



ISSN: 0976-3031

Available Online at <http://www.recentscientific.com>

International Journal of Recent Scientific Research
Vol. 8, Issue, 3, pp. 15885-15892, March, 2017

**International Journal of
Recent Scientific
Research**

DOI: 10.24327/IJRSR

Research Article

CARBON TETRACHLORIDE INDUCED HEPATORENAL FAILURE IN MALE ALBINO RATS: A DOSE RESPONSE STUDY

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DOI: <http://dx.doi.org/10.24327/ijrsr.2017.0803.0026>

ARTICLE INFO

Article History:

Received 17th December, 2016

Received in revised form 21st

January, 2017

Accepted 05th February, 2017

Published online 28th March, 2017

Key Words:

Carbon tetrachloride, Hepatorenal failure,
Oxidative Stress, Antioxidant enzyme.

ABSTRACT

Like other halogenated alkanes carbon tetrachloride or tetrachloromethane (CCl₄) induces hepatorenal injury in both acute and chronic cases in rats. Rats were sequentially administered with a dose of 115 mg/kg of body weight /day and 230 mg/kg of body weight /day respectively for 7 days and also an acute dose of 2000 mg/kg of body weight was also given for mortality, wellness parameter study. Several oxidative stress markers, biochemical parameters and liver and renal functions tests were measured. The present study was aimed to determine LD50 of CCl₄ by subcutaneous application in male rats as per the guidelines of Organization for Economic Cooperation and Development (OECD). Our results suggested that the CCl₄ induction causes hepatorenal toxicity by generation of liver fibrosis in experimental animals, at a concentration of 230 mg/kg bw/day in a time period of 7 days subcutaneous administration in male rats.

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INTRODUCTION

Recently, numerous environmental toxicants from diverse chemical categories are suspected to cause acute or chronic life threatening toxicity in humans. Carbon tetrachloride (CCl₄) is a colorless, odorant solvent which makes its entry into environment as gaseous form because of improper disposal of industrial wastes. Exposure on CCl₄ leads to Headache, dizziness, vomiting, stomach pain, light headedness, tiredness, weakness and blurred vision. The principal target organs due to carbon tetrachloride exposure in humans are on liver, kidney. The mode of toxicity of CCl₄ mostly depends on lipid peroxidation of cell membrane and generation trichloromethyl radical (CCl₃•), which then further due to low partial pressure of oxygen produces more toxic trichloromethyl peroxy radical (CCl₃O₂•) and causes detrimental irreversible ill effects in exposed individual (Ritesh et al.,2015, Boll et al., 2001). This environmental toxin has also got importance because of its repeated use to induce hepatic dysfunction (especially liver fibrosis) in many animal experimental models (Elgazar A. F., 2013).

People exposed to CCl₄ often develop patchy, fibrotic, cirrhotic liver, which by time successfully develops a renal failure along with complete hepatic damage. The day by day incidence of hepatorenal dysfunction is becoming a serious threat to modern civilization. There is immense necessity for an affordable therapy for patients who cannot afford expensive liver or kidney transplant as the only effective remedy to keep them alive.

The present study was aimed to find out the threshold level (LD50) of CCl₄, which can initiate the hepatic injury in a dose dependent manner, by subcutaneous application in male rats as per the guidelines of Organization for Economic Cooperation and Development (OECD).

MATERIAL AND METHODS

Chemicals and reagents

Carbon tetrachloride (CCl₄), Tris buffer, Sodium chloride (NaCl), Triton-X 100, potassium dihydrogen phosphate (KH₂PO₄), dipotassium hydrogen phosphate (K₂HPO₄), ethylene diamine tetra acetate (EDTA), sodium hydroxide (NaOH), chloroform, TCA, TBAPotassium hydroxide (KOH),

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methanol, alcohol, and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. 5', 5'-dithio (bis)-2-nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), were procured from Sigma (St. Louis, MO, USA). All other chemicals were from Merck Ltd., SRL Pvt. Ltd., Mumbai and were of the highest purity grade available.

Selection of animals and care

The study was conducted on 18 healthy, Wister strain male albino rats (Supplied by Ghosh animal, animal foods and animal cages Supplier, Kolkata 54) having a body weight of 100 ± 15 g. These experimental animals were acclimatized in laboratory condition for period of 2 weeks prior to the experiment. Experimental animals were housed three rats/cage in a room with temperature $22 \pm 2^\circ$ C with 12–12 h dark–light cycles by the side of a humidity of $50 \pm 10\%$. They were provided with standard diet and water *ad libitum*. The experimental animal care was provided according to the Guiding Principle for the Care and Use of Animals (Olert et al., 1993).

Experimental design

Acute toxicity study by limit test

An acute toxicity of Carbon tetrachloride was conducted by acute toxicity method as per Organization of Economic Co-operation and Development (OECD) guidelines 425 (OECD., 2001) where the used limit dose was of 2000 mg/ kg b.wt. Healthy Wister strain male rats ($n = 6$) were chosen for the acute study by random sampling method. Several wellness parameters of the experimental animals were made and recorded in durations of 30 min, 2hr, 4hr, 6hr, 8 hr, 10 hr, 12hr, 18 hr, 24 hr, 36 hr, and 48 h after the administration of said dose. Several changes with time, after application of dose for skin and fur, eyes, mucus membrane, behavioral pattern changes, tremor, convulsions, salivations, diarrhea, lethargy, sleep and mortality were recorded for 48 hr.

Main test

The main test was conducted on eighteen healthy male albino rats ($n = 18$). The doses were selected 115 mg and 230 mg/ kg b.wt., for 7 days continuously. The randomly selected experimental rats were divided into three equal groups as follows: Group 'A': control, Group 'B': CCl_4 at 115 mg/kg bw/rat/day; Group 'C': CCl_4 at 230 mg/kg bw/rat/day with basal diet and water *ad libitum* for 7 days.

Animal's sacrifice

This experimental design for threshold dose selection continued for 7 days. After 7 days, the animals were sacrificed & blood was collected from the aorta after which the liver & kidneys were collected for different biochemical and hematological analysis. Half of liver and kidney tissue were taken and wrapped with sterilized aluminum foil and stored into -20° c deep refrigerator until preparation of tissue homogenates. The other halves of liver and kidney were preserved in 10% neutral formaldehyde solution till processed for histological examination.

Separation of serum and homogenization of liver and kidney

Serum was separated by centrifugation of blood samples at $1500 \times g$ for 15 min taken without anticoagulant. The isolated

samples were kept at -80°C for the biochemical estimation of different parameters (Tripathy et al., 2012) and washed tissues were immediately homogenized in the ice-cold buffer containing 0.25 M sucrose, 1 mM EDTA, and 1 mM Tris-HCl, pH 7.4. The homogenate was first centrifuged at $600 \times g$ for 10 min at 4 C, and the supernatant was stored at -80°C for the biochemical estimation of different parameters (Tripathy et al., 2013).

Biochemical estimation

Total cholesterol, (TC)

Total cholesterol level in serum assayed according to the method of Warnick et al., 1995 by test kit.

Triglycerides (TG)

The serum level of triglyceride estimated by using test kit according to the method of Stein et al., 1995.

Serum glutamic oxaloacetic transaminases (SGOT)

The level of aspartate aminotransferase in serum measured according to the Bergmeyer et al., 1974, 2,4- DNPH Method by using Test Kit.

Serum glutamic pyruvic transaminases (SGPT)

The serum level of alanine aminotransferase estimated according to the Bergmeyer et al., 1974, 2,4- DNPH Method by using Test Kit.

Alkaline phosphatase (ALP)

The activity of alkaline phosphatase estimated according to the method of King et al., 1978 by using ALP Test Kit.

Creatinin level in serum

Serum creatinin level measured according to bonnes et al., 1945, Alkaline picrate method, by using test kit.

Urea level

Urea level in serum determined according to Coulambe et al., 1965, by using kit.

Determination of Lipid Peroxidation (MDA) Level

According to the method of Ohakawa et al. (1979), lipid peroxidation of liver and kidney homogenate was evaluated. In brief, the reaction mixture prepared with Tris-HCl buffer (50 mM, pH 7.4), tetra-butyl hydroperoxide (BHP) ($500 \mu\text{M}$ in ethanol), and 1 mM FeSO_4 . The samples were incubated at 37 C for 90 min and the reaction was stopped by adding 0.2 mL of 8% sodium dodecyl sulfate (SDS) followed by 1.5 mL of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 mL of 0.8% TBA and further heating the mixture at 95 C for 45 min. After cooling, samples were centrifuged, and TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using $1.53 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ as extinction coefficient. The MDA levels were expressed in terms of nmol/mg protein.

Determination of Super Oxide Dismutase (SOD) Activity

SOD activity of liver and kidney homogenate was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Mestro and McDonald et al. (1986). The samples

were measure at 420 nm at 25 C for 3 min. SOD activity was expressed as unit/mg protein.

Determination of Catalase (CAT) Activity

CAT activity of these tissue homogenate was measured by the method of Luck (1963). Catalase activity was calculated by using the molar extinction coefficient of 43.6 M⁻¹cm⁻¹for H₂O₂. The level of CAT was expressed as unit/mg protein.

Determination of Reduced Glutathione (GSH) Level

GSH estimation in tissue homogenate was estimated according to the method of Moron et al. (1979). The required amount of sample was mixed with 25% of TCA and then it was centrifuged at 2,000×g for 15 min. The supernatant was diluted to 1 mL with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 mL of 0.6 mM DTNB was added. After 10 minutes the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman’s reagent) was measured at 405 nm. The levels of GSH were expressed as μg of GSH/mg protein.

Determination of Glutathione Peroxidase (GPx) Activity

The GPx activity of liver and kidney homogenate was evaluated by the method of Paglia and Valentine (1967). Absorbance at 340 nm was recorded for 5 min. Values were expressed as nmol of NADPH oxidized to NADP by using the extinction coefficient of 6.2× 10³M⁻¹cm⁻¹at 340 nm. The activity of GPx was expressed in terms of nmol NADPH consumed/min/mg protein.

Histological Study

Histological analysis of liver and kidney tissue of each experimental group was performed by the method of Iranloye and Bolarinwa (2009). The animals were sacrificed and the tissues were immediately perfused in saline and formalin and were fixed for 7 days in 10% formaldehyde. Next, dehydration was carried out in ascending grade of alcohol (70%-100%).

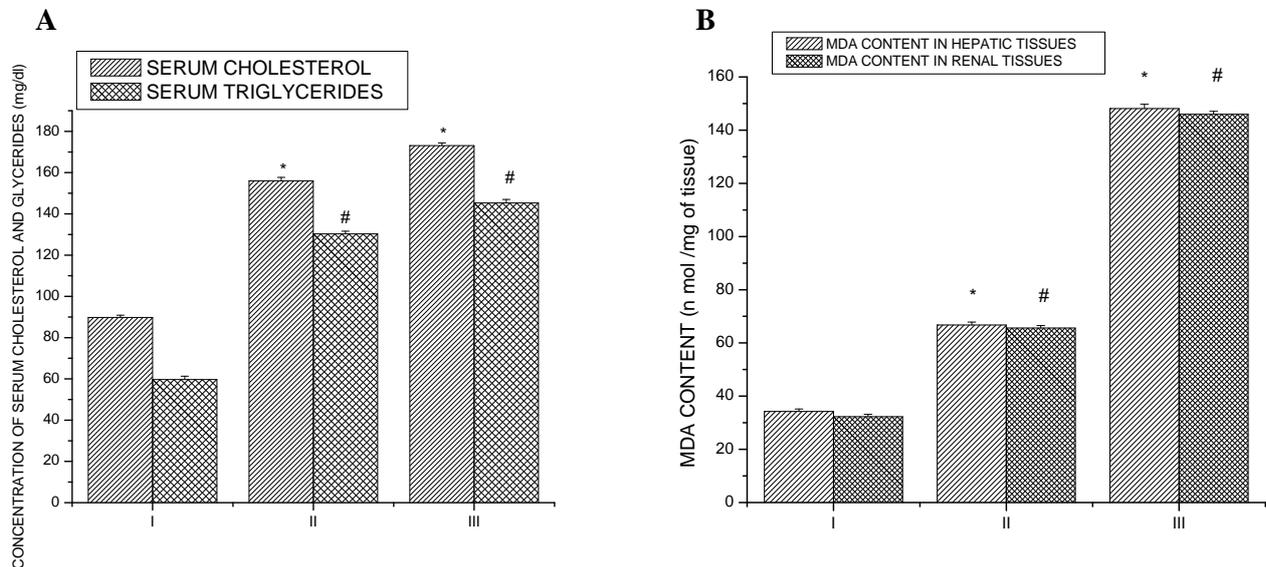


Fig. 1 Graphical presentation represents the Total cholesterol (TC) in serum (A), Total glyceride (TG) in serum (A) and MDA level in liver (B) of experimental groups. Values are expressed as mean±SEM, n=6. *,# indicates significant difference (P<0.05) compared to control group. Group I: Control, Group II: CCl₄ at 115 mg/kg bw, Group III: CCl₄ at 230 mg/kg bw.

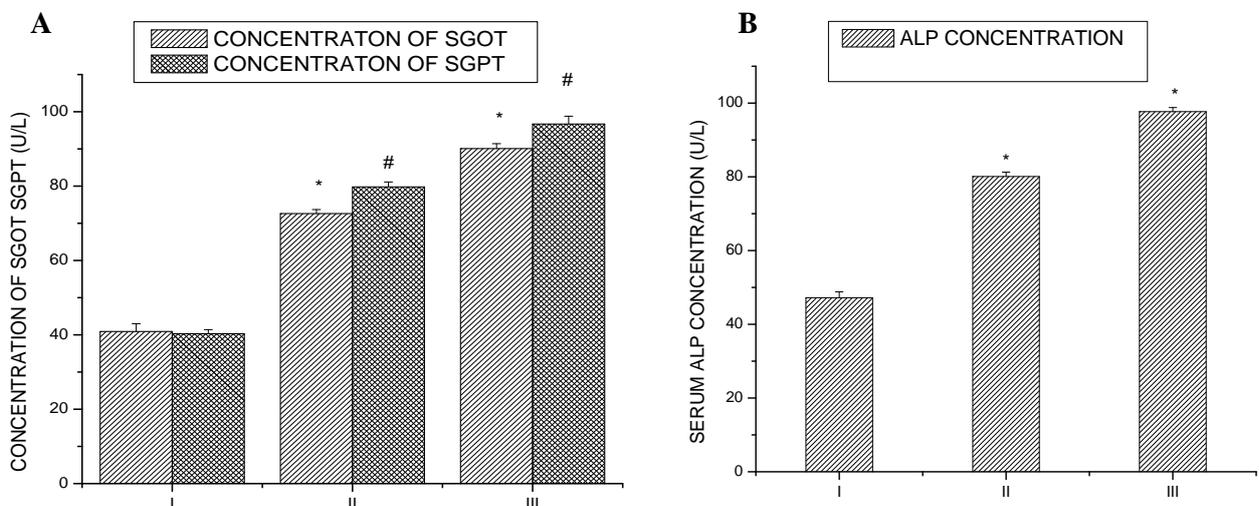


Fig. 2 Graphical presentation represents the SGOT, SGPT (A) and Alkaline phosphatase (ALP) (B) of experimental groups. Values are expressed as mean±SEM, n=6. *,# indicates significant difference (P<0.05) compared to control group. Group I: Control, Group II: CCl₄ at 115 mg/kg bw, Group III: CCl₄ at 230 mg/kg bw.

The tissues were kept in xylene overnight to remove the alcohol. Then, embedding and casting in paraffin wax with wooden block was done and sectioning of 5 μm thick carried out using a microtome. The sectioned tissues were mounted on slides using a thin film of egg albumen smeared on each side. After deparaffinization in xylene, the sections were passed through alcohol, stained with haematoxylin-eosin, and mounted in neutral DPX medium. The slides were then evaluated for pathological changes under microscope.

Data Analysis

The data were calculated and statistical analysis were done using a statistical package, Origin 6.1, Northampton, Mass, USA the software. The statistically calculated data were expressed as mean \pm SEM, $n = 6$. Comparisons between the means of control and CCl_4 administered group at 115mg/kg/bw and another CCl_4 challenged group with 230 mg/kg bw group, by one way ANOVA, $P < 0.05$, level of significance.

RESULT

Wellness Parameter Study

Several parameters like skin and fur, eyes, mucus membrane, behavioral pattern changes, tremor, convulsions, salivations, diarrhea, lethargy, sleep and mortality were recorded. It has been observed that after 2hr of application of CCl_4 at 2000 mg/kg bw salivation begins, lethargy develops in some of experimental animals at 4hr from the administration of the said dose. At 6hr, eye colour of experimental animals became extensively red. The experimental animals started losing their skin fur at 10 hr from administration of the said dose. Convulsion and tremor appeared at 10 hr. After 24 hr from the time of administration of the dose one rat died, and after 36 hr three experimental rats were died. After completion of the duration of the experiment i.e. 48 hr four experimental rats were died and 2 were still alive as shown in Table. 1. Therefore, the approximate LD50 is less than 2000 mg/kg bw.

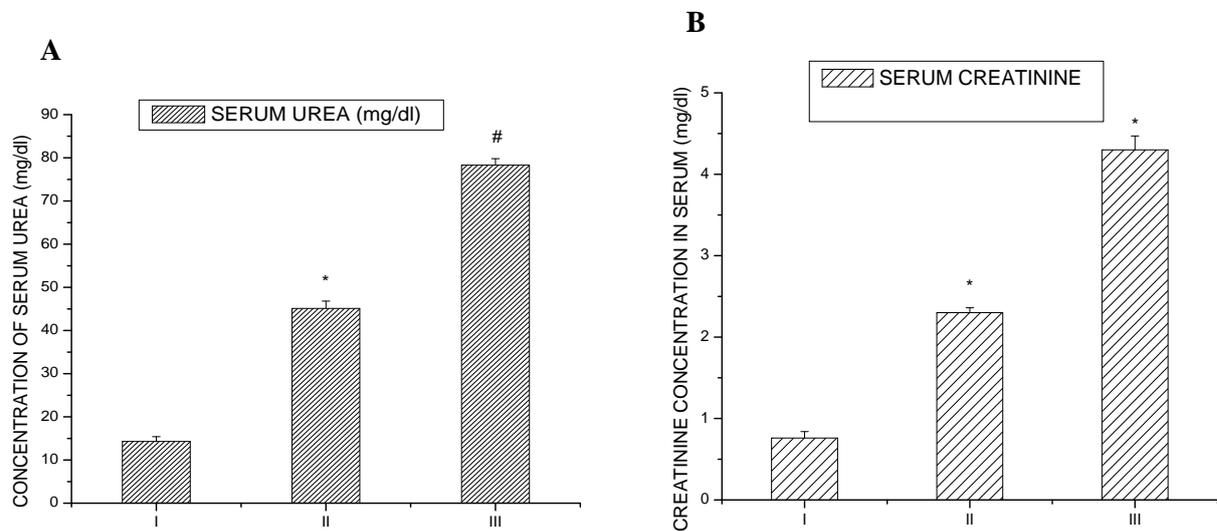


Fig 3 Graphical presentation represents the urea (A) and creatinine activity (B) of experimental groups. Values are expressed as mean \pm SEM, n=6. *,# indicates significant difference ($P < 0.05$) compared to control group. Group I: Control, Group II: CCl_4 at 115 mg/kg bw, Group III: CCl_4 at 230 mg/kg bw.

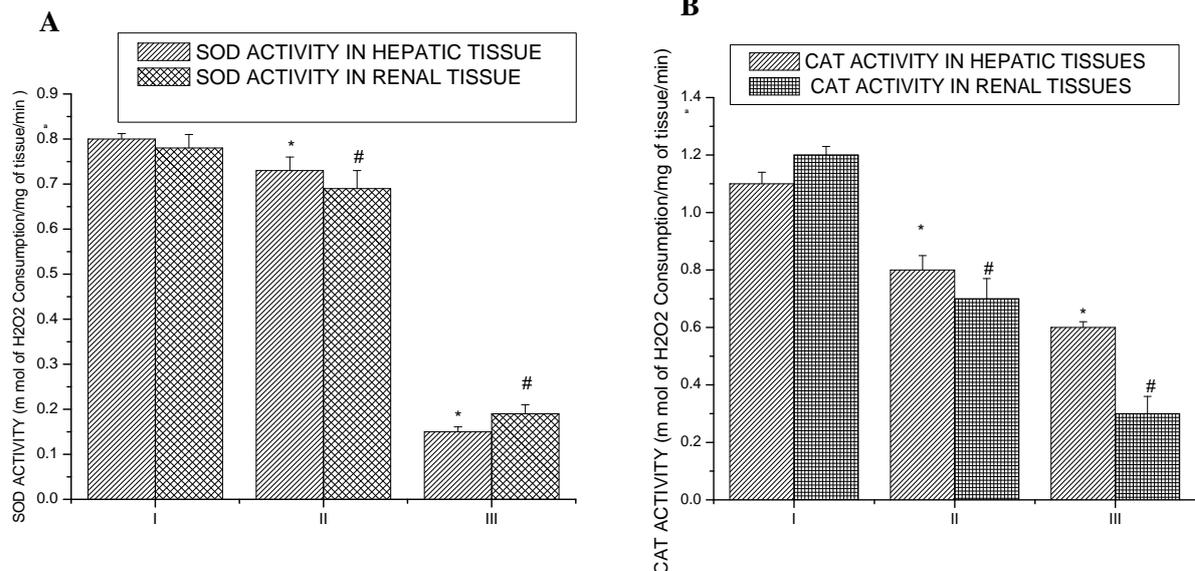


Fig. 4 Graphical presentation represents the superoxide dismutase (SOD) and catalase (CAT) activity of experimental groups. Values are expressed as mean \pm SEM, n=6. *,# indicates significant difference ($P < 0.05$) compared to control group. Group I: Control, Group II: CCl_4 at 115 mg/kg bw, Group III: CCl_4 at 230 mg/kg bw.

Table.1 Observation of different wellness parameters of rats for the limit test of carbon tetrachloride at the dosage rate of 2000mg/kg body weight/rat

Parameters	Observation Time																					
	30 min		2hr		4hr		6hr		8hr		10hr		12hr		18hr		24hr		36hr		48hr	
	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E
Skin & Fur	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	R	N	R	N	R	N	R	N	R	N	R	N	R	N
Mucus Membrane	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	Y	X	Y	X	Y	X	Y
Lethargy	X	X	X	X	X	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X
Sleep	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Convulsions	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Tremours	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Diarrhoea	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Morbidity	X	X	X	X	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y
Mortality	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Abbreviations: C- Control, D- Dead, E- Experimental, FL- Fur loss, L- Live, N- Normal, R- Red, X- No, Y- Yes.

Main Test

Determination of TC, TG, MDA

Total cholesterol, total glyceride has been increased significantly ($p < 0.05$) in serum in CCl₄ treated groups (at 115 and 230 mg/kg bw treatment). With the increment of CCl₄ administration at 230 mg/kg bw dose, the total cholesterol has also been increased by 73.71% (Fig. 1A), and total glyceride increased by 93.13% (Fig. 1A) significantly ($p < 0.05$) compared to control. MDA level due to lipid peroxidation in tissue homogenate has been found to elevate by 128.45% and 132.35% in liver and kidney respectively ($p < 0.05$), compared to control group (Fig. 1B).

Estimation of SGOT, SGPT and ALP

In serum, the SGOT, SGPT and ALP level been increased significantly ($p < 0.05$) compared to control group. At 230 mg/kg bw CCl₄ treated group, SGOT has been increased by 120.29% (Fig. 2A), SGPT has been increased by 139.95% (Fig. 2A) and ALP has been increased by 106.99% (Fig. 2B) significantly ($p < 0.05$) compared to control group.

Determination of creatinine and urea in serum

In this study, we measure the creatinine and urea level in the serum. It has been found that, creatinine concentration increased by 465.78% and 447.55% for urea significantly when compared to control ($p < 0.05$) (Fig.3A, B).

Antioxidant enzyme level

Antioxidant enzymes like SOD and CAT decreased significantly ($p < 0.05$) in tissue homogenate due to CCl₄ intoxication at 115 and 230 mg/kg bw. In group c the SOD and CAT has been decreased by 81.25% and 70.73% in hepatic cells and 68.33% and 71.31% in renal cells respectively (Fig. 4A, B).

Reduced glutathione (GSH) and glutathione peroxidase (GPx) activity

Fig. 5, shows that reduced glutathione level and glutathione peroxidase activity has been decreased in tissue homogenate. The GSH activity has been decreased by 56.88%, compared to control in liver and 53.29% in kidney ($p < 0.05$) and GPx activity has been decreased significantly ($p < 0.05$) by 44.44% in hepatic cells and 42.47% in renal cells compared to control group.

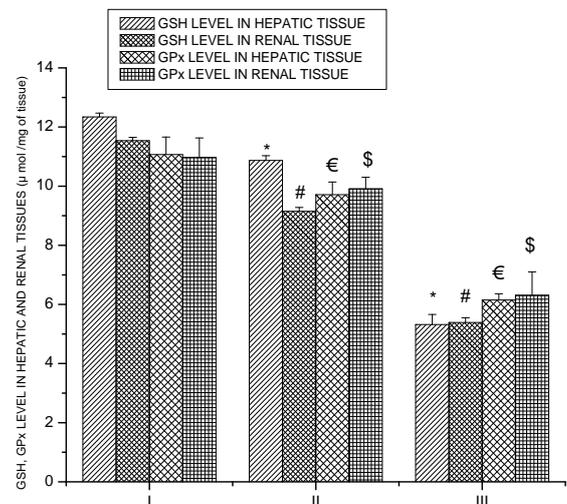


Fig. 5 Graphical presentation represents the reduced glutathione (GSH) and glutathione peroxidase (GPx) activity of experimental groups. Values are expressed as mean±SEM, n=6. *,# indicates significant difference (P<0.05) compared to control group. Group I: Control, Group II: CCl₄ at 115 mg/kg bw, Group III: CCl₄ at 230 mg/kg bw.

Histological Examinations

Histological examination of liver and kidney sections of normal rats showed normal architecture of hepatic lobules with normal central vein, portal tract, hepatocytes and sinusoids and normal glomeruli with intact Bowman’s Capsule as shown in Fig. 7A and 8A. Subcutaneous injection of CCl₄ (115 mg/kg body wt/day and 230 mg/kg body wt/day) to rats revealed fatty degeneration, coagulative necrosis, damaged hepatic sinusoids and damaged glomeruli, Bowman’s Capsule as shown in Fig. 7B, 7C and 8B, 8C.

DISCUSSION

CCl₄ induced hepatorenal toxicity changes normal liver to fibrotic and cirrhotic one along with renal dysfunction due to vasoconstrictions in renal arteries (Ortega et al., 2002). However, due to lack of therapeutic agents with low expenses causes against hepatorenal dysfunction recently this disease came to an interest. The wellness study was done not only to find out behavioral changes happens in acute exposure, but also to gather an idea about mortality rate of the exposed animals. In this study, after 48 hr duration 2 experimental animals were alive. The result of the study is quite similar to Nandi et al.,

2015, where also the same wellness parameters observed. Wellness parameters actually reflect the *in vivo* changes after application of acute dose. By the study with these parameters the duration and degree of total systemic damage due can be observed. We observed that, it took only 2 hr to commence the toxic effect of CCl₄ and after 24 hr the mortality rate decreases (Table.1).

during lipid peroxidation may well act as “second toxic messengers” of the complex chain reactions and consequently, polyunsaturated fatty acids of the membrane bilayer are converted to lipid hydroperoxides (Esterbauer et al., 1990). The cytotoxic malonaldehyde is also chemically reactive, causes cell damage (Schmidt et al., 1996). MDA, reactive electrophile species, is a marker of oxidative stress as it originates stressed

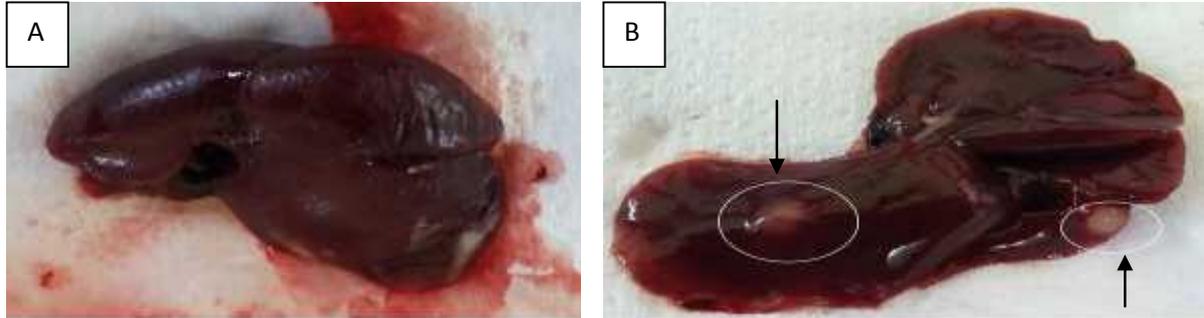


Fig 6 The images are showing the liver of different experimental groups. Group I: Control, Group III: CCl₄ at 230 mg/kg bw. After 7 days administration of CCl₄, liver cirrhosis (red round highlighted) has been found at group III.

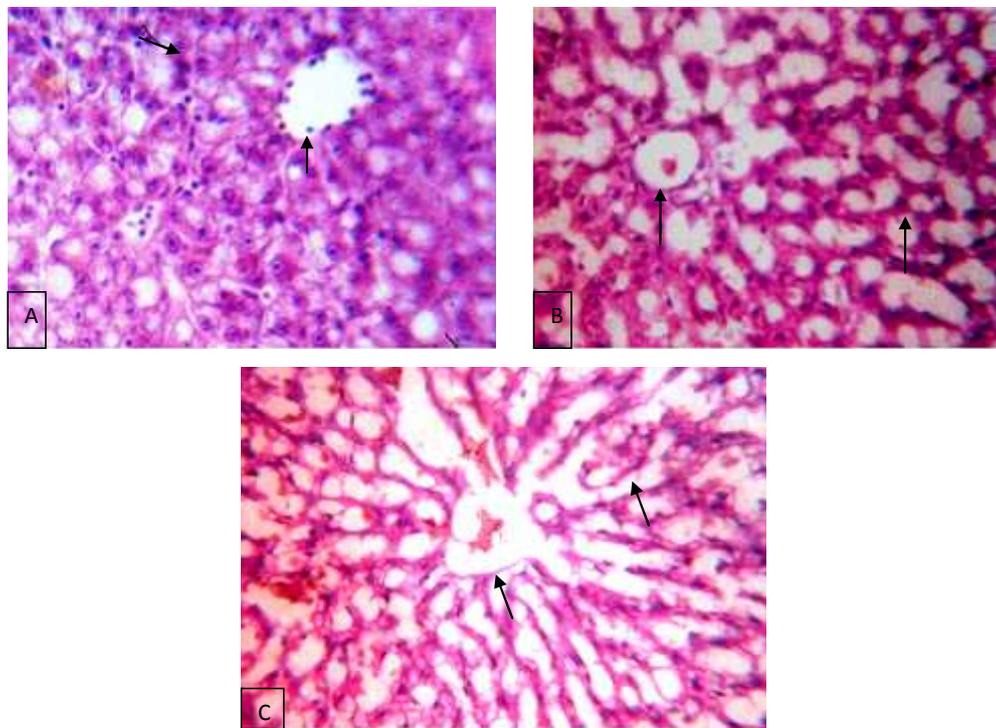


Fig 7 Section A: Showing normal histological structure of liver tissue like normal hepatic cell, central vein and sinusoids of untreated control group rats. Section B: CCl₄-treated at 115 mg/kg body wt/day for 7 days showing massive cellular disruptions as damaged hepatic cells, central vein and disrupted sinusoids compared to control gr. Section C: CCl₄-treated at 230 mg/kg body wt/day for 7 days showing massive cellular disruptions as damaged hepatic cells, central vein and disrupted sinusoids compared to control gr.

In male albino rats, 115 and 230 mg/kg bw CCl₄ for 7 days were charged. The level of total cholesterol and glyceride concentration in serum has been increased significantly ($p < 0.05$) in liver and kidney compared to control (Fig. 1A). The principle causes of carbon tetrachloride (CCl₄) induced hepatic damage are lipid peroxidation, generation of free radicals and decreased activities of antioxidant enzymes (Castro et al., 1974; Poli 1993). In this study, it has also been found that the MDA level has been elevated significantly ($p < 0.05$) with the increase of CCl₄ concentration in the tissues (Fig.1B). Initially, lipid peroxidation amplify free radicals generation in the cells, and the reactive aldehydes, generated

Here, we found that the CCl₄ exposed hepatorenal system go through the lipid peroxidation.

In this experiment, there was a marked increase in serum concentrations of SGOT, SGPT and ALP (Fig. 2) were actually sign of damaged hepatic function and impaired renal function were revealed by increased urea and creatinine concentration in serum (Fig.3).

Headed for confirmation about the oxidative stress and anti-oxidative function into the *in vivo* system, the SOD, CAT activity in the liver, kidney were measured. The results showed

that the SOD and CAT activity decreased in the liver and kidney significantly, compared to control (Fig. 4).

damage in to the hepatorenal system gradually with the elevation of dose.

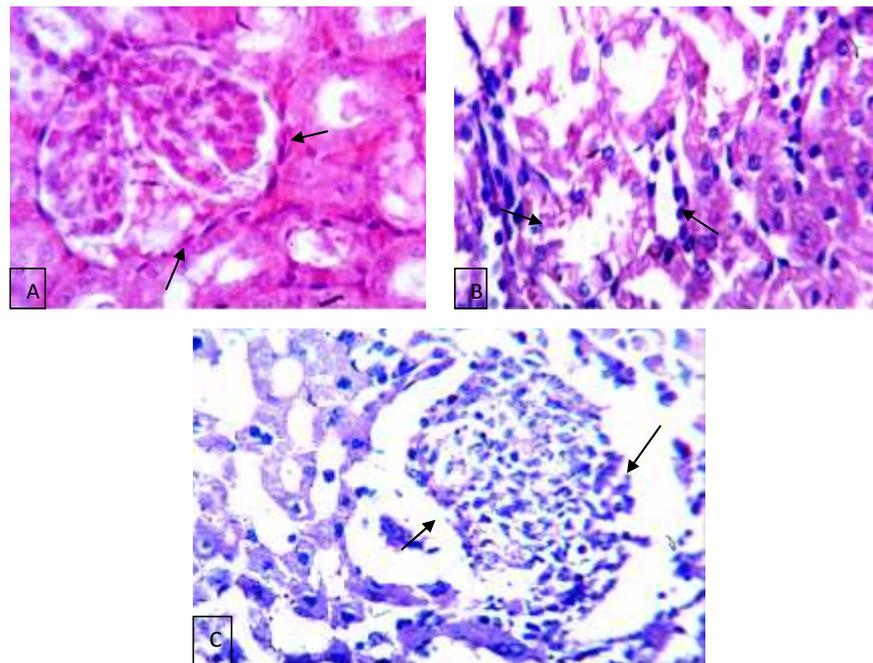


Fig 8 Section A: Showing normal histological structure of kidney tissue like normal Glomerulus and Normal Tubule of untreated control group rats. Section B: CCl₄-treated at 115 mg/kg body wt/day for 7 days showing massive cellular disruptions as damaged glomeruli, loss of Bowman's Capsules, necrosis of tubular epithelial cells or damaged tubules. Section C: CCl₄-treated at 230 mg/kg body wt/day for 7 days showing massive cellular disruptions as damaged glomeruli, loss of Bowman's Capsules, necrosis of tubular epithelial cells or damaged tubules.

It may be due to the release of enzymes into blood from the tissues during the CCl₄ intoxication. SOD is a chain breaking antioxidant agent into the host. SOD protects cells from injury by catalyzing the superoxide into oxygen and hydrogen peroxide (Yoelii *et al.*, 1975). From this study, the SOD generation in tissue, is decreased due to CCl₄ intoxication (Fig. 4A) and another antioxidant enzyme, catalase, which is found in tissues (Beyer *et al.*, 1989) also decreased due to the CCl₄ toxicity. CAT helps to defend the cell from the toxic property of hydrogen peroxide by catalyzing its disintegration into molecular oxygen and water without the production of free radicals and performs as a scavenger of H₂O₂ produced by SOD and save from the harmful effects of H₂O₂ (Iyawe *et al.*, 2010). In the study, catalase activity also decreased significantly ($P < 0.05$) in tissues of CCl₄ treated experimental groups (Fig. 4B).

In mamalian system, there are several enzymes, which are important for synthesis, turnover and maintenance of intracellular system (Tripathy *et al.*, 2012). For the fulfillment of the purpose of study, reduced glutathione (GSH) and glutathione peroxides (GPx) were measured to understand the antioxidant enzyme status as well as glutathione system in liver. In our study, it has been evaluated that the GSH and GPx activity has been reduced significantly ($p < 0.05$) in CCl₄ treated groups, compared to control (Fig. 5).

Reduced thiols (-SH) are the major antioxidants in the cell and Both GSH and protein bound thiols contributes maximum to the total thiols pool in the cell (Prakash *et al.*, 2008). Low levels of total thiols pool have been shown to be associated with various disorders with increased generation of free radicals in liver and kidney due to the CCl₄ exposure. Throughout the study, it has been evaluated that CCl₄ causes

In between two concentration of CCl₄, i.e 115 mg/kg bw and 230 mg/kg bw, maximum adverse effect has been found from 230 mg/kg bw treated group. The results obtained from different biochemical parameters totally supported by open eye examinations just after dissection (Fig. 6) as well as histological examinations (Fig. 7, 8).

CONCLUSION

From this study, it may be concluded that subcutaneous CCl₄ exposure, in albino male rats causes successful hepatorenal dysfunction by generation of liver fibrosis at 230 mg/kg bw after 7 days exposure. At this concentration, CCl₄ initiates fibrotic, cirrhotic liver and an impairment in kidney functions by creating an oxidative imbalance into cells which finally leads to hepatorenal dysfunction. This study suggest that this concentration may be followed as threshold dose of CCl₄ against male albino rats in laboratory research for further experiment. Furthermore, thorough study it is necessary to be confirm about the physiological changes and also to find out whether this dose is responsible to impair hepato-renal system.

Acknowledgement

The authors thank Department of Science and Technology (DST), Govt. of India for grant and Midnapore College, Raja N L Khan Women's College, Midnapore, University College of Science and Technology, University of Calcutta, W.B., India, for providing the facilities to execute the study.

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How to cite this article:

Saptadip Samanta et al. 2017, Carbon Tetrachloride Induced Hepatorenal Failure In Male Albino Rats: A Dose Response Study. *Int J Recent Sci Res.* 8(3), pp. 15885-15892.