzed for antioxidant status**.**

2.4 Evaluation of liver function enzymes in serum

Serum was used for the determination of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), Serum albumin and serum Bilirubin.

2.5 Determination of antioxidant status in the liver

Liver was excised after sacrificing the animals and washed with ice-cold PBS and 10% homogenate was prepared in PBS (pH 7). A part of this homogenate was used for the determination of reduced glutathione (GSH). Rest of the homogenate was centrifuged at 1,000 rpm for 10 min for removing the cell debris, unbroken cells, nuclei, erythrocytes and mitochondria. The supernatant was used for the estimation of superoxide dismutase (SOD), glutathione peroxidise (GPx) and malondialdehyde (MDA). The protein was estimated by Lowry's method.

2.6 Histopathological studies

Small sections taken from each lobe of the liver were fixed immediately in 10% neutral formalin for a period of at least 24 hr, and embedded in paraffin wax. Thin sections of 5 micron thickness were made using a microtome and later stained with haematoxylin-eosin. The histopathological examinations were carried out at Sudharma Metropolis Pathological Laboratory, Thrissur, Kerala, India.

2.7 Statistical Analysis

The results are presented as Mean ± SD of the studied group. Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons test.

3 RESULTS

3.1 Evaluation of liver function enzymes in serum

Administration of acetaminophen (150 mg/kg body weight) results in manifestation of hepatotoxicity as can be revealed from the elevated levels of serum marker enzymes (**Table.1**). The levels of serum GOT (46.06±9.4), GPT (45.5± 10.35), ALP (110.26 ± 22.16), bilirubin (0.72±0.04) and albumin (3.27±0.27) were elevated in acetaminophen treated animals GOT (250.75±11.78), GPT (258.5±12.60), ALP (213.6 ± 22.35), bilirubin (1.67±0.88) and albumin (4.91±0.12)] when compared to control, indicating liver damage. Silymarin is a known hepatoprotective agent and administration of this compound at a dose rate of 75 mg/kg body weight prevented the onset of hepatotoxicity induced by acetaminophen. Administration of FA after paracetamol treatment prevented elevated levels of serum marker enzymes in a dose dependent manner. Post treatment of FA significantly reversed the levels of GOT, GPT, ALP, bilirubin and albumin when compared to acetaminophen alone treated mice.

Table 1. Effect of ferulic acid on serum marker enzymes in paracetamol induced hepato toxicity in mice. values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. 'a' represents p< 0.001 vs PAR control; 'b' represents p< 0.01 vs PAR control

Treatments	SGOT (U/L)	SGPT(U/L)	ALP (IU/L)	BILIRUBIN (mg/dL)	ALBUMIN(g/dL)
Control	46.06±9.4	45.5±10.35	110.26±22.16	0.72±0.04	3.27±0.27
PAR(150mg/Kg)	250.75±11.78	258.5±12.60	213.6±22.35	1.67±0.88	4.91±0.12
PAR +Syl	80.5±2.64[a]	82.25±8.30[a]	162.6±24.60[a]	0.95±0.1[b]	3.82±0.16 [a]
PAR+FA(100 mg/kg)	73.25±2.98[a]	68.5±11.4[a]	152.6±20.40[a]	0.73±0.11[b]	3.6±0.02 [a]
PAR+FA(200 mg/kg)	66.25±4.34[a]	60.5±10.20[a]	134.3±10.06[a]	0.68±0.1[a]	3.3±0.1 [a]

3.2 Biochemical Measurements and Antioxidant Status

The total antioxidant activity, as a measure of antioxidant status, was significantly decreased in the liver tissue of the acetaminophen treated group (**Table.2**). Acetaminophen treatment caused a significant decrease in the level of SOD, GPx and GSH in liver tissue when compared with control group. The activities of all the antioxidant enzymes were significantly enhanced in the animals treated with FA. The post-treatment with FA (100 and 200 mg/kg) resulted in significant increase of SOD, GPx and GSH in a dose dependent manner. The silymarin treated animals also showed a significant increase in antioxidant enzymes levels compared to acetaminophen treated animals.

Generation of malondialdehyde (MDA) was measured as a marker of lipid peroxidation (LPO) and an indicator of oxidative injury. Analysis of LPO levels by thiobarbituric acid reaction showed a significant increase in the acetaminophen treated mice. The post treatment with FA (100 and 200 mg/kg) significantly prevented the increase in LPO level which was brought to near normal level. The effect of FA was comparable with that of standard drug silymarin (**Figure.2**).

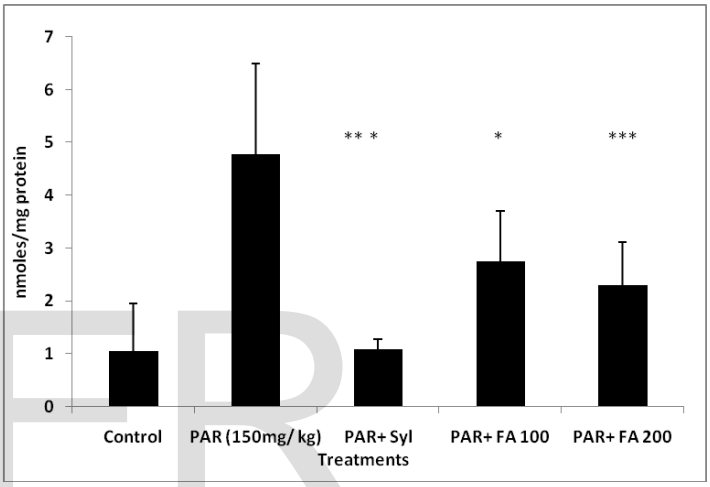

Figure 2. Effect of administration of ferulic acid on paracetamaol-induced lipid peroxidation (MDA formation) in liver tissues of mice. (* indicate p<0.05 and *** indicate p<0.001 when compared with the PAR alone treated group)

3.3 Morphological Study

Histological analysis was performed to confirm FA mediated hepatoprotection. In the untreated control animals the morphological observations of liver tissues showed normal architecture of hepatic cells with clear cytoplasm and slightly dilated central veins. Here the kupffer cells are appeared normal and all cells having normal large nuclei (**Figure.3[A]**). In acetaminophen treated animals the liver tissue shows distorted architecture. Central veins are markedly congested. There are extensive area of necrosis and haemorrhage. Nuclei are distorted and some of the hepatocytes contain vacuolated cytoplasm (**Figure.3[B]**). The silymarin treated animals also showed similar results as that of FA but here the sinusoidal spaces are compressed. And some areas

showed minimal nuclear pleomorphism (**Figure 3.[C]**). In the extract post-treated animals with FA (100 and 200 mg/kg), the normal architecture of the liver tissue can be seen (**Figure3. [D&E]**). Here central veins are slightly dilated and hepatocytes are slightly pleomorphic of them have larger nuclei. Sinusoidal space appears normal, and also the kupffer cells. Thus the histological observations supported the results obtained from liver enzyme assays.

Table 2. Effect of post administration of FA on paracetamol induced depletion of GPx, GSH, and SOD levels in liver tissue of mice. Values are expressed as mean ± sd (n = 5). Data were analyzed by using one-way anova followed by Tukey multiple comparison test. 'a' represents p< 0.001 vs PAR control; 'b' represents p< 0.01 vs PAR control

Treatments	GPx(Unit/mg protein))	GSH(n moles/mg protein)	SOD(Unit/mg protein)
Control	25.4±1.39	24.16±2.33	15.26±1.8
PAR(150mg/Kg)	9.56±2.63	8.39±1.1	5.75±0.2
PAR +Syl	12.38±1.04[a]	12.6±0.08[a]	8.73±0.17[b]
PAR+FA(100 mg/kg)	16.3±1.52[a]	17.87±0.85[a]	9.75±1.70[a]
PAR+FA(200 mg/kg)	19.25±1.70[a]	20.33±1.52[a]	11.3±1.15[a]

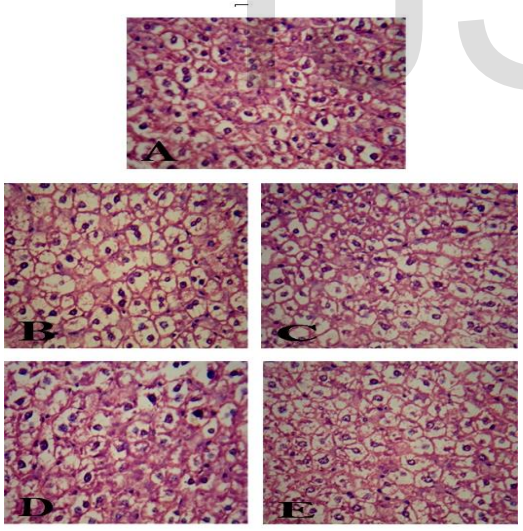

Figure.3 Histopathology. Representative sections of liver tissue of mice **(A)** untreated **(B)** PAR(150mg/kg) **(C)** PAR+Syl and **(D)** PAR +FA 100 mg/kg **(E)** PAR +FA 200 mg/kg

4 DISCUSSION

After an overdose of paracetamol, elevated levels of the toxic NAPQI metabolite are generated, which can extensively deplete hepatocellular GSH and covalently modify cellular proteins resulting in hepatocyte death. Assessment of liver function is made by estimating the activities of serum GPT, GOT and bilirubin which are present higher concentration in cytoplasm. When there is hepatopathy, these molecules leak into the blood stream in compliance with the extent of liver damage [18], [19]. Bilirubin is one of the most useful clinical clues to the level of damage of necrosis and its increase is a measure of binding, conjugation and excretory capacity of hepatocyte.

Paracetamol overdose result in toxic liver damage leading to potentially fatal, hepatic centrilobular necrosis and liver failure [8] associated with metabolic activation by the P-450 system to form a quinine imine metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), which covalently binds to proteins and other macromolecules to cause cellular damage. At low doses, NAPQI is well detoxified, mainly by conjugation with glutathione. At higher doses glutathione becomes depleted and the excess of NAPQI arylates and oxidizes hepatic proteins [20]. Oxidative stress is also a mechanism that has been postulated to be important in the development of paracetamol toxicity. Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures [21]. Assessment of liver function can be made by estimating the activities of serum Alanine amino transferase (ALT), Aspartate Amino transferase (AST) and Bilirubin which are originally present higher concentration in cytoplasm. When there is heaptopathy, these leaks into the blood stream in conformity with the extent of liver damage [18], [22]. The elevated level of marker enzymes seen in the group II, paracetamol treated mice shows the extensive liver damage induced by toxin.

The experimental results indicate that FA were able to delimit the liver damage induced by acetaminophen. The protective effect was found to be significant and can be compared with the standard hepatoprotective drug silymarin [23]. Accidental or incidental acetaminophen overdose may be associated with toxic liver damage leading to potentially fatal, hepatic centrilobular necrosis and liver failure. Oxidative stress is another mechanism that is said to

be important in the progress of acetaminophen toxicity. It was observed that the levels of cellular antioxidant enzymes and molecules are decreased significantly in paracetamol treated animals. This further results in the peroxidative damage to membranes results in the leakage of enzymes, and metabolites to circulation. In the present study, it was observed that, the animals treated with paracetamol showed elevated levels of serum markers such as SGPT, SGOT, ALP and bilirubin. Normally, a higher concentrations of SGOT and ALP are present in liver. Due to hepatocyte necrosis or abnormal membrane permeability, these enzymes are released from the cells and their levels in the blood increases. SGPT is a sensitive indicator of severe liver damage and increased level of this enzyme in non hepatic diseases is unusual [22].

Oral administration of FA exhibited a significant reduction in acetaminophen induced levels of serum GOT, GPT, ALP, and bilirubin which is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage. This could be an evidence for the protective effect of FA which helps to maintain the functional integrity of hepatic cells. More over the decrease in serum bilirubin after treatment with the FA in liver damaged mice induced by acetaminophen indicated their effectiveness in normal functional status of the liver. Thus above alterations can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. The silymarin with a dose of 75 mg/kg, body weight has also provided a better inhibition of the elevated level of SGOT, SGPT, ALP, and serum bilirubin content. Thus the data obtained from the serum parameters of the present study clearly indicates that the antihepatotoxic activity of the FA probably through the correction of cellular integrity of hepatic cell and its regeneration.

Oxidative stress is considered to be associated with many diseases, including cell damage. Glutathione is one of the most abundant tripeptide, a non-enzymatic biological antioxidant present in the liver. Its functions are concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals and maintenance of membrane protein thiols and as a substrate for glutathione peroxidase (GPx) and GST [24]. We observed that the level of GSH was decreased in association with acetaminophen treatment and administration of FA significantly increased ($P< 0.001$) the level of glutathione in a dose dependent manner.

The increase in MDA level in liver of acetaminophen treated mice suggests provoked lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Lipid peroxidation has been postulated as being a destructive process in liver injury caused by acetaminophen administration [25], [26], [9]. The post treatment with FA reversed the increase in MDA levels more significantly ($P< 0.001$). The coincidence of antioxidant activity and protective effect on liver tissues after acetaminophen administration suggest that both free radical generation and lipid peroxidation may be involved in this type of drug injury process.

SOD and GPx enzymes are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage [27], [13]. In the present study, it was observed that FA significantly increased the hepatic SOD activity in acetaminophen treated mice.

In conclusion, the results of this study suggest that in mice, post administration of FA possesses ameliorating effects on paracetamol induced hepatotoxicity. This shows that administration of FA can reduce reactive free radicals there for cause lesser oxidative damage to the tissues and improve the activities of the hepatic antioxidant enzyme. Hence it may be possible that the mechanism of hepatoprotection of FA is due to its antioxidant effect.

ACKNOWLEDGEMENTS

The authors express their gratitude to Amala Cancer Research Centre, Thrissur, Kerala, India and Sree Narayana College, Nattika, Thrissur, Kerala, india for the support as a research grant to ACT and SCAD.

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