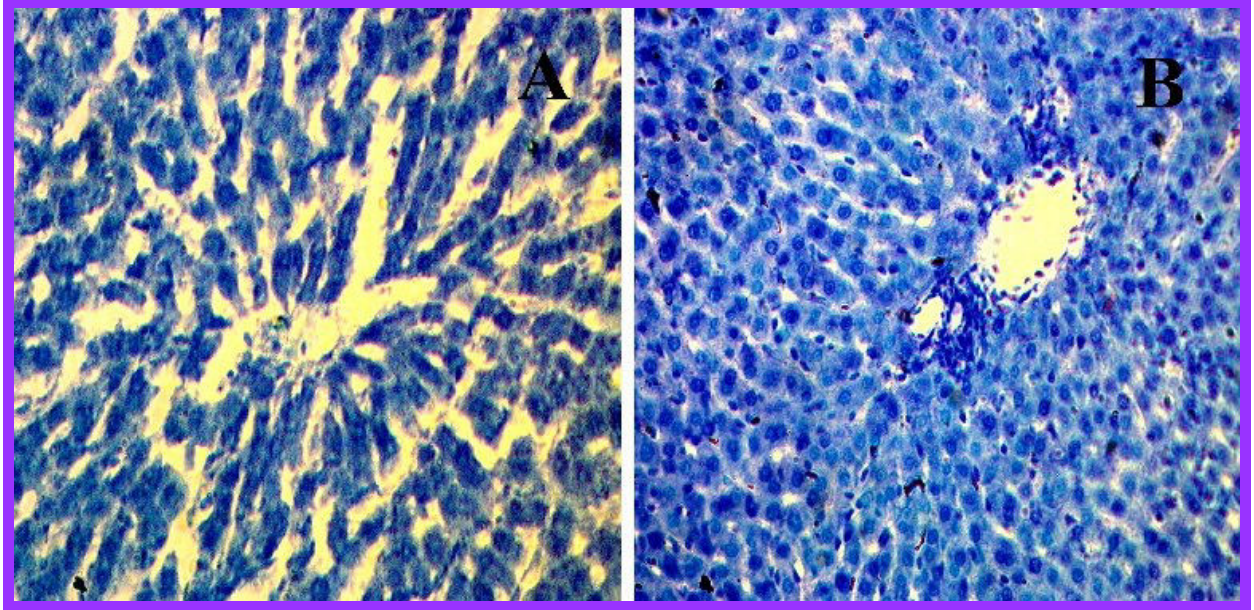

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Iron deposition causes oxidative stress, inflammation and fibrosis in carbon tetrachloride-induced liver dysfunction in rats

Hasan Mahmud Reza, Md. Abu Taher Sagor and Md. Ashraful Alam

Department of Pharmaceutical Sciences, School of Life Science, North South University, Bashundhara, Dhaka, Bangladesh.

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Abstract

The present study investigates the pathological implications of parenchymal iron overload due to CCl_4 treatment in liver dysfunction. Sixteen female rats were randomly divided into control and CCl_4 treated groups. The serum levels of transaminases, malondialdehyde, advanced protein oxidation product, catalase, and reduced glutathione concentrations in the plasma and hepatic homogenate were determined. Moreover, histopathological changes in liver sections were investigated for inflammatory cell infiltration, fibrosis and iron overload. The administration of CCl_4 resulted in increased liver marker enzymes activities and oxidative stress parameters mentioned above com-pared to control. Moreover, CCl_4 administration also decreased antioxidant enzymes activities and increased inflammatory cell infiltration and fibrosis along with iron deposition in liver of rats. These findings indicate that CCl_4 may induce hepatic fibrosis and inflammation during CCl_4 induced liver injury via iron mediated oxidative damages.

Introduction

Hepatic cirrhosis or liver fibrosis is known to be an irreversible process in liver due to chronic insult such as, diabetes, high fat diet or viral infection in the liver. Early sign of hepatic cirrhosis includes abnormal liver enzymes, nonalcoholic fatty liver disease which ultimately leads to hepatocellular carcinoma, and acute liver failure (Iwaisako et al., 2012). Carbon tetrachloride (CCl_4) is a known hepatotoxin, frequently used to induce liver fibrosis in animal models (Neubauer et al., 1998). A single dose of CCl_4 leads to centrilobular necrosis and steatosis, while prolonged administration leads to liver fibrosis, cirrhosis, and hepatocellular carcinoma. In liver, CCl_4 impairs hepatocytes directly by altering the permeability of the plasma, lysosomal, and mitochondrial membranes. Generally, CCl_4 is metabolized in liver by the mixed function oxidase system in hepatocytes via CYP2E1 which produces highly reactive free radical metabolites trichloromethyl radical ($^{\bullet}CCl_3$), causing severe centrilobular necrosis (Gowri Shankar et al., 2008). One of the major outcomes of this process is lipid peroxidation, which is capable of causing damage to the cell membrane, organelles and this process can be more dangerous while leading to release of reactive aldehydes with activation of pro-inflammatory and pro-fibrotic process (Novo and Parola, 2012). Damaging liver cells activate innate immune system like Kupffer cells which further stimulate the production of natural killer T-Cells, natural killer cells, and Helper cells which results in producing pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interferon-γ, interleukine-β (IL) (Kremer et al., 2010; Liaskou et al., 2012).

Liver fibrosis is the pathologic result of ongoing chronic inflammatory liver diseases and is characterized by hepatic stellate cell proliferation and differentiation to myofibroblast-like cells, which deposit extracellular matrix (ECM) and collagen (Friedman 2008). The activated hepatic stellate cells produce large amounts of ECM components. It has been often observed that

oxidative stress activate the apoptosis and necrosis and help the release and synthesis of pro-inflammatory and fibrogenic factors which can change hepatocyte functions, alter Kupffer cell, and promote activation of hepatic stellate cells and fibrogenesis (Novo and Parola, 2012). Iron is known to increase cellular oxidative stress via production of reactive oxygen species (ROS) by catalyzing the Fenton reaction (Li et al., 2012). Secondary iron over-load and primary hemochromatosis have received increasing attention due to its role in oxidative stress. Iron overload has been observed in patients with chronic liver diseases, including alcoholic liver disease and chronic viral hepatitis, and is a secondary side effect of repeated blood transfusions (Price and Kowd-ley, 2009; Lee and Kowdley, 2012). Previous study also suggests that iron overload in non-parenchymal Kuppfer cells was shown to induce hepatic stellate cell proliferation and activation, leading to liver cirrhosis (Pietrangelo et al., 1995; Montosi et al., 1998).

In view of the above facts, we investigated whether CCl_4 administration could induce oxidative stress through iron deposition in liver. Therefore, we determined the plasma marker enzymes activities, lipid peroxidation levels, enzymatic and non-enzymatic antioxidants along with histopathological changes in the liver for inflammation, fibrosis and iron deposition in rats treated with CCl_4.

Materials and Methods

Chemicals and reagents

CCl_4 were obtained from Merck (Germany). Thiobarbituric acid (TBA) was purchased from Sigma Chemical Company (USA). Reduced gluta-thione (GSH), and trichloroacetic acid (TCA) were purchased from J.I. Baker (USA). Alanine aminotrans-ferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase assay kits were obtained from DCI diagnostics (Budapest, Hungary), 50, 50-dithiobis-2-nitrobenzoate (Ellman's reagent) from Sigma (USA) and sodium hydroxide from Merck (Germany). All other chemicals and reagents used were of analytical grade.

Animals and treatments

Ten to twelve weeks old, 16 Long Evans female rats (150–170 g) were obtained from Animal production unit of Animal House at Department of Pharmaceutical Sciences, North South University and were kept in ordinary cages at room temperature of 25 ± 3°C with a 12 hours dark/light cycles. They have free access to standard laboratory feed and water, according to the study protocol approved by Ethical Committee of Department of Pharmaceutical Sciences, North South University for animal care and experimentation. To study the development of liver dysfunction in CCl_4 rats, all animals were equally divided into two groups (eight rats each). Animals of Group I was treated with 1 mL/kg of saline (0.85%) and olive oil (3 mL/kg) intragastrically twice a week for two weeks. Rats of Group II was treated with CCl_4 (1:3 in olive oil) at a dose of 1 mL/kg intragastrically twice a week for two weeks. Animals were checked for the body weight, food and water intake on a daily basis.

After 14 days, dose, all the animals were weighted, sacrificed, collected the blood and organs like heart, kidney, spleen and liver. Immediately after collection of the organs, they are weighted and stored at -20°C for further analysis.

Assessment of hepatotoxicity

Liver marker enzymes ALT, AST and alkaline phosphatase were estimated in plasma by using DCI diagnostics kits (Hungary) according to the manufacturers protocol.

Assessment of oxidative stress markers

For determination of oxidative stress markers, liver tissue was homoge-nized in 10 times volume of phosphate buffer containing (pH 7.4) and centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was collected and used for the determination of protein and enzymatic studies as described below.

Estimation of lipid peroxidation product (MDA)

Lipid peroxidation in liver was estimated colorimetrically measuring MDA using thiobarbituric acid described elsewhere (Niehaus and Samuelsson, 1968). In brief, 0.1 mL of tissue homogenate (Tris-Hcl buffer, pH 7.5) was treated with 2 mL of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min and cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm.

Assay of nitric oxide (NO)

NO was determined according to the method described elsewhere as nitrate and nitrite (Tracey et al., 1995). In this study, Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 5% 1-napthylamine. The reaction mixture (3 mL) containing brain homogenates (2 mL) and phosphate buffer saline (0.5 mL) was incubated at 25°C for 150 min. A pink colored chromophore was formed. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. NO level was measure by using standard curve and expressed as nmol/mL.

Estimation of advanced oxidation protein products (AOPP)

Determination of AOPP levels was performed by modification of the methods described elsewhere (Witko-Sarsat et al., 1996; Tiwari et al., 2014). Two mL

of plasma was diluted 1:5 in PBS: 0.1 mL of 1.16 M potassium iodide was then added to each tube, followed by 0.2 mL acetic acid after 2 min. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 2 mL of PBS, 0.1 mL of KI, and 0.2 mL of acetic acid. The chloramine-T absorbance at 340 nm being linear within the range of 0 to 100 mmol/L, AOPP concentrations were expressed as µmol/L chloramine-T equivalents.

Estimation of catalase (CAT) activity

CAT activities were determined by the method of Chance and Maehly (Khan, 2012) with some modifications. The reaction solution of CAT activities contained: 2.5 mL of 50 mmol phosphate buffer (pH 5.0), 0.4 mL of 5.9 mmol H_2O_2 and 0.1 mL enzyme extract. Changes in absorbance of the reaction solution at 240 nm were determined after one min. One unit of CAT activity was defined as an absorbance change of 0.01 as units/min.

Histopathalogical determination

For microscopic evalua-tion liver tissues were fixed in neutral buffered formalin and embedded in paraffin, sectioned at 5 µm and subsequently stained with hematoxylin/eosin, tolue-dine staining, sirius red and prusian blue staining were also done to evaluate the inflammation, necrosis and fibrosis in liver. Sections were studied under light microscope at 40x magnifications.

Statistical analysis

The values are expressed as mean ± standard error of mean (SEM). The results were evaluated by using the student's t test using Graph Pad Prism Software. Statistical significance was considered $p<0.5$ in all cases.

Results

Body weight of each rat was recorded every day during the experiment for all groups. It was found that the body weight did not change in both CCl_4-intoxicated rats group and control rats group (Figure 1). CCl_4 intoxicated group rats also showed significant decrease in food and water intake compare to control rats, reduction of food and water intake was again unaltered in control group (Table I).

Table I shows the effect of CCl_4 treatments on the rats' organs weight. The spleen wet weight significantly ($p < 0.05$) increased in the CCl_4-treated rats when compared with control. CCl_4-treated rats also showed slight decreased in liver wet weight, however, control group rat did not change the wet weight of the liver. Another crucial finding in this study was the reduction of kidney wet weight; however the heart wet weight was unaltered due to CCl_4 intoxication compared to control rats.

Biochemical assays of liver functions revealed that, CCl_4 induced a significant increase in plasma AST, ALT, and alkaline phosphatase activity in plasma and liver of rats compared to that of control group rats.

To determine the oxidative stress in our study, we evaluated the MDA, nitric oxide and APOP content in plasma and liver homogenates (Table II). CCl_4 induced rats showed a higher concentration of lipid peroxidation product MDA both in plasma and liver homogenates (7.9 ± 0.4 and 27.9 ± 0.5 nmol/mL in plasma and liver homogenates respectively) compared to control group (4.4 ± 0.3 and 13.6 ± 0.5 nmol/mL in plasma and liver homogenates respectively). CCl_4 treatment also increased the plasma and tissue nitrate level in rats compared to the control rats (Table II).

CCl_4 has profound effect on APOP development in plasma and liver. CCl_4 challenge significantly increased the APOP concentration in plasma and liver (242.9 ± 12.0 and 1249.3 ± 16.4 nmol/mL equivalent to chloramine-T respectively) compared to control group (112.5 ± 5.9 and 270.9 ± 15.8 nmol/mL equivalent to chloramine-T respectively). Nitric oxide measured as nitrate

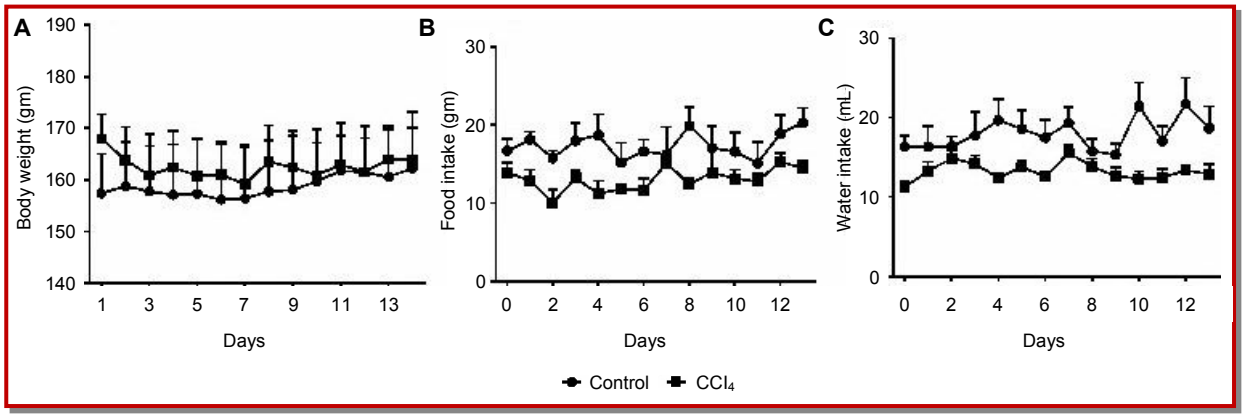

Figure 1: Effect of CCl_4 on body weight (A), food intake (B) and water intake (C) in rats. Values are presented as mean ± SEM. Student's t tests were done for statistical comparison. Values are considered significance at $p<0.05$. a vs b, control vs CCl_4

Table I

Effect of CCl_4 on body weight, food and water intake and organ weight

Parameters	Control	CCl_4
Initial body weight (g)	157.4 ± 7.6	168.0 ± 4.7
Final body weight (g)	162.2 ± 11.0	163.9 ± 6.1
Food intake/day (g)	17.3 ± 0.4[a]	13.0 ± 0.4[b]
Water intake/day (mL)	18.0 ± 0.5[a]	13.2 ± 0.3[b]
Liver wet weight (g/100 g of body weight)	3.7 ± 0.1	3.4 ± 0.3
Kidneys wet weight (g/100 g of body weight)	0.6 ± 0.0	0.5 ± 0.0
Heart wet weight (g/100 g of body weight)	0.3 ± 0.0	0.3 ± 0.0
Spleen wet weight (g/100 g of body weight)	0.3 ± 0.0[a]	0.5 ± 0.0[b]

Values are presented as mean ± SEM. Student's t tests were done for statistical comparison. Values are considered significance at $p<0.05$. a vs b, control vs CCl_4

was also increased both in plasma and liver homogenates (5.7 ± 0.4 and 57.2 ± 7.9 nmol/mL in plasma and liver homogenates respectively) compared to control rats. In addition, CCl_4 induced a significant decrease in liver antioxidant enzyme activities GSH and CAT respectively, compared to the control levels (Table II).

Inflammation in the liver was seen in rats treated with CCl_4. Massive serge of inflammatory cells was found in the centilobular part of liver section stained with H & E

Table II

Effect of CCl_4 on biochemical parameter in plasma and liver

Parameters	Groups	
	Control	CCl_4
	Plasma	
AST(U/L)	25.6 ± 1.5	41.3 ± 2.0[b]
ALT(U/L)	19.5 ± 0.9	41.8 ± 1.7[b]
ALP(U/L)	53.5 ± 2.6	80.6 ± 3.4[b]
MDA (nmol/mL)	4.3 ± 0.3	7.9 ± 0.4[b]
NO (nmol/mL)	3.7 ± 0.2	6.0 ± 0.6[a]
APOP (µmol/L chlora -mine-T equivalents)	112.5 ± 5.9	242.9 ± 12.0[b]
Catalase (U/min)	5.9 ± 0.2	3.7 ± 0.4[b]
GSH (µg/mg protein)	19.5 ± 1.0	10.8 ± 1.0[b]
	Liver	
AST(U/mg protein)	371.6 ± 14.4	520.4 ± 23.4[b]
ALT(U/mg protein)	301.7 ± 21.1	498.0 ± 28.0[b]
ALP(U/mg protein)	517.8 ± 21.2	946.5 ± 31.7[b]
MDA (nmol/mL)	13.6 ± 0.5	27.9 ± 0.5[a]
NO (nmol/mL)	13.3 ± 0.4	22.2 ± 0.9[a]
APOP (µmol/L chlora -mine-T equivalents)	270.9 ± 15.8	1,249.3 ± 16.4[b]
Catalase (U/min)	46.4 ± 2.5	24.2 ± 0.6[a]
GSH (µg/mg protein)	39.0 ± 1.2	21.4 ± 1.4[a]

Values are presented as mean ± SEM. Student's t tests were done for statistical comparison. Values are considered significance at [a]$p<0.05$. [b]$p<0.001$, control vs CCl_4

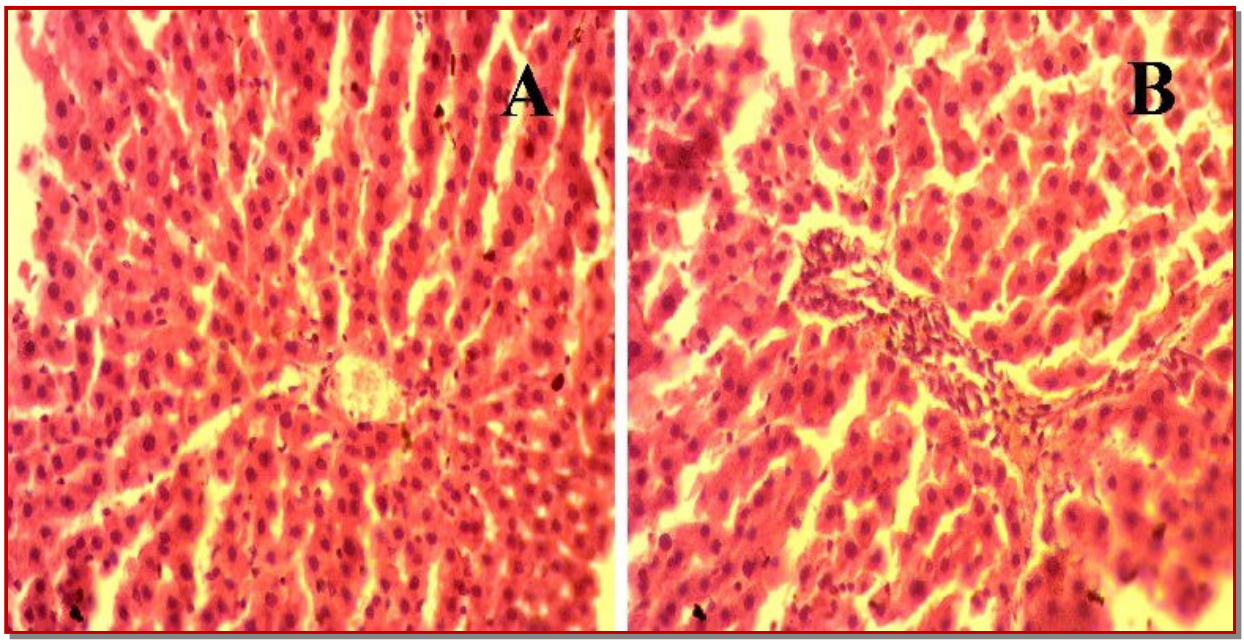

Figure 2: Hematoxylin and eosin staining showed inflammatory cell infiltration and necrosis in liver of rats treated with CCl_4. A, Control; B, CCl_4, Magnification 40x

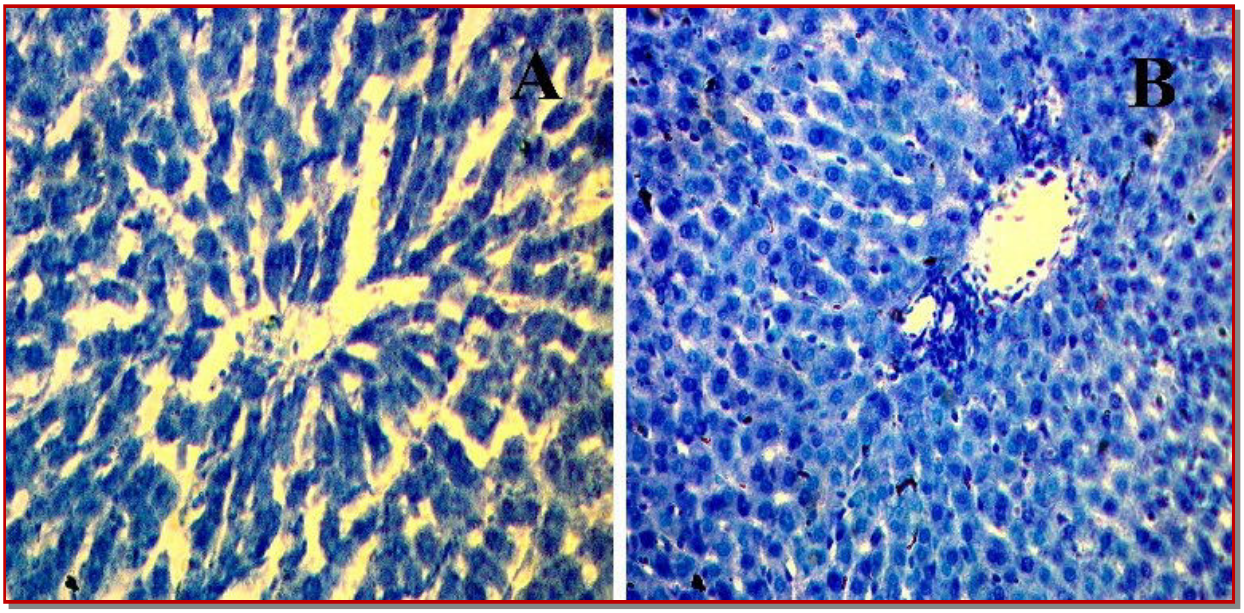

Figure 3: Toluidine blue staining showed mast cells infiltration (deep blue dots) in liver of rats treated with CCl_4. A, Control; B, CCl_4, Magnification 40x

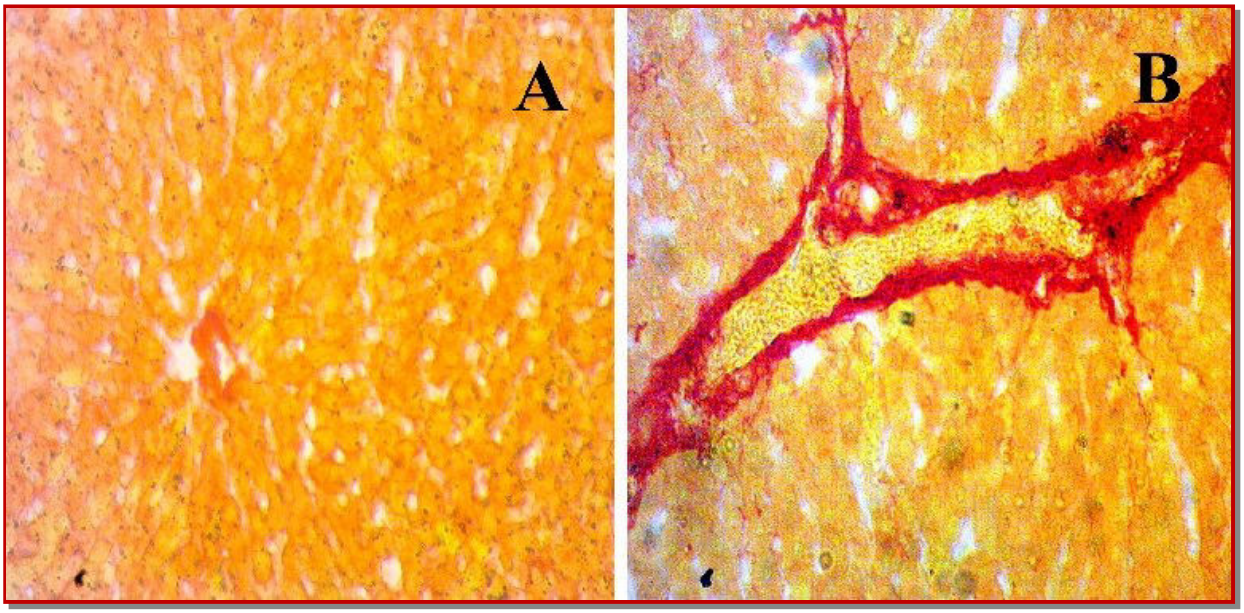

Figure 4: Sirius red staining showed fibrosis (red color) in liver of rats treated with CCl_4. A, Control; B, CCl_4, Magnification 40x

staining in CCl_4 treated rats group (Figure 2). Necrotized tissue scar and ballooning of the hepato-cytes were also seen in liver of CCl_4 treated rats. Toluidine blue staining showed mast cells infiltration in CCl_4 treated rats compared to the control (Figure 3). Liver fibrosis was evaluated histologically by visualizing the red color of collagen fibers deposition using Sirius red stain. The collagen fibers were heavily deposited around portal tracts and central veins in CCl_4-intoxicated group and extended from central vein to portal tract resulting in the formation of pseudolobules which is not seen in control rats (Figure 4). Additionally, iron deposition was also seen in liver section of CCl_4 treated rats by Prussian blue staining (Figure 5).

Discussion

CCl_4-induced toxicity and its mechanisms have been extensively investigated after oral administration to rodents. Our investigation revealed that CCl_4 treatment in rats induces oxidative stress, inflammatory cell infiltration and fibrosis in rat's liver. In the present study the hepatotoxic effects of CCl_4 were detected by

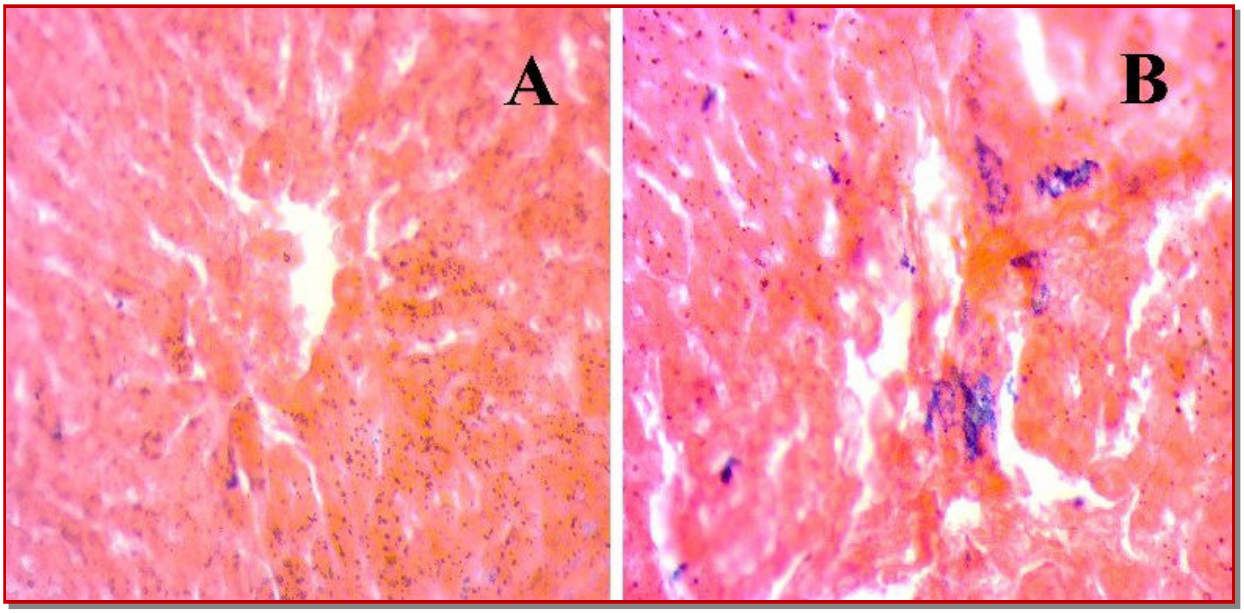

Figure 5: Prussian blue staining showed iron deposition (blue color) in liver of rats treated with CCl_4. A, Control; B, CCl_4, Magnification 40x

significant elevation in liver enzymes (AST, ALT, and alkaline phosphatase) activities. Several authors have reported a significant increase in liver enzymes in rats subjected to CCl_4 intoxication (El-Khatib and Mansour, 2001; Ali et al., 2010). Necrosis or membrane damage in liver releases these enzymes into circulation and hence it can be measured in plasma or serum. High levels of AST indicate liver damage, such as that caused by viral hepatitis as well as cardiac infarction, whereas, ALT is more specific to the liver, and is thus a better parameter for detecting liver injury (Giannini et al., 2005). Alkaline phosphatase is also associated with conditions linked to the hepatic biliary tract (Giannini et al., 2005).

Oxidative stress through generation of ROS plays an important role in the development of liver damage and increases the inflammatory responses which ultimately stimulates the production of pro-fibrogenic mediators and initiate hepatic fibrogenesis (Cui et al., 2003; Galli et al., 2005; Ghiassi-Nejad and Friedman, 2008). In our study, it was found that, administration of CCl_4 resulted in a significant depletion of liver GSH accompanied with a significant increase in lipid peroxidation product TBARS as compared to the control group. Furthermore, the activity of CAT that share in the enzymatic neutralization of free radicals was significantly decreased by CCl_4 treatment. Several studies reported that GSH level, which are capable of preferentially conjugating with the toxic metabolites and free radicals in tissues, exhausted in steatohepatitis or other hepatic injury caused by CCl_4 administration (von Montfort et al., 2012). Nitric oxide (NO) is a mediators of systemic vasodilatation, have been reported to be increased in liver cirrhosis. Some author proposed that a high level of nitric oxide is associated with CCl_4-induced acute liver injury (Tipoe et al., 2006). Moreover, clinical studies showed that serum nitrite levels in cirrhotic patients were significantly increased in comparison to that of controls (Mohammed et al., 2003).

Additionally, histopathological assessment in liver tissues of CCl_4 treated rats also revealed typical hepatotoxic effects, as described previously in the literature (Nabeshima et al., 2006; Jin et al., 2013). Inflammatory cells have been accumulated in the necrotized region and alongside the bile ducts and blood vessels in liver of CCl_4 treated rats. Mast cells infiltration was also observed in the liver section of CCl_4 treated rats in our study. However, the other type of inflammatory cells could not be identified in this study. In addition to liver -resident Kupffer cells, infiltrating immune cells have recently been linked to the development of liver fibrosis (Karlmark et al., 2009). Previous reports suggest that monocytes infiltration occurs in acute and chronic carbon tetrachloride (CCl_4)-induced liver injury in mice (Karlmark et al., 2009). During fibrosis progression, monocyte-derived macro-phages release several cytokines inducing chronic inflammation as well as directly activate hepatic stellate cells, resulting in their proliferation and trans-differentiation into collagen-producing myofibroblasts (Imamura et al., 2005; Karlmark et al., 2009).

Most crucial finding of this study was massive iron deposition in liver section in CCl_4 treated rats. The liver is the main storage organ for iron. In iron overload condition, free radical formation and generation of lipid peroxidation products are increased due to oxidative stress through an increased rate of hydroxyl free radical ($HO^{\bullet}$) generation by the Haber-Weiss reaction in tissues

(Siah et al., 2006; Novo and Parola, 2012). Iron deposition in liver is also linked with hepatic oxidative stress, inflammation and fibrosis (Pietrangelo et al., 1995). Heme oxygenase (HO) enzyme metabolizes the tissue iron. It has been reported that abnormal heme oxygenase activity may facilitates iron deposition in liver section in lab animals (Poss and Tonegawa, 1997). Moreover, hereditary hemochromatosis (which is a genetic disorder and a common cause of iron overload) patients may prone to develop hepatocellular carcinoma and estimated risk to be 100-200 fold higher in these patients (Bradbear et al., 1985; Georg et al., 1988).

In conclusion, the present study provides evidence for fibrotic effect in liver due to CCl_4 treatment which is mediated probably by iron overload induced oxidative stress and inflammation. Further studies are warranted to elucidate other molecular target for the development of fibrosis in this rat model.

Acknowledgement

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Author Info
Md. Ashraful Alam (Principal contact)
e-mail: sonaliagun@yahoo.com

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