Protective Effect of a 43 kD Protein from the Leaves of the Herb, *Cajanus indicus* L. on Chloroform Induced Hepatic-disorder

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*Cajanus indicus* is a herb with medicinal properties and is traditionally used to treat various forms of liver disorders. Present study aimed to evaluate the effect of a 43 kD protein isolated from the leaves of this herb against chloroform induced hepatotoxicity. Male albino mice were intraperitoneally treated with 2 mg/kg body weight of the protein for 5 days followed by oral application of chloroform (0.75 ml/kg body weight) for 2 days. Different biochemical parameters related to physiology and pathophysiology of liver, such as, serum glutamate pyruvate transaminase and alkaline phosphatase were determined in the murine sera under various experimental conditions. Direct antioxidant role of the protein was also determined from its reaction with Diphenyl picryl hydraxyl radical, superoxide radical and hydrogen peroxide. To find out the mode of action of this protein against chloroform induced liver damage, levels of antioxidant enzymes catalase, superoxide dismutase and glutathione-S-transferase were measured from liver homogenates. Peroxidation of membrane lipids both *in vivo* and *in vitro* were also measured as malonaldehyde. Finally, histopathological analyses were done from liver sections of control, toxin treated and protein pre- and post-treated (along with the toxin) mice. Levels of serum glutamate pyruvate transaminase and alkaline phosphatase, which showed an elevation in chloroform induced hepatic damage, were brought down near to the normal levels with the protein pretreatment. On the contrary, the levels of antioxidant enzymes such as catalase, superoxide dismutase and glutathione-S-transferase that had gone down in mice orally fed with chloroform were significantly elevated in protein pretreated ones. Besides, chloroform induced lipid peroxidation was effectively reduced by protein treatment both *in vivo* and *in vitro*. In cell free system the protein effectively quenched diphenyl picryl hydraxyl radical and superoxide radical, though it could not catalyse the breakdown of hydrogen peroxide. Post treatment with the protein for 3 days after 2 days of chloroform administration showed similar results. Histopathological studies indicated that chloroform induced extensive tissue damage was less severe in the mice livers treated with the 43 kD protein prior and post to the toxin administration. Results from all these data suggest that the protein possesses both preventive and curative role against chloroform induced hepatotoxicity and probably acts by an anti-oxidative defense mechanism.

Keywords: Antioxidant, Hepatotoxin, Hepatoprotection, Liver damage, Oxidative stress, 43 kD protein

Introduction

Herbal plants have been recently popularized in modern medicine, since many therapeutically important compounds are derived from them. Extracts prepared from these plants are used in the treatment of liver diseases like hepatitis, cirrhosis etc. *Cajanus indicus* is one such plant, which is used extensively in folklore medicine to treat hepatic disorders (Chopra *et al*., 1986). The aqueous extract of this plant is used to treat several forms of liver disorders, including jaundice and hepatomegaly (Kirtikar and Basu, 1935; Ghosh and Biswas, 1973). Despite of being aware of its beneficial actions on liver, the exact mode of action of this plant as a hepatoprotective agent is still not known. Moreover, therapies could not be devised due to insufficient standardization of the plant extract. In our laboratory, a 43 kD protein has been isolated and purified to homogeneity from the leaves of this herb and is believed to be its major hepatoprotective component. This protein has been found to be effective in reducing hepatotoxicity induced by toxins such as carbon tetrachloride, thioacetamide etc (Sarkar *et al*., 2005).

Among the various halogenated toxicants, chloroform (CHCl₃) is one of the most well known liver damaging agents (Kluwe *et al*., 1978; Smith *et al*., 1983). It finds its way into
our body through chlorine-contaminated drinking water. CHCl₃ acts as a central nervous system depressant and at large doses causes anæsthesia, analgesia and necrosis (Arena and Drew, 1986). The organs in our body that are worst affected are liver and kidney (Larson et al., 1993). The acute toxicity demonstrated by CHCl₃ is due to its bio-transformation to a nucleophilic bi-functional metabolite phosgene that reacts with glutathione to form diglutathionyl dithiocarbonate or is directly metabolized to carbodiöxide and chloride free radicals (Constan et al., 1999). Cytochrome P450-2E1 is the enzyme that catalyzes the conversion process. Phosgene, formed in the bio-transformation process of CHCl₃ binds covalently to tissue macromolecules resulting in impairment of cellular vital function. CHCl₃ like most other liver damaging agents, such as acectaminophen, alcohol or viral hepatitis release reactive oxygen species (ROS) at the site of injury (Stevens and Anders, 1981; Tomasi et al., 1985). At the molecular level, the stress caused by ROS is implicated by the activation of several inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1, arachidonic acid, metabolites such as thromboxane A₂, prostaglandins and leukotrienes (Ljungman et al., 1991). Since CHCl₃ toxicity is basically mediated by free radicals, therefore free radical scavengers such as dimethyl sulphoxide (DMSO) and amino-benzotriazole (ABT) apart from Vitamin-E and Vitamin-C have been in use in CHCl₃ insults (Achdoum, 1991).

Present study investigated the efficacy of the homogeneously prepared 43 kD protein against CHCl₃ induced liver damage in vivo. The hepatic damage caused by CHCl₃ and the reverse role played by the protein were evaluated by determining the i) levels of the enzymes maintaining liver integrity such as serum glutamate pyruvate transaminase (serum GPT) and alkaline phosphatase (ALP); ii) levels of the antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferase (GST) and iii) by measuring the level of in vivo and in vitro hepatic malondialdehyde (MDA). Moreover, direct antioxidant potential of this protein was determined from its ability to scavenge diphenyl dipicryl hydrazyl (DPPH) radical and superoxide (O₂⁻) radical and also by its ability to react with hydrogen peroxide (H₂O₂). In addition, histopathological studies were conducted from liver sections of a) normal mice, b) mice treated with CHCl₃, and c) treated with protein prior and post to CHCl₃ administration to assess the ultrastructural changes of the liver.

Materials and Methods

Materials. Cajanus indicus is a shrub belonging to the family leguminosae and sub family papilionacea. Fresh plant leaves were obtained from Bose institute Experimental farm.

Chemicals. Kits for the measurement of serum GPT and ALP were purchased from Span diagnostics Ltd., India. CHCl₃ was purchased from Sisco research laboratories (SRL), India. Beside this Sodium pyrophosphate, Collagenase type I, Dulbecco's modified eagle's medium (DMEM), Fetal bovine sera (FBS) and DPPH radical, purified catalase (from murine liver) and superoxide dismutase (from bovine liver) enzymes were made available from Sigma chemical Co. All other chemicals used in the study were of analytical grade of highest quality.

Animals. Swiss albino male mice of body wt 25 ± 2 g were used in the experiments. The animals were kept for two weeks prior to the experiment to acclimatize with the lab conditions. All of them had free access to standard diet and water ad libitum. The study was conducted in conformity with standard experimental animals study ethical protocols.

Preparation of homogeneous protein from the leaves of Cajanus indicus. The protein was purified from the leaves of young Cajanus indicus (manuscript submitted for publication). The leaves were homogenized in 20 mM tris-HCl buffer, pH 7.4 and the clear homogenate was saturated with 60% ammonium sulphate. The pellet was reconstituted and dialysed in Tris-HCl buffer, passed through DEAE Sephadex column and eluted in linear gradient of 0-1 M NaCl in tris buffer. The fraction showing hepatoprotective activity was desalted, concentrated and applied on a Sephadex G-50 column. The protein fraction showing biological activity was finally subjected to reverse phase chromatography on a C-18 hydrophobic column attached to HPLC. The molecular weight of this fraction was determined on a SDS polyacrylamide gel.

Protein estimation. Protein concentration was measured according to the method of Bradford (Bradford MM, 1976) using crystalline bovine serum albumin as standard.

Liver injury models. In order to compare the efficacy of the hepatoprotective protein in providing protection and cure against CHCl₃ induced liver damage, we divided the experimental mice broadly into two groups. The first group is the pre-treatment (protective) group and the second group is the post-treatment (curative) group.

Protective group. The pretreatment group was divided into 3 subgroups each consisted of six mice. The first one served as control mice. The second group of mice was fed orally with 0.75 ml/kg body wt for 5 days; mice in the 2nd group were fed orally with 0.75 ml/kg body wt of com oil for 5 days followed by 0.75 ml/kg body wt of CHCl₃ in com oil at 1:1 ratio for 2 days and the mice in the 3rd group were intraperitoneally injected with the protein at a dose of 2 mg/kg body wt for 5 days followed by oral feeding of CHCl₃ for two days. Mice groups were then sacrificed after 48 h of CHCl₃ administration since hepatic necrosis caused by CHCl₃ becomes visible as early as 10 h and reaches peak at 48 h (Lind et al., 2000). Blood and liver samples were collected separately for each animal. A positive control group was kept in which six mice were pretreated with 200 mg/kg body wt of Vitamin E for 5 days (Gokalp et al., 2003) followed by CHCl₃ treatment as in the protein treated models.

Curative group. It is known that liver can regenerate itself after two days of toxin injury (Thakore and Mehendale, 1994; Soni et al., 1999). To ascertain whether the protein can cause the healing of the liver faster than its natural regeneration, a time course study was...
conducted where six mice in each group were injected with the protein for 1, 2, 3 and 4 days after the administration of a single dose of the toxin for 2 days. Mice were sacrificed after 2nd, 3rd, 4th and 5th day respectively. In another set, six mice in each group were treated with the toxin only for 2 days and sacrificed after 2nd, 3rd, 4th and 5th day. In addition, six mice were kept as normal control.

Assessment of liver functions. Blood samples collected from puncturing mice heart were kept overnight to clot and then centrifuged at 3,000 x g for 10 min. Serum GPT was measured by 2,4-dinitro phenyl hydrazine (DNPH) method of Rietman and Frankel (Reitman and Frankel, 1957) and ALP was estimated by Kind and King’s method (Kind and King, 1954).

Determination of antioxidant activity in cell-free systems

Quenching of DPPH radical. The free radical activity of the protein was measured using the DPPH radical (Blois, 1958). The DPPH radical in ethanol (100 mM, 2 ml) was added to 2 ml of various concentrations (5-50 mg/ml) of the protein. The solution was then shaken vigorously and held for 30 min at room temperature in dark. The decrease in absorbance of DPPH was measured at 517 nm. Ethanol was used as the blank solution and DPPH solution in ethanol was used as control. Percent inhibition was calculated by comparing the absorbance values of the control and the protein.

Scavenger effect on H₂O₂. For the assay, different concentration of the protein (0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.05 mg/ml) was mixed separately with 700 ml, 5 mM H₂O₂ and incubated at 37°C. Disappearance of peroxide was observed at 240 nm for 10 min. 1 unit of CAT activity is that which reduces 1 µmole of H₂O₂ per minute. Purified catalase (50 µU/ml) was used as a standard.

Scavenger effect on O₂⁻ radical. Superoxide anion was generated in vitro as described by Paolitti (Paolitti et al., 1986). The assay mixture contained in a total volume of 1 ml, 100 mM triethanolamine-dithiothreitol buffer (pH 7.4), 3 mM NADH, 25 mM/12.5 mM EDTA/MnCl₂, 10 mM β-mercaptoethanol, the 43 kD protein of different concentrations (0.001 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.05 mg/ml). After 20 min incubation at 25°C, the decrease in absorbance was measured at 340 nm. Purified SOD (80 µU/ml) was used as a standard.

Estimation of lipid peroxidation products

In vivo (from liver homogenates). Lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation, was measured using the method of Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990). TBARS concentration of the samples were calculated using the extinction coefficient of MDA which is 1.36×10⁴ M⁻¹cm⁻¹ since 99% of TBARS exists as MDA.

In vitro (from hepatocytes). Hepatocytes were isolated from mice liver (Malhi et al., 2002) by the perfusion technique with collagenase type I at 37°C. The cells were suspended in DMEM containing FBS and the suspension was adjusted to obtain ~2×10⁶ cells/ml. About 1 ml of hepatocyte suspension (~2×10⁶ cells) was incubated with the protein in varying concentrations (0.001 mg/ml, 0.01 mg/ml and 0.1 mg/ml) for different sets of experiment for 30 min. CHCl₃ (10 mM) was then added and incubated for another 30 min. The normal control cells were kept in culture medium only. The toxin control was done by incubating the hepatocytes with CHCl₃ (10 mM) for 30 min. All the experiments were done in triplicates under the same conditions. For positive control, hepatocytes were incubated with α-tocopherol (Vitamin E at a concentration of 1 mg/ml) and then exposed to CHCl₃. Incubation was executed at 37°C with gentle shaking and at the end of the incubation period, hepatocyte suspensions were collected to assess the cell damage. Hepatocyte suspensions were centrifuged at 60 x g and membrane lipid peroxidation was measured from the precipitate by a colorimetric reaction with thiobarbituric acid (TBA) (Esterbauer and Cheeseman, 1990).

Determination of the levels of anti-oxidant enzymes in the liver homogenates

Estimation of CAT. The enzyme CAT converts hydrogen peroxide formed via the action of SOD on superoxide radical into water. The activity was measured in liver homogenates by the method of Bonaventura (Bonaventura et al., 1972).

Estimation of SOD. The activity of SOD was assayed following the method originally developed by Nishikimi (Nishikimi et al., 1972) and then modified by Kakkar (Kakkar et al., 1984).

Estimation of GST. GST catalyses the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. It was measured according to the method of Habig and Jakoby (Habig and Jakoby, 1974).

Liver histopathology. Liver specimens from all the experimental groups were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of about 5 µm thickness were stained with haematoxylin and eosin to study the general structure of the liver.

Statistical analysis. All the values are represented as mean ± S.D (n = 6). Student’s t-test was applied for detecting the significance of difference between groups. P values of 0.05 or less were considered significant.

Result

SDS-PAGE of the purified protein from the leaves of Cajanus indicus. Fig. 1 shows the SDS-PAGE pattern of the final step purified hepatoprotective protein from Cajanus indicus. The protein was purified to homogeneity by 5 different steps including homogenization, (NH₄)₂SO₄ precipitation, ion exchange chromatography, gel filtration and high performance liquid chromatography. A single symmetrical band appeared in the region of 43 kD, indicating the homogenous preparation of the protein. Present study was carried out using this purified protein.

To study the protective role (pretreatment) of the protein a dose-dependent study was carried out. The hepatoprotective protein was applied intraperitoneally at a dose of 150 µg/kg, 500 µg/kg, 1 mg/kg, 2 mg/kg and 2.5 mg/kg body wt prior to
chloroform administration. A concentration of 2 mg/kg body weight of the protein showed maximum hepatoprotective action as evidenced from its effect on the levels of serum GPT (Fig. 2) and antioxidant enzyme, CAT, as will be shown later. This concentration of the hepatoprotective protein has been used throughout the study.

As described previously (materials and methods) a time course study was followed with the post-treated mice. After receiving the toxin once for 2 days, the protein was administered for 1, 2, 3 and 4 days and sacrificed on 2nd, 3rd, 4th and 5th day respectively. Fig. 3 shows that maximum response was observed on serum GPT when the protein post treated mice were sacrificed on the fourth day. This is also true for liver CAT levels as will be shown later. Results for all the other studies were, therefore, presented for the fourth day.

Effect of the protein on serum GPT level. CHCl₃ treatment of the mice increased the serum GPT level (78 ± 2 units/ml serum) by approximately 2.3 fold than the normal level, (34 ± 2 units/ml of serum). Pretreatment of the protein for 5 days prior to CHCl₃ administration lowered the level to 43 ± 2 units/ml serum, while mice treated with Vitamin E effectively reduced the serum GPT level to 40 ± 2 units/ml serum. This observation is shown in the Fig. 2. As seen from the figure protein administration at a dose of 2 mg/kg body wt showed the best protective effect of the protein.

The curative effect of the protein on serum GPT level is shown in Fig. 3. After 48 hrs of CHCl₃ administration, the serum GPT value increases to 96 ± 3 units/ml serum from the normal level of 36 ± 2 units/ml serum. A single dose of protein after CHCl₃ administration had no effect on serum GPT level. Protein injected for 2 days after CHCl₃ administration reduced the serum GPT level to 75 ± 2 units/ml. Protein administration for 3 and 4 days after CHCl₃ administration
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significantly reduced the serum GPT to 40 ± 1 units/ml and 38 ± 1 units/ml respectively than their respective CHCl₃ controls, which were 55 ± 2 units/ml and 51 ± 2 units/ml of serum. Protein post-treatment of mice for 3 days and sacrificed on the 4th day had the most prominent curative effect on CHCl₃ toxicity.

Effect of the protein on ALP level. The ALP level in the serum of the normal control, CHCl₃ and protein treated prior to CHCl₃ intoxication are shown in the Fig. 4. ALP levels in the serum of the CHCl₃ treated mice increased to 58 ± 2 KA units/ml of serum indicating severe liver damage. This value was approximately 3.5 times of the normal value of 17 ± 1 KA units/ml serum. Protein pretreatment significantly reduced the level to 33 ± 1 KA units/ml serum, while Vitamin E treatment reduced the ALP value to 40 ± 1 KA units/ml serum (Pretreatment).

Post-treatment in Fig. 4 shows the ALP level in mice treated with the protein for 3 days after toxin treatment for 2 days. Best results were obtained when the protein was given for 3 days. Mice sacrificed on 4th day after protein post treatment for 3 days showed serum ALP level within the normal range of 22 ± 1 KA units/ml serum.

Effect of the protein on products of lipid peroxidation. Fig. 8 (Pretreatment) shows the in vivo level of MDA in the normal control, CHCl₃ treated, protein pretreated and Vitamin E fed mice. The increase in lipid peroxidation in CHCl₃ treated mice (68 ± 2 nmoles/gm liver tissue) was 2.4 fold over the control group (28 ± 1 nmoles/gm liver tissue). Mice pretreated with the protein for 5 days, with 2 mg/kg body wt of protein, showed significant reduction in the MDA level (24 ± 1 nmoles/gm tissue). The value of MDA obtained in the protein pretreated mice was even less than the normal controls.
Fig. 8 (Post-treatment) shows the effect of the protein on normal control, CHCl₃ treated and protein post treated mice. Mice treated with the toxin for 2 days showed MDA values of 68 ± 2 nmoles/gm tissue. Post treatment of mice with the protein after toxin treatment reduced the production of MDA to almost normal level of 37 ± 2 nmoles/gm tissue. This is the effect of the protein observed when the mice were treated with the toxin (once for 2 days) + protein administered for 3 days and sacrificed on the 4th day.

Fig. 9 shows the effect of the 43 kD protein on lipid peroxidation in hepatocytes. CHCl₃: hepatocytes exposed to CHCl₃ (10 mM) for 30 min. P1 + CHCl₃, P2 + CHCl₃ and P3 + CHCl₃: hepatocytes incubated with the protein (0.001 mg/ml, 0.01 mg/ml and 0.1 mg/ml concentration respectively) for 30 min and then exposed to CHCl₃ for another 30 min. Vit E + CHCl₃: hepatocytes first incubated with Vitamin E (1 mg/ml) and then with CHCl₃. Results have been shown as percent over control. Each value represents mean ± S.D. (*p < 0.05).

Fig. 8 (Post-treatment) shows the effect of the protein on normal control, CHCl₃ treated and protein post treated mice. Mice treated with the toxin for 2 days showed MDA values of 68 ± 2 nmoles/gm tissue. Post treatment of mice with the protein after toxin treatment reduced the production of MDA to almost normal level of 37 ± 2 nmoles/gm tissue. This is the effect of the protein observed when the mice were treated with the toxin (once for 2 days) + protein administered for 3 days and sacrificed on the 4th day.

Fig. 9 shows the effect of the protein on CHCl₃ induced lipid peroxidation in hepatocytes. Lipid peroxidation was increased significantly (110 %) in CHCl₃ treated hepatocytes over normal control cells indicating membrane damage. Protein treatment of (0.1 mg/ml) prior to CHCl₃ administration, significantly prevented that damage by reducing the MDA formation to a level even less than that of the control value.

Effect of the protein on CAT level. The dose dependent effect of the protein on CAT levels has been shown in Fig. 10. CAT activity in CHCl₃ treated mice was lower (40 ± 1 units/mg total protein) than that of normal (108 ± 3 units/mg total protein). Treatment of the mice with the protein prior to CHCl₃ treatment increased the CAT activity to 81 ± 3 units/mg total protein as compared to the CHCl₃ treated mice. The CAT activity in normal mice liver (109 ± 3 units/mg total protein) was reduced when treated with CHCl₃ (35 ± 1 units/mg total protein). As seen from the figure a dose of 2 mg/kg body wt of the protein showed the maximum hepatoprotective activity.
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Protein post-treatment for 1 and 2 days after chloroform application practically had no effect on the reduced CAT activity (Fig. 11). The CAT activity elevated to 84 ± 2 and 86 ± 4 units/mg total protein when the animals were post-treated with the protein for 3 and 4 days compared to their respective CHCl₃ controls (62 ± 2 and 76 ± 2 units/mg total protein). Fig. 11 shows that maximum effect of the protein was seen on levels of CAT when the protein post-treated mice were sacrificed on the fourth day.

**Effect of the protein on SOD level.** The normal mice showed a GST level of 17.0 ± 0.7 units/mg total protein. This was reduced by 4.0 fold in CHCl₃ treated mice (4.0 ± 0.1 units/mg total protein), while the level increased to 9.0 ± 0.1 units/mg total protein in mice treated with the protein prior to CHCl₃ feeding (Pretreatment of Fig. 13).

Post-treatment of Fig. 13 shows the effect of the protein on GST activity in liver upon its administration after CHCl₃ treatment for 2 days. After 3 days of protein treatment, the GST activity was elevated to almost normal level of 7.3 ± 0.1 units/mg total protein while its respective chloroform control showed a value of 4.0 ± 0.1 units/mg total protein.

**Effect of the protein on liver histopathology.** Liver samples were taken from normal, CHCl₃ treated and protein treated (both pre- and post-) mice. Histopathological assessment showed prominent changes including centrilobular necrosis, ballooning of hepatocytes, distribution of polymorphonuclear infiltrates surrounding the central vein, disorganization of normal radiating pattern of cell plates around central vein etc. in tissues treated with CHCl₃ (Fig. 14b). Qualitatively, the inflammation in protein treated mice liver before and after CHCl₃ administration were less severe compared to that of CHCl₃ (Fig. 14c and Fig. 14d respectively). These observations show the protective role of the 43 kD protein against CHCl₃ induced hepatic damage.
Discussion

It is well documented that exposure of living beings to any toxic metabolite constitutes stress which results in a surge of free radicals (Testai et al., 1995; Diez-Fernandez et al., 1996; Abraham et al., 1999). These free radicals can trigger cell damage through mechanisms of covalent binding to tissue macromolecules, enhanced lipid peroxidation and depleting stores of antioxidant enzymes. Reduced activity of one or more antioxidant enzymes leads to oxidative stress and damage to the liver (Kyle et al., 1987; Kurose et al., 1997). The preliminary effect of hepatotoxicity is the enhancement in the serum levels of GPT, serum glutamate oxaloacetate transaminase (serum GOT) and ALP, which are required for maintaining the integrity of the liver. When the hepatocyte membrane is damaged, these enzymes located normally in the cytosol, are released in the blood stream. Estimation of these enzymes in the serum is a useful quantitative marker for the extent of liver damage (Ansari et al., 1991).

The present study reveals the hepatoprotective activity of the herbal protein purified from *Cajanus indicus* against CHCl$_3$ toxicity. Mice treated with CHCl$_3$ showed elevated levels of the serum enzymes such as serum GPT and ALP. In cell free systems the protein shows an unique scavenging activity by reacting with DPPH which is a synthetic radical and also with superoxide radical. Moreover CHCl$_3$ treatment also produced significant elevation in the hepatic MDA level (in vivo and in vitro) and a decline in antioxidant enzymes CAT, SOD and GST. Administration of the protein at a dose of 2 mg/kg body wt prior and post to CHCl$_3$ treatment, normalized (reduced almost to the normal controls) the values of serum GPT, ALP and MDA formation both in vivo and in vitro and uplifted the level of CAT, SOD and GST. The DPPH radical, because of its odd electron gives a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in presence of a radical scavenger, its absorption vanishes and the resulting decolourisation is stoichiometric with respect to the number of electrons taken up. The 43 kD protein at various concentration was able to quench the synthetic DPPH radical. Having established a concentration based radical scavenging activity of the protein we then proceeded to investigate the ability of
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the protein to react with O₂⁻ and H₂O₂, both of which are potentially cytotoxic. Results showed that the protein could not react with H₂O₂ and potentiate its breakdown to water. However, it exhibited a SOD-like effect, inhibiting O₂⁻ formation in a dose-dependent manner.

As mentioned previously (Stevens and Anders, 1981) CHCl₃ damages the liver via production of ROS. Our results also suggest that treatment of mice with CHCl₃ caused an overall decrease in the levels of the antioxidant enzymes such as CAT, SOD and GST. CAT and SOD belong to preventive and chain breaking class of antioxidants respectively. Both these enzymes work in a coordinated manner to quench the oxygen free radicals produced in our body. Decreased levels of CAT and SOD suggest once again that CHCl₃ exerts its toxic effect by causing oxidative stress. The antioxidant defense system in our body cannot cope up with the rate of generation of these free radicals, when their levels increase beyond the optimum range. As a result, these antioxidant enzymes get used up and their content falls below the normal level. However, treatment of mice with the protein by intraperitoneal means bring back the levels of CAT and SOD to near normal levels. This may happen due to two reasons. The protein may act directly and scavenge the ROS derived by oxidation-reduction cycle within the cell or it may work in unison with the existing antioxidant enzymes and help prevent their loss during the oxidative injury caused by CHCl₃.

GST is an enzyme that participates in the detoxification process due to conjugation reaction between GSH and xenobiotics (Adang et al., 1990). It plays a critical role in protection against reactive free radicals and products of oxidative stress (Hayes and Pulford, 1995). As seen with CAT and SOD, GST activity was also reduced in mice treated with CHCl₃ indicating the use of the enzyme in combating ROS formed in an oxidative process. This 43 kD protein increased the level of GST, again suggesting its antioxidant role.

Apart from generation of ROS at the site of injury, CHCl₃ also acts as a direct hepatotoxin by disrupting tissue membranes. The commonly used indicator of tissue damage is the peroxidation of lipids on the membrane of liver in the form of aldehydic products such as MDA. Ability of CHCl₃ to cause free radical damage was indicated by the production of increased hepatic lipid peroxides measured as MDA in CHCl₃.

Fig. 14. (a) Histopathology of normal liver section stained with haematoxyline-eosin stain. CV indicates the central vein. (b) Histopathology of liver treated with CHCl₃ for 2 days and sacrificed on the 3rd day. (c) Histopathology of liver treated with the protein for 5 days and then treated with CHCl₃ for 2 days. (d) Histopathology of liver treated with CHCl₃ for 2 days and then treated with the protein for 3 days.
induced hepatotoxicity. This finding is similar to the findings as reported by Masuda [Masuda et al., 1980] that halomethanes like CHCl₃ or CCl₄ increased the levels of MDA content in liver. However, pretreatment as well as post treatment of mice in vivo with the protein mitigated this CHCl₃ induced lipid peroxidation. Treatment of mice with the protein, prior to chloroform application had a profound effect on lipid peroxidation products as it lowered the MDA level even below the normal range. Extent of lipid peroxidation in CHCl₃ treated hepatocytes also showed a similar reduction in MDA value, upon protein treatment. Result suggests that the protein plays a positive role in minimizing lipid peroxidation and act through an antioxidant mechanism.

Liver histology of CHCl₃ treated mice showed severe necrosis along the central vein. The sizes of the hepatocytes were also larger and balloon like compared to the normal mice liver. The necrosis was mostly centrilobular and extending through the whole liver lobule. On the other hand, the inflammations in the livers of both protein pre- and post-treated mice were less necrotic. These results again show the protective nature of the protein against the hepatic damage caused by CHCl₃.

In most studies on xenobiotic induced hepatotoxicity, the protecive agent has been shown to be powerful to protect the liver when administered prior to the toxicant. Our study however reveals that the protein has got equal potential as a hepatoprotective agent in both pretreated (preventive) and post treated (curative) mice. Time course study of the protein as a curative agent showed that the efficacy of the protein increased proportionally with the number of days, with the peak effect reaching on the 4th day. Thus the results showed that post treatment of the mouse treated mice with the protein showed an additive effect and it actually accelerates the self-healing process of the liver. In the time course study when the protein was initially administered for 1 and 2 days, subtle changes were observed relative to the CHCl₃ control mice in the levels of serum GPT and ALP, but there were practically no changes in the levels of the antioxidant enzymes. This finding shows that the protein may not be able to induce antioxidant mechanism of the liver quickly (when the protein is administered for 2 days only) as it does so with the liver specific serum marker enzymes. This fact is also revealed from another study done in our laboratory by taking thioacetamide as the toxic (Sarkar et al., 2005).

In conclusion, we would like to mention the probable mechanism by which the protein exerts its hepatoprotecive action against CHCl₃ induced toxicity could be attributed to the enhanced supply of antioxidant enzymes or by the acceleration of detoxification and excretion. Taken together, the results of this present study demonstrate that the protein might have a therapeutic role in preventing CHCl₃ induced hepatotoxicity possibly through its free radical scavenging properties. The results encourage further in vitro and in vivo studies to elucidate its efficacy as a hepatoprotective molecule.

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References


Hayes, J. D. and Pullford, D. J. (1995) The glutathione S
transferrin supergene family: Regulation of glutathione S
transferrin and the contribution of the isoenzymes to cancer
chemoprotection and drug resistance. Critical Reviews
spectrophotometric assay of superoxide dismutase. Ind. J.
Biochem. Biophys. 21, 130-132.
Kind, P. R. N. and King, E. J. (1954) Estimation of plasma
phosphate by determination of hydrolyzed phenol with anti-
Selective modification of the renal and the hepatic toxicities of
chloroform by induction of drug-metabolizing enzyme systems
Kurose, I., Higuchi, H., Miwa, S., Saito, H., Watanabe, N.,
Hokari, T., Hirotawa, M., Takahashi, M., Zeki, S., Nakamura, T.,
mediated apoptosis of hepatocytes exposed to acute ethanol
Superoxide dismutase and catalase protect cultured hepatocytes
from the cytotoxicity of acetaminophen. Biochem. Biophys.
Res. Commun. 149, 889-896.
hepatoxic and nephrotoxic effects of chloroform in male F-
344 rats and female B6C3F1 mice. Fundam. Appl. Toxicol. 20,
302-315.
Hepatoprotection by dimethyl sulfoxide. III. Role of inhibition
of the bioactivation and covalent bonding of chloroform.
Ljungman, A. G, Grum, C. M., Deeb, G. M., Bolling, S. F. and
Morgannoth, M. L. (1991) Inhibition of cytochrome
metabolite production attenuates ischemia-reperfusion lung
Isolation of human progenitor liver epithelial cells with
extensive replication capacity and differentiation into mature
on the hepatoxic action of chloroform and relaxed
halogenomethanes in normal and Phenobarbital pretreated
Nishi, M., Rao, N. A. and Yagi, K. (1972) The occurrence of
superoxide anion in the reaction of reduced phenazine
Commun. 46, 849-854.
sensitive spectrophotometric method for the determination of
154, 536-541.
the determination of serum glutamic oxalacetic and glutamic
curative role of a 43 kD protein from the leaves of the herb
Cajanus indicus on thioacetamide induced hepatotoxicity in
Mechanism of chloroform nephrotoxicity. I. Time course of
chloroform toxicity in male and female mice. Toxicol. Appl.
Pharmacol. 70, 467-479.
Sonni, M. G, Raniah, S. K., Muttat, M. M., Clewell, H. and
Mehendale, H. M. (1999) Toxicant inflected injury and
stimulated tissue repair are opposing toxicodynamic forces in
diethyl maleate and Phenobarbital treatments on the
Interact. 37, 207-215.
Testai, E, Di Marzio, S., di Domenico, A., Piccardi, A. and
Thakore, K. N. and Mehendale, H. M. (1994) Effect of
Phenobarbital and mirex pretreatments on carbon tetrachloride
Tomasi, A., Albano, E., Biasi, F., Slater, T. F., Vannini, V. and
Dianazini, M. U. (1985) Activation of chloroform and related
trihalomethanes to free radical intermediates in isolated
hepatocytes and in the rat in vivo as detected by the ESR-spin