A Protein from *Cajanus indicus* Spreng Protects Liver and Kidney against Mercuric Chloride-Induced Oxidative Stress

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Mercuric chloride (HgCl_2) is a widespread environmental toxin that affects mainly liver and kidney. The present study has been carried out to investigate the protective action of a protein (the CI protein) isolated from the herb, *Cajanus indicus* Spreng against HgCl_2 induced renal and hepatic toxicities in mice. Intraperitoneal administration of HgCl_2 at a dose of 5 mg/kg body weight for 1 d significantly reduced the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Moreover, it also depleted the glutathione to oxidized glutathione (GSH/GSSG) ratio. In addition, HgCl_2 increased the activities of serum marker enzymes (namely, glutamate pyruvate transaminase, GPT and alkaline phosphatase, ALP), creatinine, blood urea nitrogen and serum tumor necrosis factor alpha ($\text{TNF-}\alpha$) level along with hepatic and renal lipid peroxidation. Besides, application of HgCl_2 to hepatocytes increased reactive oxygen species production and reduced the total antioxidant activity of the treated hepatocytes. Treatment with the CI protein intraperitoneally at a dose of 2 mg/kg body weight before or after HgCl_2 administration showed that it could scavenge free radicals *in vitro* and protect the alterations of the antioxidant molecules and the other parameters used in this particular study. Histological studies also revealed a milder lesion in kidney and liver samples of the CI protein treated mice compared to mice treated with HgCl_2 alone. Effects of a known antioxidant *N*-acetylcysteine have been used to compare its action to that of the CI protein.

Key words mercuric chloride; oxidative stress; hepato-renal disorder; *Cajanus indicus*; CI protein; tissue protection

There is growing evidence that heavy metals, in general, and mercurial compounds, in particular, are toxic to humans. Large populations are currently exposed to low levels of mercury owing to the use of mercury (Hg) pesticides in agriculture or as components of batteries in fluorescent light bulbs. Hg exists in a wide variety of physical and chemical states, each of which has unique characteristics for target organ specificity. Exposure to Hg vapor and to organic Hg compounds specifically affects the central nervous system, while the kidney, liver and gastrointestinal tract is the target organ for inorganic Hg compounds.

Reactive oxygen species (ROS) have been involved as mediators in ischemic, toxin and immune mediated tissue damages.^{1–3)} Stacey and Kappas⁴⁾ reported the role of oxidative stress in mercury toxicity. Their view was also supported by the findings of Woods *et al.*⁵⁾ that mercuric chloride (HgCl_2) imposes a loss of antioxidant potential in terms of glutathione (GSH) and glutathione peroxidase (GPx) and promotes free radical formation *via* thiol complexation. The increase in ROS generation is also accompanied by a decrease in the antioxidant enzymes catalase and glutathione peroxidase. Besides, lipid peroxidation has also been proposed to be occurring in hepatic and renal tissues following parenteral administration of HgCl_2 .^{6,7)} Hg(II) can also inactivate a number of enzymes by blocking the functional sites through binding to sulfhydryl groups, which are part of catalytic or binding domains.⁸⁾ Covalent binding to sulfhydryl groups by Hg(II) alters protein conformation, creates protein adducts through modification of side chains and finally leads to changes in protein shape and activity.⁹⁾

Nevertheless, the evidence for pathogenic role of HgCl_2 induced oxidative stress is confusing as some studies do not point to increased peroxide formation in kidneys.^{10,11)} Moreover, administration of several antioxidants, too, failed to provide protection in HgCl_2 -induced increase in renal lipid

peroxidation.¹²⁾

A 43 kDa protein (the CI protein) molecule¹³⁾ was isolated and purified in our laboratory from the leaves of *Cajanus indicus* Spreng, a popular herbal plant in several tropical countries. In India the traditional uses of *Cajanus indicus* Spreng are believed to be in treatment for liver disorders, jaundice, hepatomegaly diabetes, hypoglycemia and cardiovascular disease.^{14,15)} It has been found to be effective in reducing hepatotoxicity induced by toxins such as fluoride, cadmium chloride, chloroform, thioacetamide, *etc.*, both *in vivo* and *in vitro*.^{16–22)}

N-Acetylcysteine (NAC) can act as a source of thiols, stimulate GSH synthesis, enhance GPx activity, promote detoxification, and act directly on reactive oxidant radicals. Evidence indicates that NAC is useful as a chelating agent in the therapy of acute heavy metal poisoning.

In the present study we, therefore, set our aim to compare the protective effects of the CI protein with that of NAC against HgCl_2 induced acute hepatic and renal tissue toxicity. For this purpose, HgCl_2 -induced hepatotoxicity was evaluated by determining the level of serum marker enzymes, glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP). Renal dysfunctions in terms of creatinine and blood urea nitrogen (BUN) were also measured from the serum in all the experimental mice groups. In addition, tumor necrosis factor-alpha ($\text{TNF-}\alpha$) was measured from the serum of treated mice groups. Intracellular ROS production and total antioxidant power was also measured from HgCl_2 and HgCl_2 +CI protein treated hepatocytes. The activities of endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were measured from liver and kidney tissue homogenates. Further, the extent of lipid peroxidation and glutathione to oxidized glutathione (GSH/GSSG) ratio were also estimated from hepatic and renal samples. To assess the ultra

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structural changes of the liver and kidney, histological studies were conducted from the liver and kidney sections of normal mice, mice treated with HgCl_2 alone, and mice treated with the CI protein both prior and post to HgCl_2 administration. Effect of a non relevant protein, bovine serum albumin (BSA), on the HgCl_2 -induced hepatic and renal toxicity was also included in the study.

MATERIALS AND METHODS

Chemicals Kits for the measurement of serum GPT, ALP, BUN and creatinine were purchased from Span diagnostics Ltd., India. Sodium pyrophosphates, Bradford reagent, bovine sera albumin (BSA), 2',7'-dichlorodihydro fluorescein di-acetate (H_2DCFDA) were made available from Sigma Chemical Company (St. Louis, MO, U.S.A.). HgCl_2 and all other chemicals used in the study were obtained from Sisco research laboratory, India.

Animals Swiss albino male mice of body weight 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* Spreng The CI protein was purified from the leaves of young *Cajanus indicus* Spreng plants.¹³⁾ Briefly, fresh young leaves were homogenized in 20 mM Tris-HCl buffer, pH 7.4 and were saturated with 60% ammonium sulphate. The pellet was reconstituted and dialysed in Tris-HCl buffer, pH 7.4; passed through DEAE Sephadex column and eluted using a linear gradient of 0–1 M NaCl in Tris-HCl buffer, pH 7.4. The fraction showing hepatoprotective activity was desalted, concentrated and applied on a Sephadex G-50 column. The protein fraction showing maximum biological activity was finally subjected to a reverse phase chromatography on a C-18 hydrophobic column attached to HPLC. Homogeneity of preparation and the molecular weight of the protein were determined using SDS polyacrylamide gel electrophoresis.

Protein Estimation Protein concentration was measured according to the method of Bradford²³⁾ using crystalline bovine serum albumin (BSA) as standard.

Experimental Design Swiss albino male mice were divided into eight groups, each consisting of 6 animals.

Mice were intraperitoneally injected with:

- i) 0.9% NaCl for the Control (C) group,
- ii) a single dose of 5 mg/kg mercuric chloride (HgCl_2) for a day and decapitated after 1, 2 and 3 d-Hg group; (this dosage has been determined from previous studies)²⁴⁾
- iii) the CI protein at a dose of 2 mg/kg body weight for 3 d before HgCl_2 application for a day and decapitated after 1, 2, and 3 d for the protein-Hg group
- iv) the CI protein at a dose of 2 mg/kg, for 1, 2 and 3 d respectively after HgCl_2 administration for a day and sacrificed after 24 h from the last treatment.
- v) *N*-acetylcysteine at a dose of 150 mg/kg for 3 d before HgCl_2 injection for a day and sacrificed after 1, 2, and 3 d for NAC-Hg group
- vi) *N*-acetylcysteine at a dose of 150 mg/kg, for 3 d after

HgCl_2 injection for a day and sacrificed after 1, 2, and 3 d for Hg-NAC group

- vii) mice were treated with 2 mg/kg body weight of BSA before HgCl_2 injection for a day and sacrificed after 1, 2, and 3 d, in the same way as that of the protein for BSA-Hg group
- viii) mice were treated with 2 mg/kg body weight of BSA after HgCl_2 injection for a day and sacrificed after 1, 2, and 3 d, in the same way as that of the protein for Hg-BSA group

Assessment of Liver and Kidney Functions Blood samples collected from puncturing mice heart were kept overnight to clot and then centrifuged at 3000 *g* for 10 min. Serum GPT was measured by 2,4-DNPH method of Reitman and Frankel,²⁵⁾ ALP was estimated by Kind and King's method.²⁶⁾ Blood urea nitrogen (BUN) was determined spectrophotometrically from serum samples using the method of diacetylmonooxime, DAM.^{27,28)} Creatinine was measured according to the method of Bonsnes and Taussky.²⁹⁾

Measurement of Serum TNF- α TNF- α levels were determined from sera of mice treated 5 mg/kg body weight of HgCl_2 alone or after the protein treatment (2 mg/kg body weight) using sandwich ELISA.³⁰⁾ Rat anti-mouse TNF- α monoclonal antibody (mAb) (IMGEX, India) was diluted to 2 mg/ml in 0.1 M NaHCO_3 , pH 8.2. Wells of a 96 flat-bottomed probind assay plate (Becton Dickinson) were coated with 50 mg/well of the capture antibody and incubated overnight at 4 °C. The plates were washed 2 times in 350 ml of PBS Tween-20. Three hundred fifty milliliters of blocking agent (1 $\times$ PBS-Tween 20 with 5% BSA) was added per well and incubated for 2 h at room temperature and washed 2 times. Standard recombinant mouse TNF- α (Genzyme) diluted serially in the blocking agent to generate a standard curve and samples were added at the rate of 100 ml/well in triplicate and incubated at 4 °C overnight and washed 4 times. The serum (sample) concentration used in the assay was 10 ng/well. Biotinylated anti-mouse TNF- α clone mp6-XT3 diluted to 1 ml/ml in the blocking agent was added and washed 6 times. Then 4×10^3 /ml of the peroxidase-conjugated streptavidin diluted 1:3 v/v in the blocking agent was added at 100 ml/well, incubated for 30 min at room temperature and washed 8 times. Finally 20 mg of a substrate, phenylene diamine, in 25 ml of 0.1 M citric acid, 25 ml of 0.2 M $\text{NaH}_2\text{PO}_4/2\text{H}_2\text{O}$ and 1 ml of 30% H_2O_2 was added at the rate of 100 ml/well and the color was allowed to develop at room temperature for 90 min. The color reaction was stopped using 3 N H_2SO_4 and read at OD 450 nm using a microplate reader. TNF- α values were calculated from the standard curve as mean $\pm$ S.D. The sensitivity of the assay was 50 pg/ml.

Hepatocyte Isolation Hepatocytes were isolated from mice liver¹⁹⁾ 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 *ca.* 2×10^6 cells/ml. About 1 ml of hepatocyte suspension (*ca.* 2×10^6 cells) was incubated with HgCl_2 (0.5 μM) alone or in combination with the CI protein in varying concentrations (0.05, 0.1, 0.5, and 1) for different sets of experiment for different time intervals.

Cell Viability Assessment Briefly at 2 h after treatment with varying concentrations of the CI protein and HgCl_2 (0.5 μM), the media were removed and cell viability was de-

terminated using the MTT assay as described elsewhere.³¹⁾

Measurement of Intracellular ROS Production Cells were loaded either with HgCl₂ (0.5 μ M) alone or with HgCl₂ and the CI protein (0.5 mg/ml) for 2, 4, 8 h and at the end of the incubation period they were incubated again with 20 μ M DCFDA for 30 min at 37 °C and intracellular ROS production was detected using the fluorescent intensity of the oxidant sensitive probe H₂DCFDA.³²⁾

Assay of Antioxidant Power of Hepatocytes: Ferric Reducing/Antioxidant Power (FRAP) Assay Briefly, 50 μ l (nearly 1×10^5 cells) of normal as well as experimental hepatocytes were treated with HgCl₂ (0.5 μ M) alone or with HgCl₂ and the CI protein (0.5 mg/ml) for 2, 4, 8 h. The cell suspension was added to 1.5 ml freshly prepared and pre-warmed (37 °C) FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM tripridyltriazine, (TPTZ) in 40 mM HCl and 20 mM FeCl₃·6H₂O in the ratio of 10 : 1 : 1) and incubated at 37 °C for 10 min. The absorbance of the sample was read against the reagent blank (1.5 ml FRAP reagent + 50 μ l distilled water) at 593 nm.³³⁾

Estimation of Lipid Peroxidation Product from Liver and Kidney Homogenates Lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation was measured using the method of Esterbauer and Cheeseman.³⁴⁾ Experimental samples containing 1 mg protein each were mixed with 1 ml TCA (20%), 2 ml TBA (0.67%) and heated for 1 h at 100 °C. After cooling, the precipitate was removed by centrifugation. The absorbance of each sample was measured at 535 nm against a blank containing all the reagents except the sample. TBARS concentration of the liver and kidney samples were calculated using the extinction coefficient of MDA which is $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ since 99% of TBARS exists as MDA.

Assay of Anti-oxidant Enzymes The CAT activity was measured in various liver and kidney homogenates following the method of Bonaventura.³⁵⁾ About 5 μ g protein from each of the liver and kidney homogenates were mixed with 2.1 ml of 7.5 mM H₂O₂ and time scan was performed for 10 min at 240 nm and the temperature, 25 °C. The disappearance of peroxide depending on the CAT activity was observed. One unit of CAT activity is defined as the amount of enzyme, which reduces 1 μ mol of H₂O₂ per minute.

The SOD activity was assayed following the method originally developed by Nishikimi *et al.*³⁶⁾ and then modified by Kakkar *et al.*³⁷⁾ About 5 μ g protein of liver and kidney homogenates were mixed with sodium pyrophosphate buffer, Phenazine methosulphate (PMT) and Nitro Blue tetrazolium (NBT). The reaction was started by the addition of NADH. Then the reaction mixture was incubated at 30 °C for 90 s and stopped by the addition of 1 ml of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm. One unit of SOD activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in 1 min under the assay condition.

In all the experimental samples, the GPx activity were measured according to the method of Flohe and Ginzler³⁸⁾ using H₂O₂ and NADPH as substrates. The conversion of NADPH to NADP⁺ was observed by recording the changes in absorption intensity at 340 nm and one unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μ mol NADPH per minute.

Estimation of GSH and GSSG The glutathione status was assessed by the method of Hissin and Hilf.³⁹⁾ This method is based on the reaction of *O*-phthaldehyde (OPT) as a fluorescent reagent with reduced glutathione (GSH) at pH 8.0 and oxidized glutathione (GSSG) at pH 12.0, which involves excitation at 350 nm and fluorescence at 420 nm. For the GSSG estimation, GSSG was complexed to *N*-ethylmaleimide (NEM) to prevent interference of GSH with the measurement of GSSG. The values were expressed as nmol/mg protein and the ratio of GSH/GSSG has been determined.

Liver and Kidney Histopathology Liver and kidney specimens from the normal, HgCl₂-treated, the CI protein+Hg-treated and Hg+the CI protein-treated mice were fixed in 10% buffered formalin (10 ml of 40% formaldehyde, 0.4 g NaH₂PO₄·2H₂O, 0.65 g Na₂HPO₄ and 90 ml distilled water, pH 7.4) and were processed for paraffin sectioning. Sections of about 5 μ m thickness were stained with haematoxylin and eosin to study the structural changes of the liver and kidney.

Statistical Analysis All the values are represented as mean $\pm$ S.D. ($n=6$). Statistical comparison between groups were analysed by ANOVA and *post hoc* comparisons were done with Bonferroni corrections. *p* values of 0.05 or less were considered significant.

RESULTS

Effect of the CI Protein on Serum GPT, ALP, Creatinine and BUN Level Parenteral administration of HgCl₂ increased the level of serum GPT, ALP, urea (BUN) and creatinine levels in serum while administration of the CI protein before and after HgCl₂ application lowered the levels of ALP, BUN and creatinine, respectively (Table 1).

Effect of the CI Protein on Serum TNF- α Level TNF- α concentration in serum following HgCl₂ treatment increased rapidly with time. At 24 h the concentration reaches to 58 ± 2.3 pg/ml serum (Fig. 1), which further increases to a peak value of 70 ± 3.4 pg/ml serum by 48 h. However, by 72 h the concentration is slightly lowered but is much higher than the basal level. Pretreatment of mice with the CI protein (2 mg/kg body weight) for 3 d following HgCl₂ administration prevented the increase in serum TNF- α .

Effect of the CI Protein on Cell Viability Figure 2 shows that as the CI protein concentration is increased (from 0.05 to 1.0 mg/ml), there is a corresponding dose dependent increase in viability of cells treated with HgCl₂. While hepatocytes treated with HgCl₂ alone had a viability of only 31%, CI protein treatment gradually increased the viability and 94% hepatocytes were viable when treated with 1 mg/ml protein. However, after increasing the CI protein concentration from 0.5 to 1.0 mg/ml there was not much significant increase in viability of hepatocytes. Therefore the CI protein concentration of 0.5 mg/ml has been used for measuring ROS formation and FRAPS studies.

Effect of the CI Protein on ROS Production HgCl₂ treatment increases the fluorescence intensity of the dye DCFDA in treated hepatocytes by 1.8 fold after 2 h of treatment and it increases it further by 2.4 fold after 8 h of treatment, compared to control hepatocytes (Fig. 3) indicating increased ROS formation in HgCl₂-treated hepatocytes. How-

Table 1. Effect of the CI Protein Pre- and Post-treatments on GPT, ALP, BUN and Creatinine Level in Blood Serum against HgCl_2 Intoxication (5 mg/kg Body Weight)

Pre-treatment

Serum parameters	N	Hg ₁	Hg ₂	Hg ₃	P+Hg ₁	P+Hg ₂	P+Hg ₃	NAC ₃ +Hg	BSA ₃ +Hg
GPT (IU/l)	32±1.7	84±4.3*	80±4.2*	77±3.9*	79±4.0	63±3.3	40±1.9**	41±1***	82±4.3
ALP (KAunits/ml serum)	15±1.0	36±1.7*	34±1.8*	30±1.7*	31±1.7	23±1.3	19±1.1**	18±0.9***	33±1.8
BUN (Urea/100 ml serum)	17±0.8	41±2.3*	38±1.9*	35±1.7*	37±1.9	26±1.5	19±0.9**	17±0.9***	38±2.0
Creatinine (mg/l serum)	3.0±0.3	9.4±0.9*	9.0±0.8*	8.1±0.6*	8.0±1.0	5.0±0.7	4.0±0.6**	5.0±0.4***	7.0±0.5

Post-treatment

Serum parameters	N	Hg ₁	Hg ₂	Hg ₃	Hg+P ₁	Hg+P ₂	Hg+P ₃	Hg+NAC ₃	Hg+BSA ₃
GPT (IU/l)	32±1.7	84±4.4*	80±4.3*	77±3.9*	80±4.1	68±3.5	50±1.0**	37±1.9***	87±4.4
ALP (KAunits/ml serum)	15±1.0	36±1.7*	34±1.8*	30±1.7*	30±1.3	25±1.3	18±0.9**	18±1.1***	32±1.7
BUN (Urea/100 ml serum)	17±0.9	41±2.2*	38±1.9*	35±1.7*	38±2.0	25±1.4	18±1.0**	19±1.1***	40±2.0
Creatinine (mg/l serum)	3.0±0.3	9.4±1*	9.0±0.9*	8.1±0.6*	9.0±0.9	7.0±0.5	4.0±0.5**	5.0±0.2***	8.0±1.0

N: level in normal-control mice, Hg₁, Hg₂, Hg₃: levels in HgCl_2 treated mice and sacrificed after 1, 2, 3 d respectively. P+Hg₁, P+Hg₂, P+Hg₃: levels in which the CI protein was given at a dose 2 mg/kg body weight for 3 d prior to HgCl_2 administration and sacrificed after 1, 2, 3 d respectively. NAC₃+Hg: levels in which NAC was given at a dose 150 mg/kg body weight for 3 d prior to HgCl_2 administration. BSA₃+Hg: levels in which BSA was given at a dose 2 mg/kg body weight for 3 d prior to HgCl_2 administration. Hg+P₁, Hg+P₂, Hg+P₃: levels in which the CI protein was given at a dose of 2 mg/kg body weight for 1, 2, 3 d respectively after HgCl_2 administration for a day and sacrificed a day after the last treatment. Hg+NAC₃: levels in which NAC was given at a dose 150 mg/kg body weight for 3 d after HgCl_2 administration. Hg+BSA₃: levels in which BSA was given at a dose 150 mg/kg body weight for 3 d after HgCl_2 administration. Data represents mean±S.D., n=6 (*p, **p and ***p<0.05). *p indicates statistically significant difference of the values of serum markers of HgCl_2 -treated mice with respect to normal control; **p indicates the same with respect to the values of serum markers of the CI protein pre- and post-treated mice respectively with respect to HgCl_2 -treated mice and ***p indicates the same for the values of serum markers of NAC treated mice with respect to HgCl_2 -treated mice.

Fig. 1. Serum Levels of TNF- α Following Intraperitoneal Administration of 5 mg/kg Body Weight of HgCl_2 (Closed Squares) or Pretreated with 2 mg/kg of the CI Protein for 3 d before HgCl_2 Administration (Closed Circles)

Each value represents mean±S.E. for three different experiments performed in triplicates.

Fig. 2. Dose Dependent Effect of the CI Protein on HgCl_2 (5 μM) Induced Loss of Cell Viability for 2 h

Each value represents mean±S.E. for three different experiments performed in triplicates. ^ap indicates statistically significant difference of cell viability of HgCl_2 -treated mice with respect to normal control; ^bp indicates statistically significant difference of cell viability of the CI protein-treated mice with respect to HgCl_2 treated mice.

Fig. 3. Time Dependent Effect of the CI Protein on HgCl_2 Induced Generation of ROS for 6 h

CON: ROS generation in untreated cell. Hg₁, Hg₂, Hg₃: ROS generation in HgCl_2 treated cells at a concentration of 5 μM of HgCl_2 for 2, 4, 8 h respectively. P+Hg₁, P+Hg₂, P+Hg₃: ROS generation in the CI protein treated (0.5 mg/ml of protein) hepatocytes along with 5 μM of HgCl_2 for 2, 4, 8 h respectively. Each column represents mean±S.D., n=6 (^ap, ^b₁p, ^b₂p<0.05). ^ap indicates statistically significant difference of ROS generation in HgCl_2 -treated mice with respect to normal control; ^b₁p, ^b₂p indicates the same with respect to the ROS generation of the CI protein co-treated mice along with HgCl_2 for 2, 4, 8 h respectively with respect to their respective HgCl_2 -treated hepatocytes alone.

ever, hepatocytes cotreated with the CI protein and HgCl_2 reduced the HgCl_2 induced increase in fluorescence intensity of DCFDA showing reduced generation of ROS.

Effect of the CI Protein on FRAP HgCl_2 caused a significant diminution in FRAP value in a time dependent manner in isolated hepatocytes compared to hepatocytes kept in medium only (Fig. 4). A gradual reduction in FRAP value was observed with increase in time and a minimum value was obtained after 4 h of HgCl_2 treatment. After 8 h of HgCl_2 treatment the FRAP values slightly increase, though it was much less compared to control. However, the antioxidant power of hepatocytes measured as FRAP value increased significantly at all time points upon co-incubation with the CI protein along with HgCl_2 .

Effect of the CI Protein on Products of Lipid Peroxida-

tion After administration of HgCl_2 , enhancement of lipid peroxidation in the liver and kidney was observed. The CI protein treatment before and after HgCl_2 application protected the tissues against the increase in MDA levels and brought them back to the control values (Figs. 5A, B, respectively). The protective effects of the CI protein and NAC seemed to be similar.

Effect on Antioxidant Enzymes No significant alteration in CAT was observed in livers of HgCl_2 , the CI protein treated prior or post to HgCl_2 treatment or in NAC treated groups of mice (Fig. 6A). However, the activity of CAT was significantly decreased in the kidney tissue of HgCl_2 treated mice (Fig. 6B) and maximum reduction was observed after 2 d of treatment. Thereafter CAT activity slightly increased after 3 d of treatment indicating natural recovery. However, the CI protein pre-treatment arrested this reduction (Fig. 6B) significantly, while the same effect was not that prominent in

protein post-treatment (Fig. 6B). On the other hand NAC treatments both before and after HgCl_2 addition increased the CAT activities in kidney.

Treatment with mercury resulted in significant reduction in SOD values of liver and kidney of mercury treated mice. The CI protein pre-treatment resulted in enhanced SOD activity in the tissues examined. In liver tissues the extent of increase on protein pre-treatment was slightly more compared to normal control (Fig. 6C). In the kidney of the CI protein-pre-treated group, significant restoration was observed after the 3rd day of treatment (Fig. 6D). In case of the CI protein post-treatment the recovery was almost close to the normal in liver (Fig. 6C) while in kidney although there was an increase in SOD activity, the level remained low compared to normal (Fig. 6D). NAC treatments both before and after HgCl_2 addition, however, increased the SOD activities in both liver and kidney significantly.

GPx activity in liver was significantly lowered in mercury treated mice (Fig. 6E). In kidney samples though there was a reduction in GPx activity upon HgCl_2 administration it was not significant compared to normal control (Fig. 6F). Application of the CI protein to mice before or after HgCl_2 application significantly restored the GPx activity in liver after the 3rd day of treatment, (Fig. 6E) while in kidney there was only a slight increase in activity on protein pre-treatment or post-treatment (Fig. 6F) groups. NAC treatments both before and after HgCl_2 addition had significant effect in increasing the SOD activities in both liver and kidney.

Effect of the CI Protein on GSH/GSSG Ratio HgCl_2 treatment decreased the GSH/GSSG ratio significantly in the mice liver and kidney homogenates. The CI protein pre or post-treatment did increase the GSH/GSSG ratio in liver (Fig. 7A) and kidney (Fig. 7B) tissues after the 3rd day of treatment, though the levels in the liver tissue were not as prominent as observed in the kidney tissue. However, after treatment with NAC the GSH/GSSG ratio in both liver and kidney were very close to the control and significantly higher than the Hg(II) group.

Fig. 4. Time Dependent Effect of the CI Protein on HgCl_2 Induced Reduction in Total Antioxidant Power (FRAP Values) of Hepatocytes

CON: FRAP values in untreated cells. Hg₁, Hg₂, Hg₃: FRAP values in HgCl_2 treated cells at a concentration of $5 \mu\text{M}$ of HgCl_2 for 2, 4, 8 h respectively. P+Hg₁, P+Hg₂, P+Hg₃: FRAP values in the CI protein treated (0.5 mg/ml of protein) hepatocytes along with $5 \mu\text{M}$ of HgCl_2 for 2, 4, 8 h respectively. Each column represents mean $\pm$ S.D., $n=6$ (^a p , ^b p , ^c p < 0.05). ^a p indicates statistically significant difference of FRAP values in HgCl_2 -treated mice with respect to normal control; ^b p , ^c p indicates the same with respect to the FRAP values of the CI protein co-treated mice along with HgCl_2 for 2, 4, 8 h, respectively with respect to their respective HgCl_2 -treated hepatocytes alone.

Fig. 5. Effect of the CI Protein Pre-treatment and Post-treatment against HgCl_2 Intoxication on MDA Content in Liver (A) and Kidney (B)

N: values in normal-control mice, Hg₁, Hg₂, Hg₃: levels in HgCl_2 treated mice and sacrificed after 1, 2, 3 d respectively. P+Hg₁, P+Hg₂, P+Hg₃: levels in which the CI protein was given at a dose 2 mg/kg body weight for 3 d prior to HgCl_2 administration and sacrificed after 1, 2, 3 d respectively. NAC₃+Hg: levels in which NAC was given at a dose 150 mg/kg body weight for 3 d prior to HgCl_2 administration. BSA₃+Hg: levels in which BSA was given at a dose 150 mg/kg body weight for 3 d prior to HgCl_2 administration. Hg₁, Hg₂, Hg₃: levels in HgCl_2 treated mice and sacrificed after 1, 2, 3 d respectively. Hg+P₁, Hg+P₂, Hg+P₃: levels in which the CI protein was given at a dose of 2 mg/kg body weight for 1, 2, 3 d respectively after HgCl_2 administration for a day and sacrificed a day after the last treatment. NAC₃+Hg: levels in which NAC was given at a dose 150 mg/kg body weight for 3 d after HgCl_2 administration. BSA₃+Hg: levels in which BSA was given at a dose 150 mg/kg body weight for 3 d after HgCl_2 administration. Data represents mean $\pm$ S.D., $n=6$ (^a p , ^b p , ^c p , ^d p < 0.05). ^a p indicates statistically significant difference of the indices of oxidative stress of HgCl_2 -treated mice with respect to normal control; ^b p and ^c p indicate the same with respect to the indices of oxidative stress of CI protein pre- and post-treated mice respectively with respect to HgCl_2 -treated mice; ^d p and ^e p indicate the same for the indices of oxidative stress of NAC pre- and post-treated mice, respectively with respect to HgCl_2 -treated mice.

Fig. 6. Effect of the CI Protein Pre-treatment and Post-treatment against HgCl₂ Intoxication on Oxidative Stress Index, CAT, Activity in Liver (A) and Kidney (B); SOD Activity in Liver (C) and Kidney (D) and GPx Activity in Liver (E) and Kidney (F)

Legend is the same as Fig. 5, except here the data are presented for CAT, SOD and GPx activities in liver and kidney.

Fig. 7. Effect of the CI Protein Pre-treatment and Post-treatment against HgCl₂ Intoxication on GSH/GSSG Ratio in Liver (A) and Kidney (B)

Legend is the same as Fig. 5, except here the data are presented for GSH/GSSG ratio in liver (A) and kidney (B).

Fig. 8. Histopathology of Liver Sections Stained with Haematoxylin-Eosin Stain ($\times 200$)

(A) Histopathology of normal liver section. (B) Histopathology of liver treated with HgCl_2 for a day and sacrificed after 48 h. (C) Histopathology of liver treated with the CI protein for 3 d and then with HgCl_2 for 1 d and sacrificed after 48 h. (D) Histopathology of liver treated with HgCl_2 for a day and then treated with the CI protein for 3 d.

Effect of the CI Protein on Liver and Kidney Histopathology Histopathological analyses showed prominent changes in tissues treated with HgCl_2 compared to protein pre- or post-treated tissues. In the liver tissue, HgCl_2 induced necrosis along the central vein and disorganization of normal radiating pattern of cell plates around it (Fig. 8B). Besides, there was elongation and subsequent tear along the central vein in HgCl_2 -treated liver sections. CI protein pre-treatment showed better recovery compared to post-treatment as it almost reversed the changes observed in HgCl_2 treated liver (Figs. 8C, D). In the HgCl_2 -treated renal sections, severe damage characterized by complete tubular necrosis with disruption of tubular basement membrane was observed (Fig. 9B). However, administration of the CI protein prior and after HgCl_2 administration showed a considerable improvement in kidney (Figs. 9C, D) morphology.

DISCUSSION

In the present study, acute administration of HgCl_2 caused toxic effects in the kidney and liver of the experimental animals and this damage was associated with the increase in serum GPT, ALP, BUN, creatinine and $\text{TNF-}\alpha$ as well as lipid peroxides. HgCl_2 also reduced hepatocytes viability, increased ROS formation and decreased the overall antioxidant power in hepatocytes. In addition, a significant reduction was observed in CAT, SOD, GPx and GSH/GSSG ratio. HgCl_2 insult resulted in severe damage in liver and kidney as revealed from histological studies. Treatment of mice with the CI protein, however, seemed to afford protection against this noxious stimulus.

Usually free radical mediated tissue injury is associated with increased circulating levels of various cytokines such as $\text{TNF-}\alpha$, Interleukin (IL)-1 β and IL-6. Therefore, it can be proposed that antioxidants or free radical scavengers counter-

Fig. 9. Histopathology of Kidney Sections Stained with Haematoxylin-Eosin Stain ($\times 200$)

(A) Histopathology of normal kidney section. (B) Histopathology of kidney treated with HgCl_2 for a day and sacrificed after 48 h. (C) Histopathology of kidney treated with the CI protein for 3 d and then with HgCl_2 for 1 d and sacrificed after 48 h. (D) Histopathology of kidney treated with HgCl_2 for a day and then treated with the CI protein for 3 d.

act the oxidative stress produced by the toxins. In our study, increased $\text{TNF-}\alpha$ levels of HgCl_2 -treated mice appeared to decrease following the CI protein treatment also supports the notion that the protein ameliorates oxidative injury caused by HgCl_2 probably through its antioxidant property.

Lipid peroxidation by Hg(II) has been demonstrated in the rat tissues, as well as in isolated rat hepatocytes, and it has been suggested that the cell membrane permeability may be affected by this process.^{40,41} Consistent with these reports we found that mercury administration indeed increased MDA formation in both the liver and kidney tissues in mice while the CI protein treatment before and after mercury administration reversed the phenomenon.

The principal toxic effects of Hg(II) arise from alterations of mitochondrial inner membrane, which is the principal site of cellular production of reactive oxidants. Therefore, HgCl_2 treatment may be accompanied by an oxidative stress-like condition.^{24,42} It was found from our study that HgCl_2 induced generation of ROS peaked at 4 h, (as evidenced in DCFDA fluorescence) and the increase, was attenuated by the administration of the CI protein and HgCl_2 for the same time. Similarly, the total antioxidant power of hepatocytes was increased when cells were incubated with the CI protein along with HgCl_2 than HgCl_2 alone. Thus, the results suggest that the CI protein possesses an antioxidative role in HgCl_2 induced generation of ROS and it increases the FRAP activity as well. Moreover, HgCl_2 -treatment reduced the levels of several antioxidant defense molecules in liver and kidney. Though there was no significant alteration in CAT activity in livers of HgCl_2 treated mice, its activity nonetheless decreased in kidneys. SOD activity, on the other hand, was reduced significantly in both liver and kidney of HgCl_2 -treated mice. Again GPx activity was also lowered prominently in livers and kidneys of HgCl_2 administered mice. Treatment with the CI protein markedly ameliorated HgCl_2 -induced

acute hepatic and renal oxidative stress since its administration increased the activity of CAT, SOD and GPx in both the liver and kidney.

The participation of oxidative stress in the HgCl₂ associated nephrotoxicity was suggested initially by Girardi *et al.*⁴³⁾ and Gstraunthaler and Pfaller.⁴⁴⁾ They showed that HgCl₂ administration reduced renal GSH content and depletion of GSH resulted in increased severity of HgCl₂-induced acute renal failure. Bando *et al.*⁴⁵⁾ have also suggested that Hg(II) administration selectively depletes mitochondrial GSH in liver making it more susceptible to oxidative damage, especially during increased free radical production. Our results also showed that depletion of tissue GSH/GSSG ratio, as observed in the HgCl₂-induced hepatic and renal injury, is one of the major factors that permit lipid peroxidation and subsequent tissue damages. Since administration of the CI protein prevented the depletion of hepatic and renal GSH/GSSG ratio, it appears that the protective effect of the CI protein involves the maintenance of antioxidant capacity in protecting the hepatic and renal tissues against oxidative stress.

In conclusion, the outcome of our present study demonstrates that the CI protein, with its potent antioxidant properties, seems to be a highly promising agent in protecting hepatic and renal tissues against HgCl₂-induced oxidative damage. It reduces HgCl₂ stimulated TNF- α formation in serum, ROS generation in hepatocytes and increases its overall antioxidant potential. The CI protein also abrogates HgCl₂-induced rise in membrane lipid peroxidation and increases the levels of antioxidant enzymes like SOD, CAT and GPx. The depleted GSH/GSSG ratio in HgCl₂ treated mice was also increased by pre or post treatment with the protein. Additionally, hepatic and renal tissue architecture was also improved in the mice pre- or post-treated with the CI protein compared to mice treated with HgCl₂ alone. In this study it was also demonstrated that the CI protein and NAC, in the doses used, might afford the same degree of protection against acute HgCl₂ toxicity.

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