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Papain Mitigates Carbontetrachloride Induced Hepatotoxicity in Rats via Attenuation of Oxidative Stress

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Abstract

Liver plays a central role in transforming and clearing toxic substances present in the body. Excessive exposure of liver to toxic agents results in hepatic damage. Certain medicinal agents in therapeutic value cause hepatotoxicity in rats due to which withdrawn from the market. In the present study, we evaluated the protective effects of papain (PN) against carbon tetrachloride (CCl_4) induced hepatotoxicity in rats. Briefly, the rats were divided into four groups of seven animals in each group. Group 1 served as normal control and received water as vehicle. Group 2 received CCl_4 0.5 ml/kg intraperitoneally. Group 3 received PN 80mg/kg (p.o, daily) in phosphate buffer for 7 days and CCl_4 0.5ml/kg i.p on 7th day. Group IV received PN 80mg/kg (p.o, daily) in phosphate buffer with disodium edentate (EDTA) for 7 days and CCl_4 0.5ml/kg i.p on 7th day. The protective effect of PN was measured biochemically and histologically in blood/liver and liver respectively. Treatment with PN significantly ($p<0.001$) reversed CCl_4 induced oxidative hepatic damage which was measured by estimation of superoxide dismutase (SOD), lipid peroxidation (MDA), reduced glutathione (GSH). CCl_4 induced elevation in serum hepatic markers (like AST, ALP, ALT, total bilirubin) were significantly reverted in rats by pretreatment with PN. The biochemical observations were paralleled by histopathological findings in rat liver both in CCl_4 and treatment groups. In conclusion, Papain attenuates CCl_4 induced hepatic damage by mitigating oxidative stress which was confirmed by histology suggesting its use as a protective agent against hepatotoxicants.

Keywords Hepatotoxicity, oxidative damage, protective role, papain

Introduction

Liver plays a central role in transforming and clearing toxic substances present in the body. Certain medicinal agents in therapeutic value cause hepatotoxicity in rats due to which withdrawn from the market [1]. Liver diseases are major cause of mortality and morbidity worldwide and emerging as a major public health challenge that requires the development of new therapeutic options [2,3]. Excessive exposure of liver to xenobiotics, drugs, infections results in hepatic damage. Chemicals like paracetamol, carbon tetrachloride (CCl_4), nitrosamines, and polycyclic aromatic hydrocarbons damage the liver significantly [4,5]. It is believed that reactive oxygen species (ROS) play an important role in the pathology of liver disease's and its progression [6]. Excessive ROS productions overwhelms the antioxidant defense mechanism, generative oxidative stress and are able to react highly with biological molecules such as lipids, proteins and DNA, resulting in their damage [7].

Carbon tetrachloride (CCl_4), a xenobiotic

undergoes biotransformation in liver by cytochrome p450 enzyme generate highly reactive trichloromethyl radical (CCl_3^-) which rapidly reacts with oxygen to form the highly reactive trichloromethylperoxyl radical (CCl_3OO^-). CCl_3OO^- molecule rapidly reacts with lipids (particularly PUFA) to form lipid peroxidation products. The free radical mediated lipid peroxidation is one of the main mechanisms of hepatic injury by CCl_4. Besides, these free radicals overwhelms the antioxidant defensive mechanism by decreasing the levels of superoxide dismutase (SOD), glutathione reductase (GSH) and catalase (CAT). Previous reports suggested that natural substances from medicinal plants and their phytochemicals exhibited strong antioxidant activity that could act against hepatic toxicity caused by various toxicants [8-11].

Papain (PN), an endolytic plant cysteine protease enzyme which is found in papaya (Carica papaya L.). PN enzyme belongs to the papain superfamily, as a proteolytic enzyme. It is of crucial importance in many vital biological processes in all living organisms [12]. It has a long history of being used to treat injuries, other causes of trauma and allergies [13]. It has previously been reported to have significant analgesic and anti-inflammatory activity against symptoms of acute allergic sinusitis like headache and toothache pain without side effects [14,15].

Therefore, this study was undertaken to evaluate the hepatoprotective effect of papain in CCl_4-induced hepatic injury in rats through biochemical and histological assessments.

Materials and Methods

Animals

Adult healthy Swiss Albino rats of either sex (120-150g) were used. The animals were acclimatized for the laboratory conditions for a period of ten days i.e. room temperature (27±3°C), relative humidity (65±10%), and 12h light/dark cycle. All animals were fed with rodent-pellet diet and water was allowed *ad libitum* under strict hygienic conditions.

Drugs and chemicals

Papain (PN) was obtained as a free gift sample from Enzyme Biosciences, Gujarat, India.

Carbon tetra chloride, all the chemicals used were of analytical grade and were procured from Merck or Sigma or S.D. Fine Chemicals.

Experimental design

Rats were randomly divided into 4 groups with 7 animals in each group.

Group I received only vehicle (water p.o, daily) and served as normal control (NC) group;

Group II received only CCl_4 0.5ml/kg i.p and served as CCl_4 control group;

Group III received papain (PN) 80mg/kg (p.o, daily) in phosphate buffer for 7 days and CCl_4 0.5ml/kg i.p on 7th day.

Group IV received papain (PN) 80mg/kg (p.o, daily) in phosphate buffer with EDTA for 7 days and CCl_4 0.5ml/kg i.p on 7th day.

At the end of experimental period (7 days of treatment) the animals were sacrificed by cervical dislocation. The blood samples were collected by cardiac puncture immediately and the liver tissue were collected, homogenized and used for further analysis.

Serum biochemical estimations

The serum biochemical for liver function such as Alanine transaminase (SGPT), Aspartate aminotransferase (SGOT), Alkaline phosphatase (ALP), Total Bilirubin, Total Protein, Creatinine, Urea were estimated by using commercially available kits.

Liver tissue preparation

The animals in all groups (three from each group) were sacrificed by cervical decapitation under anesthesia and livers were quickly

dissected out, homogenized in 50mM phosphate buffer (pH 7.0) containing 0.1mM EDTA to yield 5% (w/v) homogenate. The homogenate was centrifuged at 10,000 rpm for 10min at 0^0C in cold centrifuge, the resulting supernatant was used to determine further biochemical studies.

Determination of protein content

Protein was determined according to the method of Lowry et al., [16] using bovine serum albumin (BSA) as standard protein.

Measurement of malondialdehyde (MDA) content

Malondialdehyde (MDA) formation was estimated by the method of Okhawa et al., [17]. Briefly, 200ml of supernatant was added to 50µl of 8.1% sodium dodecyl sulphate, vortexed and incubated for 10min at room temperature. 375µl of thiobarbituric acid (0.6%) was added and placed in a boiling water bath for 60min and then the samples were allowed to cool at room temperature. A mixture of 1.25ml of butanol: pyridine (1.5: 1 ratio), was added, vortexed and centrifuged at 1000rpm for 5min. The optical density of the colored layer was measured at 532nm on a Spectrophotometer against reference blank and the rate of MDA formed is expressed as nmol of MDA formed/h/mg protein.

Measurement of reduced glutathione (GSH) content

Reduced glutathione content were measured according to the method of Ellman, [18]. Briefly, 0.75ml of supernatant was mixed with 0.75ml of 4% sulphosalicylic acid and then centrifuged at 1,200rpm for 5min at 4^0C. From this 0.5ml of supernatant was taken and added to 4.5ml of 0.01M DTNB, and the yellow color developed was read Spectrophometrically at 412nm immediately. The GSH content was calculated as nmol GSH/mg protein.

Measurement of superoxide dismutase (SOD) activity

SOD activity was measured according to the method of Misra and Fridovich, [19] by monitoring the auto-oxidation of (-) – epinephrine at pH 10.4 for 4min at 480 nm. Briefly, 100µl of brain supernatant was added to 880µl of 0.05M carbonate buffer containing 0.1mM EDTA (pH10.4), and 20µl of 30mM epinephrine (in 0.05% acetic acid) was added to the mixture and the optical density values were measured at 480nm for 4min on a UV-Visible Spectrophotometer, activity is expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50% which is equal to 1 unit. The SOD activity is expressed as U/mg protein.

Histopathological analysis

1 animal from each group were anaesthetized and sacrificed by rapid decapitation. The livers were removed and immersed in cold saline solution for 10 minutes followed by overnight fixation with 10% formalin at room temperature, and sectioned into standard coronal slices 4–5 mm thickness and embedded in paraffin blocks, liver sections of 4–6 µm thickness were stained with hematoxylin and eosin.

Statistical analysis

All values were expressed as mean ± standard error of mean (SEM). Data was analyzed by one way analysis of variance (ANOVA) with post-hoc Dunnett's test using Graph Pad Prism software (Version 5.0). A value of $p<0.05$ was considered as statistically significant.

Results

Serum biochemical markers of hepatotoxicity

CCl_4 group showed significant ($p<0.001$) increase in serum ALT, AST, ALP, total bilirubin and total protein levels when compared with normal control group. PN (in buffer) and PN (in buffer with EDTA) treated group showed significantly ($p<0.001$) decreased levels of ALT (Fig. 1), AST (Fig. 2), ALP (Fig. 3) and total bilirubin (Fig. 4) when compared with CCl_4 group.

Antioxidant enzymes

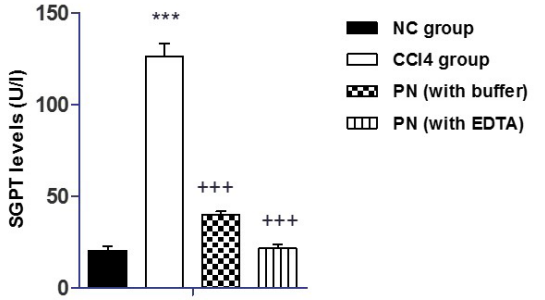

Figure 1. Effect of PN on Alanine transaminase (ALT) levels

Values are expressed as mean ± SEM (n=6). Statistical difference was analyzed by one way ANOVA followed by post hoc Dunnett's test. *** (p< 0.001) vs Normal Control (NC) group; +++(p< 0.001) vs CCl_4 group.

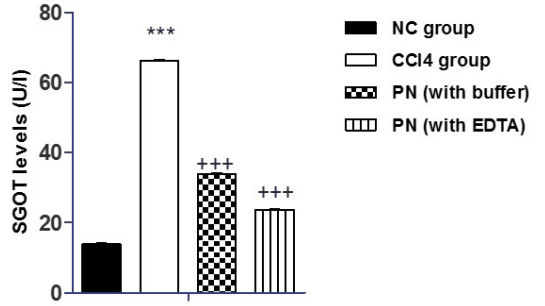

Figure 2. Effect of PN on Aspartate aminotransferase (AST) levels

Values are expressed as mean ± SEM (n=6). Statistical difference was analyzed by one way ANOVA followed by post hoc Dunnett's test. *** (p< 0.001) vs Normal Control (NC) group; +++(p< 0.001) vs CCl4 group.

Liver GSH levels were significantly (p<0.001) decreased in CCl_4 treated group when compared with NC group. PN (with buffer) and PN (with EDTA) treated group showed significantly (p<0.001) increased GSH levels when compared with CCl_4 group (Fig. 5).

SOD levels were found reduced significantly (p<0.001) in the CCl_4 group as compared with the NC group. PN (with buffer) and PN (with EDTA) treated group showed significantly (p<0.001) increased levels of SOD when compared with CCl_4 group (Fig. 6).

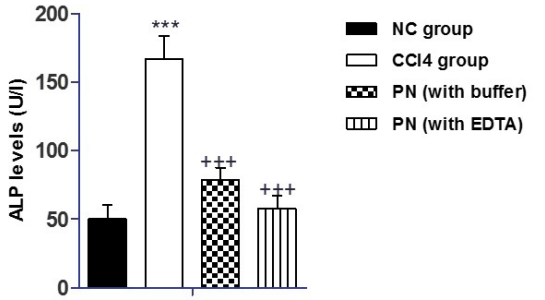

Figure 3. Effect of PN on Alkaline phosphatase (ALP) levels

Values are expressed as mean ± SEM (n=6). Statistical difference was analyzed by one way ANOVA followed by post hoc Dunnett's test. *** (p< 0.001) vs Normal Control (NC) group; +++(p< 0.001) vs CCl_4 group.

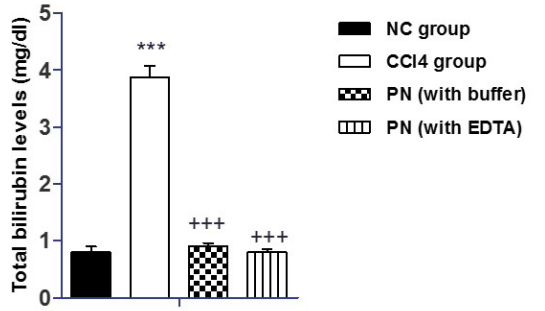

Figure 4. Effect of PN on Total Bilirubin levels

Values are expressed as mean ± SEM (n=6). Statistical difference was analyzed by one way ANOVA followed by post hoc Dunnett's test. *** (p< 0.001) vs Normal Control (NC) group; +++(p< 0.001) vs CCl_4 group.

Lipid peroxidation - Oxidative stress marker

A significant (p<0.001) increased in MDA levels was found in CCl_4 group when compared with normal control group. PN (with buffer) and PN (with EDTA) treated group showed significantly (p<0.001) decreased MDA levels when compared with CCl_4 group (Fig. 7).

Histopathological examinations

Hematoxylin and eosin stained sections of liver in CCl_4 group showed significant degenerative changes like congestion with high vacuolated tissue (10x magnification). PN (with EDTA)

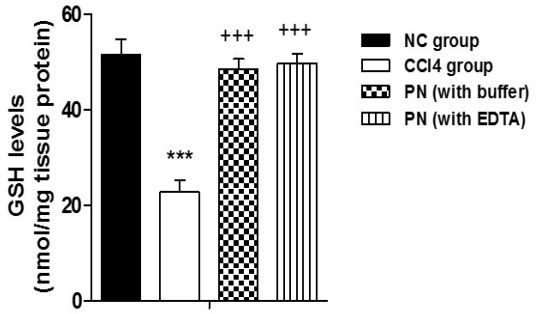

Figure 5. Effect of PN on liver reduced glutathione (GSH) levels

Values are expressed as mean ± SEM (n=6). Statistical difference was analyzed by one way ANOVA followed by post hoc Dunnett's test. *** (p< 0.001) vs Normal Control (NC) group; +++(p< 0.001) vs CCl_4 group.

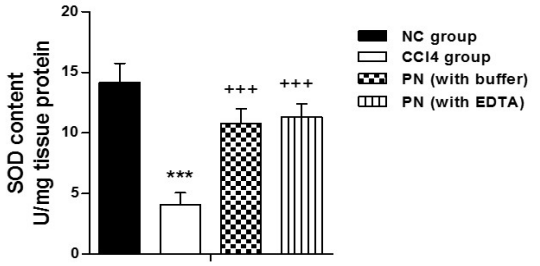

Figure 6. Effect of PN on liver reduced superoxide dismutase (SOD) content

Values are expressed as mean ± SEM (n=6). Statistical difference was analyzed by one way ANOVA followed by post hoc Dunnett's test. *(p<0.05), *** (p< 0.001) vs Normal Control (NC) group; +++(p< 0.001) vs CCl_4 group.

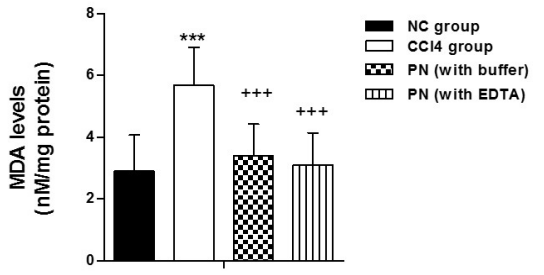

Figure 7. Effect of PN on liver malondialdehyde (MDA) content

Values are expressed as mean ± SEM (n=6). Statistical difference was analyzed by one way ANOVA followed by post hoc Dunnett's test. *** (p< 0.001) vs Normal Control (NC) group; +++(p< 0.001) vs CCl_4 group.

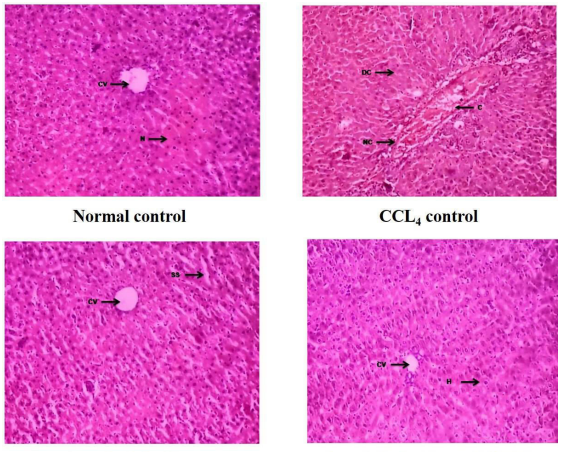

Figure 8. Effect of PN on histological changes in liver

treated group showed regenerative changes in with no vacuolation similar to normal cyto-architecture (10x magnification) (Fig. 8).

Discussion

Excessive exposure of liver to xenobiotics results in hepatotoxicity, which is a major public health problem. CCl_4 is one such xenobiotic and gets distributed throughout tissues soon after its exposure in liver. Cytochorome P450 starts biotransformation of CCl_4 and generates reactive metabolite trichloromethyl radical (CCl_3^-), readily converted to trichloromethyl peroxyradical ($CCl3OO^-$) in presence of oxygen. This highly reactive free radical causes hepatocellular damage [20].

Our study demonstrated that CCl_4 caused a significant increase in levels of AST, ALT and ALP which are known biomarkers of hepatic damage [8,9]. Previous studies have shown that during liver damage, these cytosolic liver marker enzymes would leak out from the swollen and necrotic hepatocytes into blood circulation thereby resulting in elevated level of these enzymes [8,9,20,21]. This process has been associated with massive centrilobular

necrosis, degeneration and cellular infiltration found in liver. Several studies reported that CCl_4 induced hepototoxicity is reduced by decreasing the activities of the liver marker enzymes [20-22]. Pretreatment of PN reversed the toxicity induced by CCl_4 as the elevated serum AST, ALT and ALP activities were reduced significantly. We found elevated serum bilirubin in CCl_4 rat group which was found reduced significantly in PN pretreated rats. Bilirubin has been defined as a conventional indicator of liver diseases. Its restoration may be due to the promotion of its glucuronidation.

Hepatoprotection via oxidative stress has been achieved though radical scavenging, through the activation of antioxidant enzymes, such as SOD and GSH. We found that both SOD and GSH were increased significantly by PN pretreatment in the CCl_4 injured groups, compared with the vehicle-treated CCl_4 groups. Consistent with SOD and GSH, MDA, a marker of lipid peroxidation, was suppressed in the PN pretreated CCl_4 injured group. These findings suggest that PN induced hepatoprotection is associated with radical scavenging effects, through both the SOD and GSH antioxidant enzymes, in accordance with the suppression of lipid peroxidation. CCl_4 induced biochemical changes in oxidative stress markers was significantly attenuated by PN through upregulation of GSH and SOD levels and down regulation of MDA levels [8,9,20-22]

Histopathological profile of liver of control animals showed normal liver architecture, whereas the liver section of animals treated with CCl_4 showed distorted liver architecture with more hepatocytes showing degenerative changes and necrosis. The liver section of animals treated with PN showed significant restoration of architecture of hepatocytes compared with hepatotoxicity induced group. The histopathological reports confirmed the hepatoprotective effect of Papain in CCl_4 induced hepatotoxicity in rats.

By considering the above results, it can be concluded that Papain attenuates CCl_4 induced hepatic damage by mitigating oxidative stress which was confirmed by histology.

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